Testing the relationship between gut permeability, elevation of systemic lipopolysaccharides and chronic disease

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Abstract

The aim of my thesis was to test whether an increase in the permeability of the gut is accompanied by an increase in the level of systemic lipopolysaccharides (LPS), also referred to as endotoxin. These two parameters were firstly concurrently determined in healthy women after the treatment with a single dose of aspirin which is thought to temporarily increase the paracellular permeability of the intestine. Gut permeability and the levels of systemic LPS in healthy women were then compared with those in women with Crohn's disease (CD) as the latter are thought to have chronically elevated paracellular permeability of the gut. Both groups also ingested a high fat drink which is reported to results in the elevation of systemic LPS. In addition, faecal calprotectin, a biomarker of ongoing inflammation in the gut, and LPS-binding protein (LBP), a proposed indirect biomarker for the exposure to LPS in the systemic circulation, were determined both in healthy women and in those with CD.

Data indicated that both temporary and chronic increase in the paracellular permeability of the small intestine can be reliably determined by the 3-h excretion of lactulose. Further the combination of levels of faecal calprotectin and 3-h excretion of lactulose and mannitol is the most sensitive tool to distinguish between healthy subjects and those with CD. Hence, it is evident that the combination of those three parameters can be used to assess gut health. In contrast, the current available methods for the direct assessment of the systemic level of LPS/endotoxin i.e. the Limulus Amebocyte Lysate (LAL) assay for the quantification of endotoxin or ELISAs for the quantification of LPS, are not reliable as the former is interfered by constituents of serum and the latter failed to detect LPS from sources other than those provided from the manufacturer of the kit. Hence, studies suggesting that the consumption of high fat meals lead to elevations of systemic endotoxin and those suggesting that levels of

systemic endotoxin is associated with the onset of metabolic syndrome are questionable. It is therefore advisable to repeat those studies when accurate methods for the quantification of LPS/endotoxin in the systemic circulation are available.

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List of Abbreviations

AOAH	Acyloxyacyl hydrolase
CD	Crohn's disease
CD14	Cluster of differentiation 14
CRP	C-reactive protein
DA	Dispersing Agent
DCs	Dendritic cells
ELISA	Enzyme-linked immunosorbent assay
GALT	Gut-associated lymphoid tissue
HDL	High-density lipoprotein
HF	High fat
IAP	Intestinal alkaline phosphatase
IBD	Inflammatory bowel disease
Ig A	Immunoglobulin A
IL-1β	Interleukin-1β
INFβ	Interferon β
IRAK1	Interleukin-1 receptor-associated kinase 1
IRF3	Interferon regulatory factor 3
KO	Knock out
LAL	Limulus Amebocyte Lysate
LBP	Lipopolysaccharide-binding protein
LDL	Low density protein
LMR	Lactulose - Mannitol - Ratio
LPS	Lipopolysaccharide
LRW	LAL reagent water
LTA	Lipoteichoic acid
MALP-2	Macrophage-activating lipoprotein 2
MAPK	Mitogen-activated protein kinase
MD-2	Myeloid differentiation factor 2
MyD88	Myeloid differentiation factor 88
NF-kB	Nuclear factor kB
OD	Optical density
O-PS	O-polysaccharide
OS	Oligosaccharide
PPC	Positive Product Control
PPs	Payer's patches
SHIP	SH-2 containing inositol phosphatase
TG	Triglyceride

CHAPTER 1 INTRODUCTION

TESTING THE RELATIONSHIP BETWEEN GUT PERMEABILITY, ELEVATION OF SYSTEMIC LIPOPOLYSACCHARIDES AND CHRONIC DISEASE

1.1 General overview of the topic

The human intestine is inhabited by 10^{14} individual bacteria, a number that is greater than the total number of cells in the human body. It is estimated that 500 to 1,000 bacterial species, predominately from the phyla Bacteroidetes and Firmicutes, colonize the gastrointestinal tract [2].

Under normal physiological conditions humans live in a symbiotic relationship with these bacteria. Whilst the presence of bacterial products such as lipopolysaccharides (LPS), also referred to as endotoxin, in the lumen is thought to aid in the development of a balanced immune system, high concentrations of LPS in the systemic circulation can lead to septic shock which is deleterious to the host [3]. Further, recent data suggest that persistent low levels of circulating endotoxin may be associated with the development of metabolic diseases such as insulin resistance, type 2 diabetes, atherosclerosis and cardiovascular disease [4-6] and that its concentration in the systemic circulation can be modified by diet [7-10]. Hence, diet-mediated rise in circulating levels of LPS/endotoxin may be responsible for post-prandial inflammation and thus may be a risk factor in the development of metabolic disorders. However, it is still unclear how bacterial products such as LPS/endotoxin are translocated from the gut lumen into the systemic circulation. Some data indicate that LPS uptake is mediated by lipid rafts and chylomicrons during the process of fat digestion and absorption (Figure 1-1) [11, 12]. On the other hand, results of mice models suggest that the intestinal permeability is increased during the absorption of dietary fat [13]. Further, augmentation of the permeability of the gut is associated with increased levels of systemic endotoxin in obese mice (Figure 1-1) [14].

1.2 Aim and objectives of the thesis

The aim of my PhD project was to test the relationship between levels of systemic LPS/endotoxin and the permeability of the gut, namely the paracellular and transcellular permeability (Figure 1-1), in health and in Crohn's disease. There are no published studies that concurrently determined those two parameters in human subjects.

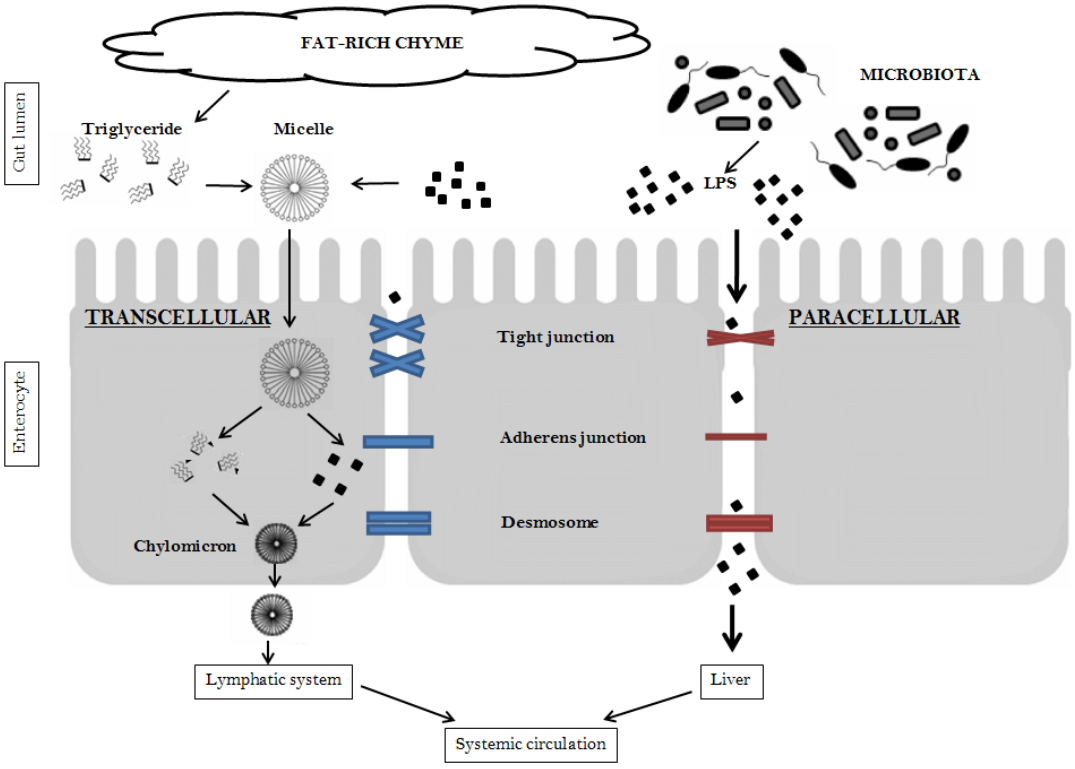

Figure 1-1. Schematic overview of the proposed absorption routes of LPS from the gut lumen into the systemic circulation.

Left enterocyte, the transcellular route; between the two enterocytes on the right hand side, the paracellular route (modified from Figure 1 Kelly et al. [1]).

1.2.1 Primary objectives

- Evaluation of methods for the determination of LPS/endotoxin in blood samples.
- Investigation whether the quantification of LPS/endotoxin in human blood and the assessment of the gut permeability by the lactulose-mannitol test, can be concurrently determined.
- Investigation whether either a temporary increase in the paracellular permeability, as induced by a single dose of aspirin, or a persistent increase in the paracellular permeability, as observed in subjects with Crohn's disease (CD), is accompanied by an augmentation of the levels of systemic LPS/endotoxin.
- Investigating the effect of the consumption of a single fatty drink on both the permeability of the gut and the levels of systemic LPS/endotoxin in healthy subjects and in those with inactive CD.
- Describing the relationship between systemic levels of LPS and endotoxin.

1.2.2 Secondary objectives

- Evaluation of an assay for the quantification of LPS-binding protein (LBP) in human sera.
- The quantification of systemic levels of LBP and of inflammation markers such as faecal calprotectin and systemic C-reactive protein, in healthy subjects and in those with CD.
- Describing the relationship between the permeability of the gut and either LBP or inflammatory markers.

1.3 Structure of the thesis

The introduction chapter is followed by a literature review that describes the structural key features of LPS and its function in Gram-negative bacteria as well as the interactions between host and LPS in both the gut lumen and the systemic circulation. Further, possible absorption routes of LPS are explained in more detail in the literature review. Chapter three provides an overview of available methods for the quantification of LPS/endotoxin and for the assessment of gut permeability, as well as a general description of the methods that were used. The evaluation of two commercially available ELISAs for the quantification of LPS, and of one commercially available Limulus Amebocyte Lysate (LAL) assay for the determination of endotoxin assay, that has been conducted prior to the pilot study, is explained in chapter four 'Method evaluation – Pilot study' followed by the description and results of the pilot study in chapter five. Since data of the pilot study indicated that the chosen LAL assay was not reliable a LAL assay from another manufacturer were evaluated and modified prior to the second study, referred to as the Intralipid study. The experimental work associated with the evaluation and modification of the latter LAL assay is described in chapter six 'Method evaluation – Intralipid study'. This chapter also includes the evaluation of an ELISA for the quantification of LBP in serum. The experimental work and the results of the Intralipid study are presented in chapter seven. Additional work regarding the partition of endotoxin into Intralipid in aqueous emulsions was conducted and is described in chapter ten 'Note – endotoxin-spiked Intralipid'. Data obtained in the Intralipid study raised questions regarding the reliability of both the LPS – ELISA and the LAL assay that were used in the study. Hence, additional experimental work was conducted for the evaluation of the LPS – ELISA which is described in detail in chapter nine 'Technical Note: LPS – ELISA'. Further modifications in the pre-treatment of serum samples prior to the LAL assay and the

evaluation of another LBP - ELISA were required. The latter are explained in chapter ten 'Method evaluation – Crohn's disease study'. The study design and results of the third study, referred to as the Crohn's disease study, are presented in chapter eleven. Given the fact that there were major technical issues with the LAL assay regarding the quantification of endotoxin in blood i.e. serum and plasma, a summary on the determination of endotoxin in human blood and on reported levels of systemic endotoxin in health and disease is provided in chapter twelve. The general conclusion is given in chapter thirteen 'Discussion'.

1.4 References

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CHAPTER 2 LITERATURE REVIEW

THE CHARACTERISTICS AND FUNCTION OF BACTERIAL LIPOPOLYSACCHARIDES AND THEIR ENDOTOXIC POTENTIAL IN HUMANS

This chapter is published in part as:

Gnauck A, Lentle RG, Kruger MC. The characteristics and function of bacterial lipopolysaccharides and their endotoxic potential in humans. International Reviews of Immunology. 2015. doi: 10.3109/08830185.2015.1087518

Alterations compared to the published paper were made in section 2.6.2. Section 2.7 was added to the paper to provide background information about gut permeability and to explain the hypotheses of LPS translocation from the gut lumen into the systemic circulation. See Appendix V for the published literature review.

DRC 16

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GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Anne Gnauck

Name/Title of Principal Supervisor: Prof Roger G. Lentle

Name of Published Research Output and full reference:
Gnauck A, Lentle RG, Kruger MC. The characteristics and function of bacterial lipopolysaccharides and their endotoxic potential in humans. Int Rev Immunol. 2015. doi: 10.3109/08830185.2015.1087518

In which Chapter is the Published Work: Chapter 2

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GRS Version 3– 16 September 2011

Abstract

The aim of the literature review is to provide background information regarding the complex cross-talk between enteral commensal bacteria and the human immune system via LPS. At the beginning, I explain the structural characterisation of the LPS molecule and its function in the bacteria. I then examine how LPS is recognised by various elements of the human immune system and the signalling pathways that are activated by the structure of the LPS molecule and the effect of various concentrations. This is followed by the discussion of general sequelae of this signalling in the gut associated and systemic immune systems i.e. the neutralisation of LPS and the development of tolerance to LPS. Further, the potential health risk of the diet-mediated persistent low levels of systemic endotoxin and the possible routes at which LPS can enter the systemic circulation are discussed.

2.1 Introduction

The human intestine is inhabited by 10^{14} individual bacteria. Hence bacteria cells outnumber the total number of cells in the human body. It is estimated that 500 to 1,000 bacterial species, predominately from the phyla Bacteroidetes and Firmicutes, colonize the gastrointestinal tract. Minor species of gut-colonizing bacteria including those from the phylum Proteobacteria notably from the genera *Escherichia*, *Salmonella*, *Vibrio* and *Helicobacter*, and from the phyla Verrucomicrobia, Actinobacteria (notably the genus *Bifidobacterium*), Fusobacteria and Cyanobacteria [7]. Under normal physiological conditions humans live in a symbiotic relationship with these bacteria. The human gut associated lymphoid tissue (GALT) is fine-tuned by 'cross-talk' between the host and commensal enteral bacteria promoting tolerance of their presence and their products as well controlling the composition of the mucosal biofilm [7].

Cross-talk between the enteral microbiota and the human host is essential for the development and maintenance of the human gastrointestinal and systemic immune system [7]. The presence of the bacterial cell membrane component lipopolysaccharide (LPS) from Gram-negative bacteria in the lumen is thought to aid in the development of a balanced immune system as it may influence the maturation of regulatory T cells in the GALT [8] and facilitate the destruction of invading organisms. Conversely, high concentrations of LPS in the systemic circulation can lead to septic shock which is clearly deleterious to the host [9]. Further, recent data suggest that persistent low levels of circulating LPS may be associated with the development of metabolic diseases such as insulin resistance, type 2 diabetes, atherosclerosis and cardiovascular disease [10-12]. Together these findings indicate that both the location and the concentration of LPS determine whether the subsequent immune response is beneficial or harmful to the host.

2.2 The structure of the outer membrane of Gram-negative bacteria

The cellular structure of Gram-negative bacteria such as species of the genus *Bacteroides*, *Escherichia* and *Salmonella* differs from that of Gram-positive bacteria such as *Lactobacilli* and *Clostridia* [7]. In addition to the (inner) cytoplasmic membrane and the peptidoglycan layer, Gram-negative bacteria possess a secondary outer membrane (Figure 2-1). Again, Gram-negative bacteria have a significantly thinner peptidoglycan layer than do Gram-positive bacteria. The inner membrane of Gram-negative bacteria is, like most biological membranes, a symmetric phospholipid bilayer. Conversely the outer membrane is inherently asymmetric comprising an inner phospholipid component and an outer lipopolysaccharide (LPS) component. Hence, the outer membrane differs both chemically and structurally from that of other cell membranes [13].

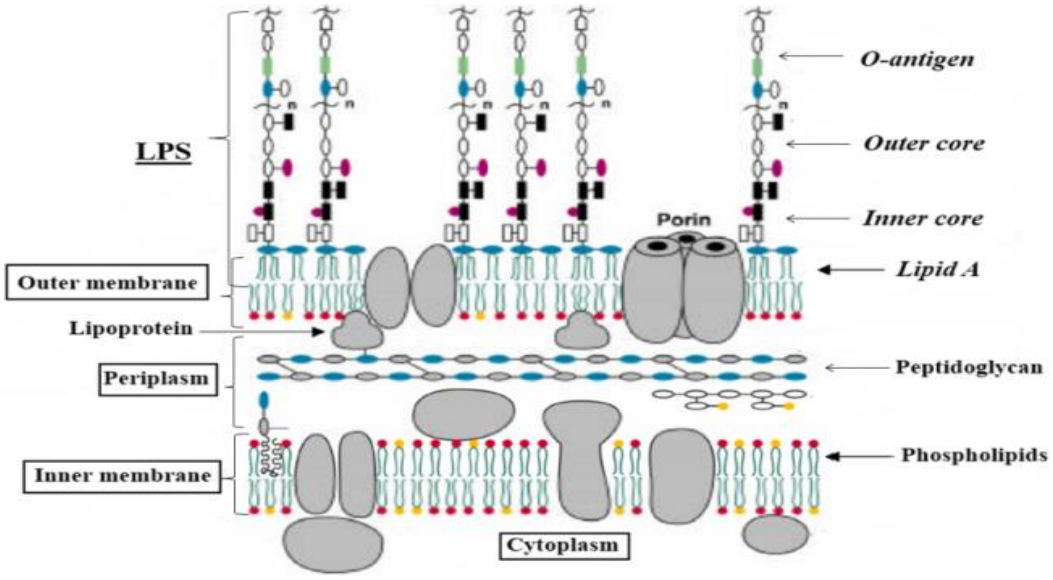

Figure 2-1. Model of the inner and outer membrane of Gram-negative bacterium *E. coli* K-12.
Showing how lipid A anchors the LPS molecule in the outer layer of the outer membrane (modified Figure 1 from Raetz and Whitfield [1]).

The outer layer of the outer membrane of *Escherichia coli* and that of other enteric bacteria contains around 10^6 molecules of LPS that comprise three quarters of the surface of the bacterial cell. Hence, LPS constitutes the bulk of the outer layer of these

organisms [2]. The LPS component is thought to regulate the level of permeability of outer membrane in response to changes in the environment and thus enables Gram-negative bacteria to survive in a diverse range of conditions and to cope with a range of chemical and physical stressors that include antimicrobial molecules, changes in temperature, pH and in oxygen concentration [13].

Some *Bacteroides* species i.e. *B. fragilis* are able to express zwitterionic polysaccharide moieties such as polysaccharide A (PSA) in the outer layers of their outer membranes and form an outer capsule [14].

2.2.1 Characterisation of lipopolysaccharide (LPS)

The LPS molecule consists of lipid A, a core oligosaccharide (OS) and an O-antigen/ O-polysaccharide (O-PS)[1]. The lipid A component is linked to OS in the inner core and serves to anchor the LPS molecule within the outer layer. The OS of the outer core binds to O-antigen (Figure 2-1 and Figure 2-2)[1].

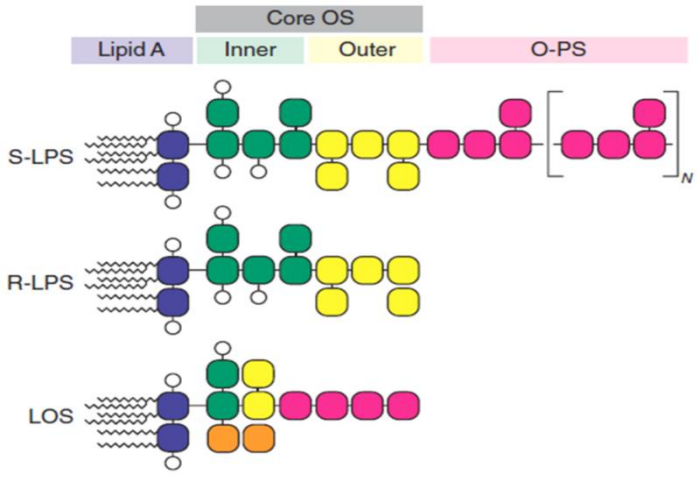

Figure 2-2. Schematic overview of the structural components of lipopolysaccharides (LPS) and lipooligosaccharides (LOS).

Smooth-LPS (S-LPS) consists of lipid A, a core oligosaccharide (OS) and an O-polysaccharide (O-PS)/ O-antigen. Rough-LPS (R-LPS) lacks O-PS and in some cases also parts of the core OS. LOS lacks O-PS but has a oligosaccharide extension attached to its core OS (Figure 1 from Tran and Whitfield [2]).

LPS molecules are classified as smooth(S)- or rough(R)-LPS according to the presence and characteristics of the core OS and the O-antigen. LPS molecules in species of Enterobacteriaceae, Pseudomonadaceae, Pasteurellaceae and Vibrionaceae contain all

three structural components [1] and are called S-LPS [2]. Bacteria with defects in the mechanism by which the O-antigen or the core OS are assembled, produce truncated LPS molecules, termed R-LPS (Figure 2-2). LPS molecules extracted from S-LPS producing bacteria are heterogeneous with respect to their O-antigen chain length and relative proportion of R-LPS.

A broad range of pathogenic Gram-negative bacteria including mucosal pathogens, lack the O-antigen component but still possess oligosaccharide extensions to their inner core OS forming structures that are termed lipooligosaccharides (LOS) (Figure 2-2) [2]. LPS molecules from a range of Gram-negative bacteria contain similar lipid A and inner core OS components but show a wide variation in the outer core OS and in the O-antigen components [2].

2.2.1.1 Lipid A

Lipid A, a glucosamine-based phospholipid, anchors the LPS molecule within the hydrophobic outer layer of the outer cell membrane in most Gram-negative bacteria species (Figure 2-1) [1]. Lipid A is initially synthesized in the cytoplasm and at the interface between the cytoplasm and the inner layer of the (inner) cytoplasmic membrane [13]. Biosynthesis of the LPS molecules commences with the linkage of a precursor lipid A with four acyl chains, termed lipid IV_A, to two units of the saccharide KDO (see Figure 2-3 for more details). Further saccharide units are added to this basic structure to form the lipid A- OS complex [1]. The lipid A- OS complex is then transported to the outer layer of the cytoplasmic membrane [15] where it is linked to the O-antigen [13]. In general, one lipid A – core OS unit anchors one O-antigen [2]. Additional species-specific lipid A modifications can occur in the outer layer of the cytoplasmic membrane or in the inner layer of the outer membrane before the

molecule is finally transported to the outer layer of the outer membrane [15]. The formation of the KDO - lipid A complex is required for bacterial growth.

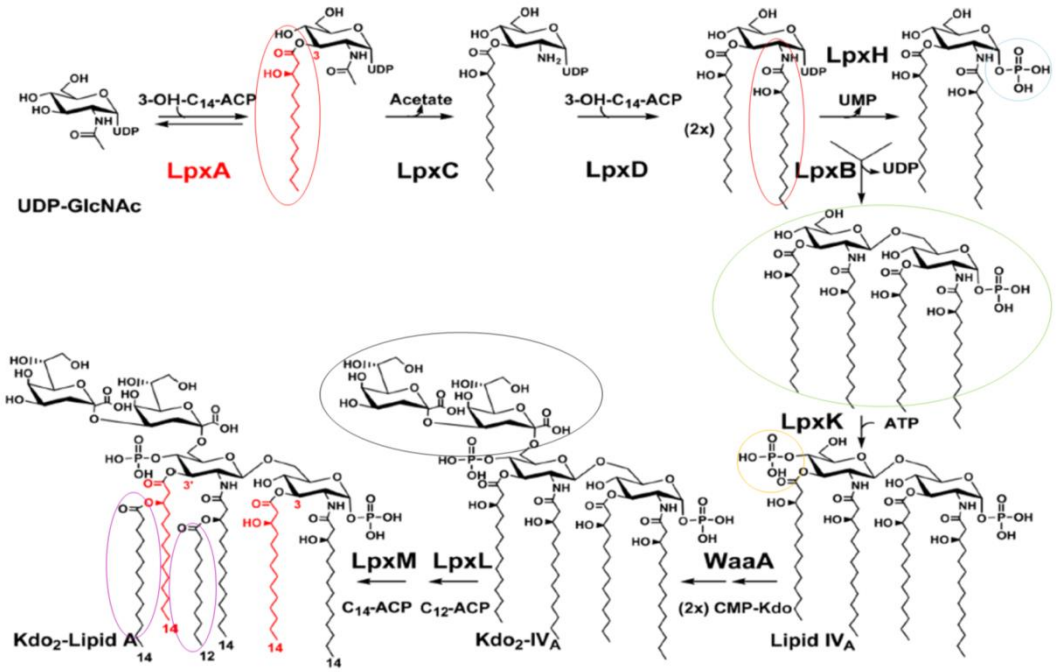

Figure 2-3. Structure and biosynthesis of Kdo2-lipid A in *E. coli* K-12.

During the initial stage of synthesis, two fatty acids are added to UDP-GlcNAc (red circles) leading to the formation of UDP-2,3-diacyl-GlcN which then is dephosphorylated at position 1 (blue circle). The resulting UMP-2,3-diacyl-GlcN is then linked to another UDP-2,3-diacyl-GlcN so that a molecule with 4 primary acyl chains and one phosphate group is formed (green circle). A second phosphate group is then added at position 4' (orange circle) to form the precursor lipid A termed lipid IV_A. Lipid IV_A is subsequently linked with two units of the saccharide KDO at position 5' (black circle) to form the basic tetra-acylated lipid A – KDO complex. During modification of lipid A subsequently up to two secondary fatty acids can be linked to the primary fatty acids at position R3' and R2' (pink circle) to form penta- or hexa-acylated lipid A (modified Figure 2 from Raetz and Whitfield [1]).

2.2.1.2 Core oligosaccharide

The biosynthesis of oligosaccharide residues takes place in the cytoplasm. The core oligosaccharide (OS) of S-LPS consist of two segments – the inner and the outer core OS. Most Gram-negative bacteria form an inner core composed of 3-Deoxy-D-manno-oct-2-ulosonic acid (KDO) and L-glycero-D-manno-heptose residues that are linked at position 5' of lipid A. The outer core forms the attachment site to the O-antigen [13] and consists mainly of hexose units but there are considerable variations in the component saccharides.

2.2.1.3 O-antigen (O-polysaccharide)

The O-antigen and the O-polysaccharide (O-PS), respectively, are constructed of two to six saccharide units [13]. The simplest O-antigens consist of repeating disaccharide units in which the component monosaccharide units are linked by single glycosidic linkages. More complex O-antigens consist, either of larger homopolysaccharide repeats that are linked by a specific sequence of different glycosidic linkages, or of heteropolysaccharide units interspaced with saccharide and non-saccharide moieties such as CO-acetyl groups and amino acids [2]. Hence, variations in constituent saccharide units, in the type of linkage between saccharide units and the complete or partial substitution of saccharide units by non-sugar moieties, result in a diverse range of O-antigens.

Biosynthesis of the repeating oligosaccharide units of the O-antigen precursors occurs in the inner layer of the cytoplasmic membrane. The completed O-antigen precursors are subsequently transported to the outer layer of the cytoplasmic membrane where they are polymerized to form an O-antigen. Concomitantly, the O-antigen is linked to the lipid A- OS complex (see above) in the interface between the cytoplasmic membrane and the periplasm. The mature LPS molecule is then transported into the newly assembled outer membrane via a series of cassette transporters [13].

2.3 The Function of the lipopolysaccharide in Gram-negative bacteria

The structural properties of the LPS molecule and its high concentration in the outer layer of the outer membrane reflect its function in maintaining the integrity of the cell membrane and in mediating interactions of the organism with its environment [2].

2.3.1 The barrier function

The inner part of the LPS molecule, containing lipid A and the inner core OS, is responsible for maintaining the barrier function of the outer cell membrane [2]. The LPS molecules are predominantly bound together by electrostatic interactions between phosphate groups of the component lipid A moieties and magnesium ions located in the outer membrane. Further, in the hydrophilic region of LPS, the core OS, there is hydrogen-bonding between the sugars of the core OS in adjacent LPS molecules, whilst the hydrophobic regions, the lipid A moiety within LPS molecules, interact hydrophobically with those in adjacent LPS molecules. These various interactions generate a compact arrangement of LPS molecules within the outer layer of the outer cell membrane [13].

A repertoire of structurally different lipid A's is required to enable the integrity of the membrane of the bacteria cell to be maintained under differing growth conditions [2]. The modification of lipid A by incorporation of suitable secondary acyl chains allows bacteria to "adapt" to such environmental variations as change in pH and temperature and the presence of antimicrobial peptides [15]. The acylation pattern is species-specific and varies with respect to both the numbers of acyl chains and the length (and degree of saturation) of fatty acids [6]. Thus, for example the lipid A of LPS from *Escherichia coli* has a hexa-acylated subunit bearing two phosphate groups and most commensal intestinal bacteria are able to produce and incorporate 3-hydroxymyrisate or 3-hydroxylaureate as primary acyl chains with laureate, myristate or both as

secondary acyl chains in order to enhance their resistance to antimicrobial peptides produced by the host [6]. The presence of the secondary acyl chains renders the outer membrane of *E. coli* impermeable to bile acids enabling the organism to colonize the gut. In contrast, species of Gram-negative bacteria that can live in water such as *Pseudomonas aeruginosa* mainly synthesize lipid A with a single secondary acyl chain (five acyl chains in total). Hence their outer membrane is more permeable to bile acid and to host antimicrobial peptides so that they are unable to survive in the lumen of the gut [6]. A number of pathogens such as *Yersinia pestis* are able to alter the acylation patterns of their lipid A moieties in response to temperature changes so as to maintain the membrane integrity whilst they colonize the host [16]. Hence, the ability to modify their lipid A moieties allows Gram-negative bacteria to survive under a range of different conditions [1].

2.3.2 The protection of the organism

The outer part of LPS, containing the outer core OS and O-antigen, varies greatly in structure [2]. The O-antigen extends outwards from the cell surface to form a protective shield [1] so that its glycan chain can influence the extent of the surrounding unstirred layer and thus limit the access of molecules in the general environment to the base of the outer membrane. The O-antigen can also act as a binding site for bacteriophages and for products of the hosts immune system [13]. Long chain O-antigens may also contribute to the virulence of human pathogens [1] as they often contain virulence factors [2].

The structure of O-antigens varies widely, according to environmental situations even within a species. Hence, for example *E. coli* has around 170 different O-serotypes [2] that can modulate the properties of the outer membrane of the organism and its interaction with the hosts immune system.

2.4 The endotoxic potential of LPS in humans

The lipid A component of LPS is the ligand of the human pattern recognition receptor Toll-like receptor 4 (TLR4) that is expressed by immune and other cells. Hence, the lipid A components of the LPS molecule are responsible for the induction of an innate immune response in both immune and other cells [1, 2]. However, the pro-inflammatory potential of lipid A, also referred to as its endotoxicity, varies between Gram-negative bacterial species according to its structure [2, 17-20]. Again, the magnitude of the inflammatory response to a particular LPS can vary with the dose that is received by the host [21].

2.4.1 The recognition of LPS by the TLR4/MD-2 complex

2.4.1.1 The Toll-like receptor 4 and its pattern of expression

The Toll-like receptor 4 (TLR4) is a transmembrane protein containing extracellular leucine rich repeats (LRR), a transmembrane domain and an intracellular Toll/IL-1 receptor (TIR) domain [22]. TLR4 is predominantly expressed by endothelial cells [23] and immune cells such as the macrophages and lymphocytes in peripheral blood [24] and in lower concentration by immune cells within the lamina propria [24-26]. TLR4 can also be expressed in small quantities by enterocytes in the small intestine and colonic mucosa of healthy humans [25, 26].

However, TLR4 alone cannot recognise LPS. Hence, it must associate with the myeloid differentiation factor 2 (MD-2) before it can induce an immune response to a LPS challenge. The MD-2 can either be a membrane-bound (m), as occurs in immune cells, or in a soluble (s) form. Non-immune cells such as epithelial and endothelial cells can express sMD-2 receptors [2]. Both forms of MD-2 can associate with TLR4 to form a heterodimer that initiates a complex, ligand specific, signalling after binding

with LPS [27]. However, these cells cannot by themselves mediate a response to LPS as it must be presented in the appropriate molecular context (see below).

2.4.1.2 The transport route of LPS to the TLR4/MD-2 complex

LPS cannot directly bind to the TLR4/MD-2 complex, LPS must be correctly 'presented' to the receptor complex. Both, membrane-bound (m) and soluble (s) forms of CD14 have the capacity to bind and correctly present LPS molecules to both membrane-bound and soluble forms of MD-2. Immune cells such as macrophages express mCD14 so they can 'directly' interact with LPS. In contrast, in non-immune cells, i.e. endothelial and epithelial cells, sCD14 must present LPS to sMD-2 which then interacts with the sMD-2 receptors on the surface of non-immune cells [2]. However, both forms of CD14 have normally a low affinity for LPS. However, this affinity is dramatically increased in the presence of LPS-binding protein (LBP) [28].

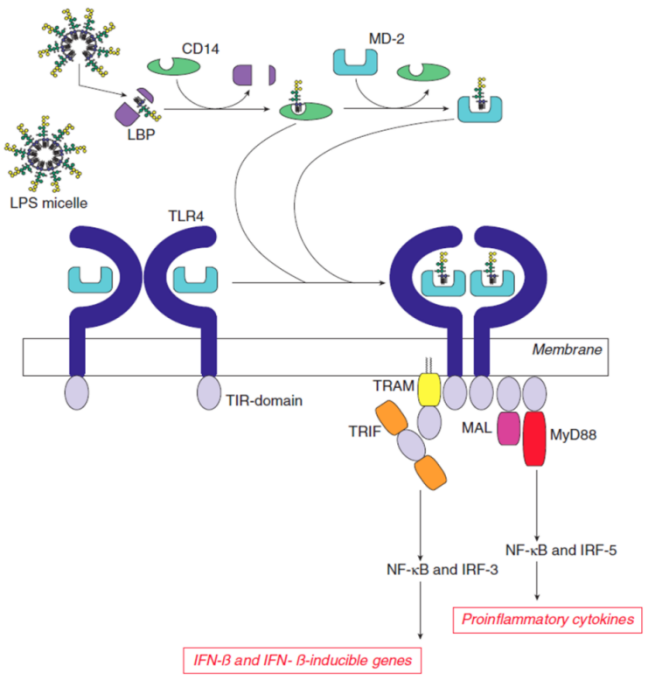

Figure 2-4. Schematic steps involved in the recognition of circulating LPS.

LPS-binding protein (LBP) sequesters single LPS molecules from the LPS aggregate and transfers it to CD14. CD14 acts to transfer the bound LPS to membrane-bound or to soluble MD-2. LPS-loaded MD-2 induces the TLR4 to dimerize and activates the TLR4-mediated signalling cascade. TLR4 activation either induce a MyD-88 dependent or TRIF–dependent pro-inflammatory immune response (Figure 8 from Tran and Whitfield [2]).

LBP can bind with a single LPS molecule either from an LPS aggregate or from fatty micelles [2] and present them to m/sCD14 (Figure 2-4) [28]. Hence, the LBP-

mediated transport of LPS to m/sCD14 is important in the induction of the LPS-dependent immune response [29]. LBP can also mediate the binding of LPS to high-density lipoproteins (HDL) whereupon it can no longer induce an immune response in circulating immune cells [30]. Hence, LBP-mediated binding of LPS to HDL somehow neutralises its pro-inflammatory potential (discussed below). Thus LPS presentation by LBP can either promote or reduce the pro-inflammatory potential of LPS. In this respect it is hypothesised that during the first 10 minutes of exposure LBP mediates the transport of LPS to CD14 and after this it is more likely to present LPS to HDL [30].

2.4.1.3 Interactions between the LPS and the TLR4/ MD-2 complex

In contrast to non-specific binding of LPS by LBP and CD14, both forms of MD-2 are able to interact specifically with LPS molecules based on the configuration of their acyl chains and their allied phosphate groups in the contained lipid A [2]. Hence, the structural form of the lipid A moiety, notably the presence of six acyl chains, governs the interaction with MD-2 and the subsequent change in its conformation [22, 27].

The hydrophobic pocket of MD-2 is able to accommodate four acyl chains. The presence of a fifth acyl chain induces a structural change in MD-2 that allows it also to be accommodated in the pocket, but a sixth acyl chain cannot be accommodated and must remain on the surface of the MD-2 protein [22, 27]. This projecting sixth acyl chain interacts with a specific phenylalanine residue on the TLR4 monomer [27]. The presence of a sixth acyl chain, whilst not inducing accommodation in the pocket, induces structural changes in the F126 loop of MD-2 that promotes the hydrophilic interaction of MD-2 with TLR4. The latter structural change allows the TLR4/MD-2 complex to interact with another TLR4 monomer to form a TLR4 dimer (Figure 2-4).

Meanwhile, the structural change induced by the fifth acyl chain that leads to its accommodation in the pocket, allows for ionic interaction between the phosphate groups and the positively charged residues of two TLR4 monomers which further facilitates the dimerization of the TLR4/MD-2 complex [27]. It is noteworthy that, hydrogen bonding between the phosphate groups of the lipid A unit and the TLR4 dimer can only occur when the lipid A contains two phosphate groups [22].

In contrast, the accommodation of a tetra-acylated lipid A in the pocket leaves the F126 loop of MD-2 exposed to the surrounding solvent [27]. The tetra-acylated form of lipid A is not acylated with a secondary fatty acid at position R3'. Hence, the exposure of the loop enables the establishment of hydrogen bonds between the 3-OH of R3' of lipid A (Figure 2-3) and the F126 loop of MD-2 [22]. The differing types of interactions of LPS with MD-2 and TLR4 according to the form of the contained lipid A can influence the character of the subsequent TLR4 downstream signalling [22, 27].

Interactions between LPS and the TLR4/MD-2 complex can also be mediated by carbohydrate moieties within the inner core of LPS but they do not have such a powerful promoting function as those described above [27].

2.4.1.4 TLR4-mediated signalling pathways

The ligand bound TLR4/MD-2 complex ultimately activates both the MyD88 (myeloid differentiation factor 88)-dependent and the TRIF (TIR-domain-containing adaptor inducing interferon-β)-dependent signalling pathways. As these two pathways have overlapping functions, fine tuning of their relative contributions is necessary to avoid an excessive pro-inflammatory response. As both pathways are only activated by

the TLR4 receptor, the modulation of the activation of the signalling cascades is critical for the homeostasis of the intestinal and the systemic immunity [31].

In general, the binding of LPS to the TLR4/MD-2 complex allows for interactions between the intracellular TIR domain of the TLR4 receptor and the intracellular TIR domain-containing adapter molecules such as MAL (MyD88 adaptor-like protein) and TRAM (TRIF-related adaptor protein). The TLR4-bound adapter molecule MAL then recruits MyD88 which subsequently activates the MyD88-dependent pathway [32]. The dissociation of MAL from the TLR4 receptor, followed by its degradation, enables the translocation of the TLR4/TRAM complex from the cell membrane into endosomes situated within the cytoplasm. This internalisation of the TLR4/TRAM complex induces the recruitment of TRIF and activates the TRIF-dependent signalling cascade (Figure 2-5) [33].

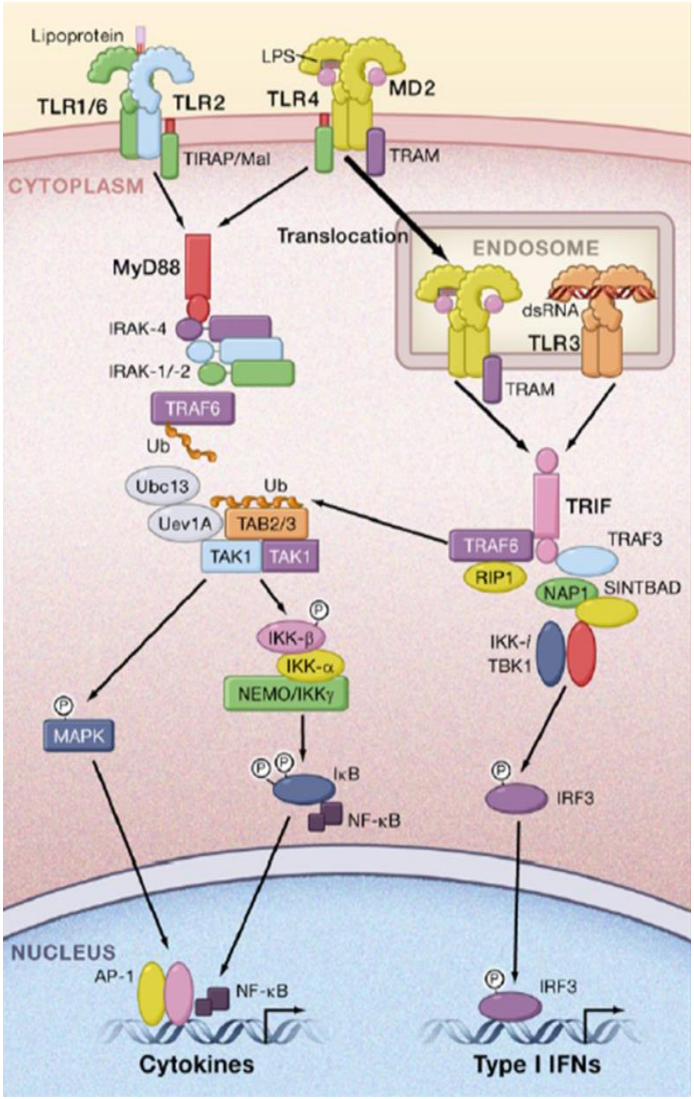

Figure 2-5. TLR4-mediated signalling pathway.

The interaction between LPS and the TLR4/MD-2 complex at the cell membrane activates MyD88-dependent downstream signalling, leading to the activation of the transcription factors NF-κB and AP-1. Both transcription factors mediate the production of pro-inflammatory cytokines. The internalisation of the TLR4 receptor induces the TRIF-dependent pathway which activates the transcription factor IRF3 and the expression of the IFNβ and type I interferon-inducible genes and/or induces the delayed activation of the transcription factors NF-κB and AP-1 (Figure 1 from Takeuchi and Akira [5]).

A cascade of molecules is involved in the downstream signalling from both TLR4 signalling pathways. The signalling cascade of the MyD88-dependent pathway includes the following steps. Firstly, IRAK4 (interleukin-1 receptor-associated kinase 4) is recruited to the TLR4-associated MyD88 and subsequently initiates the phosphorylation of IRAK1 [32]. IRAK-1 then induces the TRAF6-mediated downstream signalling cascade that results either in the activation and translocation of the transcription factor NF-κB (nuclear factor κB), and/or the activation of the transcription factor activator protein (AP)-1 by the MAPK (mitogen-activated protein kinase) signalling cascade (Figure 2-5) [31]. Both transcription factors can promote the expression of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β and mediate a pro-inflammatory response to the LPS challenge [5].

The TRIF-dependent pathway can induce a delayed activation of the NF-κB and the MAPK pathway via TRAF6 downstream signalling, and/or support the TRAF3-mediated activation and translocation of the transcription factor IRF (interferon regulatory factor)3 which in turn stimulates the gene expression of interferon (INF)γ and type I interferon-inducible genes (Figure 2-5). The IRF3-mediated pathway is essential for the clearance of microorganisms by host antiviral and antibacterial peptides [31].

2.4.2 The structure of lipid A determines the endotoxicity of the LPS molecule

As described above, the structure of the lipid A moiety influences the character of the subsequent interaction with the TLR4/MD-2 complex [22, 27]. Since LPS molecules with a hexa-acylated lipid A subunit induce a stronger pro-inflammatory immune response than do those with four acyl chains (Figure 2-6), it is assumed that

dimerization of TLR4/MD-2 is required before the activation of the TLR4 downstream signalling pathways can take place [22].

As most pathogenic Gram-negative bacteria including *Helicobacter pylori*, *Yersinia pestis*, *Francisella tularensis*, *Chlamydia trachomatis* and *Legionella pneumophila* express LPS molecules that contain tetra-acylated lipid A and induce a reduced TLR4-mediated pro-inflammatory immune response in comparison to that from hexa-acylated LPS produced by commensal organisms such as non-pathogenic *E. coli*, it may be that lipid A modification enables pathogens to evade the recognition by the TLR4/MD-2 complex and avoid the stimulation of the hosts pro-inflammatory immune system [2, 6, 9]. However, it remains to be seen why the host should evolve an immune response that activates a pro-inflammatory response to enteral commensal bacteria but not to pathogens.

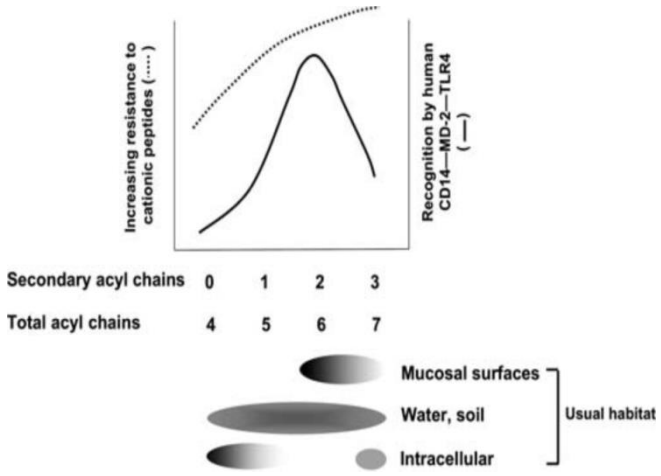

Figure 2-6. The relationship between the amount of secondary acyl chains, the resistance against host antibacterial peptides and the TLR4 recognition as well as the habitat of Gram-negative bacteria.

Hexa-acylated lipid A bearing four primary and two secondary acyl chains and is the most endotoxic form of lipid A as it induces the greatest production of the pro-inflammatory cytokine TNF-α. Gram-negative bacteria use hexa-acylated lipid A to form highly impermeable outer membrane that has a high resistance to host released antimicrobial peptides and is therefore well adapted to the condition within the lumen of the gut (Figure 1 from Munford and Varley [6]).

Evidence suggests that although tetra- and hexa-acylated LPS interact differently with MD-2 and TLR4 [22, 27] both can initiate TLR4-mediated signalling pathways. However, both types of LPS molecules can induce other feedback loops in host cells depending on their structural differences [19]. For example, both tetra- and hexa-

acylated LPS molecules activate the expression of cell surface activation markers in dendritic cells (DCs) in a similar manner when given at physiologic high doses (100 ng/ml) to either mice or humans. However, the outcomes of this activation differs in the two species; 30 min after the stimulation with tetra-acyl LPS there is stronger NF-κB translocation and activation of the TLR4-dependent signalling in mouse DCs and macrophages than occurs after stimulation with hexa-acyl LPS. The stronger activation of the NF-κB pathway by tetra-acylated LPS also leads to a greater up-regulation in gene expression of TNF-α and IL-12, for up to 4 hours post-stimulus. However, there is a concomitant increase in the post-transcriptional degradation of the two pro-inflammatory cytokines which results in a reduction in the release of TNF-α and IL-12. The latter process accounts for the weaker pro-inflammatory immune response induced by tetra-acylated LPS compared to that from hexa-acylated LPS [19] and not because tetra-acylated LPS has not been inefficiently detected by the immune system per se.

Not only does the acylation pattern of the lipid A moiety affect the activation of the TLR4 signalling but also the number of associated phosphate groups. Hexa-acylated lipid A moieties with two phosphate groups strongly activate both the MyD88-dependent and the TRIF-dependent pathways whereas hexa-acylated lipid A moieties with a single phosphate group strongly activate the TRIF-dependent pathway but activate the MyD88-dependent pathway only weakly. This pattern of activation, termed 'TRIF bias' is associated with less endotoxicity [31] as it ultimately induces the expression of antiviral and antibacterial peptides that are involved in the clearance of bacteria rather than the induction of inflammation.

Again the site at which LPS is phosphorylated influences the immune response. Hence, penta-acylated, 1-monophosphorylated LPS moieties from the enteral commensal

Bacteroides thetaiotaomicron, induce stronger NF-κB activation than does LPS from pathogenic *Porphyromonas gingivalis* which is phosphorylated at position 4' [34].

Hence, the structure of lipid A influences the balance in activation of the MyD88-dependent and TRIF-dependent pathways and the extent of the ultimate inflammatory response [22, 27]. All in all, LPS molecules bearing a lipid A subunit with four primary and two secondary acyl chains (hexa-acylated lipid A) along with two phosphate groups are highly endotoxic as they induce the greatest expression of the pro-inflammatory cytokine TNF-α. Less endotoxic LPS molecules that do not contain these moieties promote another immune response [19] and may compete with other more endotoxic forms at the sites that activate the immune response [2].

2.4.3 The effect of the total dose of LPS on TRL4-signalling pathways

Not only the structure of the lipid A moieties but also the size of the dose of LPS, dictate the levels of activation of the MyD88-dependent and TRIF-dependent signalling cascades (Table 2-1) [21].

The concentration of LPS extracted from *E. coli* that is necessary to induce a strong pro-inflammatory immune response i.e. production of TNF-α, *in-vitro* is around 10 ng/ml [35]. High physiological concentrations of LPS from *E. coli*, i.e.10 - 100 ng/ml, are known to induce both the MyD88- and the TRIF-dependent pathways and to robustly up-regulate TLR4-mediated production of IL-6 and TNF-α [36, 37] by macrophages [35, 38]. The subsequent expression of anti-inflammatory cytokines such as IL-10 and AP-20 together with other pro-tolerant mechanisms are thought to mediate a negative feedback loop which then down-regulates the initial pro-inflammatory immune response [36].

In contrast to the response to such high concentrations of LPS, challenge with 'subclinical' levels of *E. coli* LPS (< 10 ng/ml) induces a weak pro-inflammatory response that fails to activate the MyD88-dependent PI3K signalling cascade that is necessary to activate the anti-inflammatory feedback loops [21] and to induce the expression of the anti-inflammatory cytokine IL-10 in murine macrophages [39]. Instead in murine macrophages at least, low concentrations of LPS induce the translocation of the TLR4 complex into mitochondria where it up-regulates the production of mitochondrial reactive oxygen species (ROS) [21]. These ROS stabilize activating transcription factor (ATF)2 and reduce the pro-inflammatory immune response. The failure of low concentrations of LPS to induce this secondary phase of negative feedback is likely responsible for the continued expression of low levels of pro-inflammatory mediators [21]. At all events the activation of the alternative TLR4-dependent signalling cascade that results from challenge with low concentrations of endotoxic LPS induces a continuing low grade rather than the 'large-but-short' acute inflammatory response induced by high concentrations of LPS.

2.4.4 The immune response to intact Gram-negative bacteria

As noted hitherto, the endotoxic potential of LPS is determined by the lipid A that it contains. Since lipid A is incorporated into the outer layer of the outer cell membrane to anchor the LPS molecule in the membrane, it is not directly exposed to the environment [1]. It has therefore been assumed that only solubilised LPS and not LPS components in the outer membrane can act as endotoxins [1, 2]. Hence, the immunomodulatory effects of LPS have been generally evaluated using purified solubilised LPS [35-43]. However, more recent studies indicate that entire Gram-negative bacteria can also activate the hosts immune response [8, 44]. Again,

formalin-killed bacteria in which the LPS remains bound to the bacterial cell membrane have a similar immunomodulatory effect to that of soluble LPS [45, 46]. Hence, it seems that lipid A within the bacterial membrane can be recognised *in-situ* by the host immune system.

The magnitude of the immune response to LPS *in-situ* may differ from that to soluble LPS. Hence, recent data indicates that the stimulation of HT-29 intestinal epithelial cells with soluble LPS derived from *E. coli* induces less expression of genes related to pro-inflammatory immune response and to barrier function than does stimulation with live whole *E. coli* [47]. Thus it appears that the complex cross-talk between intestinal epithelial cells and live commensal bacteria may involve a number of bacterial components other than LPS. However, whether the greater expression of pro-inflammatory genes leads to up-regulation of the secretion of cytokines has not been investigated. Further, the proportion of increased effect on pro-inflammatory gene expression that results from lysis of the whole live or formalin killed bacteria within the cell medium has not been determined.

The relative importance of other bacterial species or their products has been demonstrated in a study in which Caco-2 cells were interposed between a layer of luminal bacteria and an underlying layer of DCs. In this model, the co-stimulation by *E. coli* (or its LPS) and intact live lactic acid producing bacteria prevented the production of TNF-α by DCs [48].

2.5 Variations in the response to LPS according to cell type

The response to LPS varies according to the type of cell that is exposed to it. Intestinal mucosal cells such as enterocytes are hyporesponsive to LPS in the lumen in that high concentrations do not promote the production of pro-inflammatory cytokines [49]. Nevertheless, the presence of LPS in the lumen can activate mechanisms that reduce the endotoxic potential of LPS [44] and minimize its penetration into gut associated lymphoid tissue (GALT) and the systemic circulation [50].

In contrast, high concentrations of LPS in the systemic circulation lead to hypotension, coagulopathy and organ failure, a potentially fatal condition termed septic shock. Septic shock results from a gross and unregulated pro-inflammatory immune activation in macrophages and vascular endothelial cells [9]. Somewhat lower concentrations of circulating LPS can induce moderate fever, stimulate the immune system and promote microbial killing. Subclinical levels of LPS in the circulation may be associated with prolonged mild or subclinical inflammation [21]. Hence, the pro-inflammatory or endotoxic potential of LPS is determined by the site at which LPS recognition takes place and its concentration. As discussed hitherto, the endotoxicity of LPS at a particular site also depends on the chemical structure of the LPS molecule [2]. Typical mediators of these endotoxin-induced pro-inflammatory reactions are tumor necrosis factor (TNF)-α and various interleukins (IL) such as IL-1β, IL-6 and IL-12 [38, 51-53].

2.5.1 Reactions of mucosal cells to luminal LPS

The intestine comprises the largest surface at which there is interaction between the host and the environment [7]. It is thought that LPS is continually released into the gut lumen from on-going lysis of contained Gram-negative commensal bacteria. Such

lysis is mediated by defensive secretions such as antimicrobial peptides and bile acids. Small amounts of LPS are also released during the process of bacterial division [51]. About 1 g of membrane-unbound LPS is contained within the lumen of intestine at any given time. The host has developed a finely tuned monitory and response system to modulate the population of normal commensal enteral microbiota as well as invading pathogens and their secretions e.g. LPS [44, 50].

2.5.1.1 The hyporesponsiveness of enterocytes on exposure to the luminal LPS

2.5.1.1.1 The pattern of expression of the TLR4/MD-2 complex by the intestinal epithelium

The induction of an immune response to LPS in intestinal epithelial cells (IECs) depends on their ability to recognise it. In general, the LPS receptor TLR4 is expressed at low levels by small intestinal and colonic enterocytes in healthy humans [25, 26]. The TLR4 receptor is also expressed by mature crypt cells [24] i.e. Paneth cells [54] but this expression is reduced or halted at least in the colon as epithelial cells mature and move toward the lumen [24]. The TLR4 co-adapter molecule MD-2 is not expressed in mature colonic crypt cells of healthy humans [25] nor in colonic HT-29 crypt cell lines [23]. The general hyporesponsiveness of mature IECs is likely based on the down-regulation of the TLR4/ MD-2 receptor complex [24]. The treatment of HT-29 cells with the T cell-derived pro-inflammatory cytokines IFN-γ and TNF-α, up-regulates the expression of TLR4 mRNA in these cells [23, 42]. Further, treatment with INF-γ is able to induce the expression of the MD-2 gene [23]. Subsequent stimulation of these cells with LPS then induces the secretion of the pro-inflammatory cytokine IL-8 [23, 42]. Together these findings suggest that the expression of TLR4 and MD-2 by mature IECs as well as the TLR4-mediated immune response is dynamically regulated in response to the surrounding cytokine profile.

This mechanism likely explains why patients with inflammatory bowel diseases (IBD) show an elevated expression of the TLR4/MD-2 complex [55]. However, whilst the expression of TLR4 is increased compared with that of normal subjects in the apical surface of enterocytes of subjects with Crohn's disease, its expression is relatively increased at the basolateral surface of granular cells of subjects with ulcerative colitis [26]. The increase in TLR4 expression at either of the two sites in patients with IBD in combination with an altered pattern of expression of other TLRs [26] may explain their enhanced responsiveness to bacteria and their products including LPS [55] generating a vicious circle that results in protracted inflammation of the intestine.

2.5.1.1.2 The development of tolerance to intestinal LPS

Stimulation of naïve human intestinal cell lines such as HT-29 cells with bacterial products including LPS, reduces the expression of TLR4 [56], indicating that, on first contact with bacterial LPS, TLR4 receptor production is down-regulated.

Whilst both primary adult and foetal murine enterocytes express the TLR4/MD-2 complex in their cytoplasm, adult IECs do not generate a pro-inflammatory immune response on exposure to LPS whereas foetal IECs do produce such a response. Hence, the age-dependent tolerance in IECs to LPS is not the result of a simple down-regulation of the production of the TLR4/MD-2 complex. It seems that exposure of murine neonatal IECs to LPS during vaginal delivery induces a TLR4-dependent NF-$_{K}$B activation followed by a decrease in levels of the protein IRAK-1 via a post-transcriptional mechanism. This reduction in the level of IRAK-1 is not seen in neonatal IECs taken from mice born by caesarean section [49]. Further, there is no alteration in the levels of IRAK-1 phosphorylation inhibitors such as Toll-interacting protein (Tollip) and sMyD88 in these cells. Since the down-regulation of IRAK-1 must

then inhibit any downstream signalling via the TLR4 receptor (see above) it seems that the postnatal down-regulation of IRAK-1 expression in IECs is solely responsible for the development of tolerance to luminal LPS by enterocytes [49].

In contrast to IECs obtained from adult murine intestine, intestinal cell lines which have not undergone post-natal priming with LPS, develop tolerance after subsequent stimulation with LPS through the overexpression of Tollip [57]. Tollip interacts with IRAK-1 and inhibits its phosphorylation by IRAK-4 subsequently supressing TLR4-mediated downstream signalling [58].

Hence a number of pathways can induce tolerance to LPS by intestinal epithelial cells depending on the timing of first contact with this agent. Such tolerance, induced either by reduced expression of the TLR4/MD-2 complex or by inhibition of TLR4 downstream signalling, may avoid an overstimulation of the host immune system by luminal LPS and facilitate the postnatal microbial colonization of the gut.

2.5.1.2 The innate immune response to colonizing luminal commensal bacteria

2.5.1.2.1 Dendritic cell responses to luminal LPS

Hyporesponsiveness of adult enterocytes to luminal LPS could inhibit antigen-presentation to T cells in the submucosa and maintain general tolerance to this material in the intestinal lumen [24-26]. However, luminal bacterial antigens such as LPS are also scavenged by Microfold (M)-cells that cover the luminal surfaces of Payer's patches (PP). These cells transport luminal antigens, including LPS, into the sub-epithelial dome where they are sampled by dendritic cells (DCs) or destroyed by macrophages [59]. However, macrophages in the GALT seem to be hyporesponsive to the immune challenge with LPS [49]. Hence, the immune response in the GALT to

LPS is likely to be mediated solely by DCs. This immune response can be boosted by a subclass of intestinal DCs that can extend their processes into the intestinal lumen, a process which allows them to collect antigenic material directly from the lumen [48].

Interestingly, in a two layer transwell model comprising Caco-2 cells and underlying immature DCs, only the direct application of Gram-negative bacteria such as *E. coli* (or its LPS) was able to induce the maturation of DCs and the subsequent production of low levels of TNF-α via TLR4. However, the DCs that lie beneath Caco-2 enterocytes had a different pattern of expression of surface activation markers and expression of cytokines compared to those that were in direct contact with Gram-negative bacteria [48]. Hence, it seems that molecules expressed by enterocytes influence the immune response of the underlying DCs to luminal bacteria.

Again, the co-stimulation with either *E. coli Nissle*, or its LPS, and lactic acid bacteria (LAB) prevented maturation of underlying TNF-α producing DCs in the same transwell model but this effect was observed only with live and not with killed LAB [48]. Hence, the cytokine profile in underlying DCs that are induced by luminal LPS, can be modified in the presence of other bacterial components.

2.5.1.2.2 The secretion of antimicrobial peptides by enterocytes and Paneth cells

Presentation of LPS to lymphocytes by DCs can lead to the maturation of Immunoglobulin (Ig) A-producing B cells [60]. The IgA secreted by these cells is subsequently taken up via the basal regions of enterocytes and released into the lumen. This IgA, termed secretory(s)IgA, combines with epitopes of bacteria within the lumen 'smothering' them without inducing a pro-inflammatory response [60]. Interestingly, Gram-negative *Bacteroides* species appear to be more efficient in activating such IgA secretion than are Gram-positive *Lactobacillus* species [61]. The lower IgA-

responsiveness to Lactobacillus species may allow these and other Gram-positive bacteria species of the genus *Clostridium* and *Enterococcus* to colonize the epithelial surfaces and the mucus layer, whilst colonisation by Gram-negative bacteria of the genus *Bacteroides* and the family of Enterobacteriaceae including *Escherichia* and *Salmonella* species is restricted to the intestinal lumen [7]. The differences in the species of commensal bacteria that inhabit the apical surfaces of enterocytes from those that exist solely within the lumen may also limit the exposure of M-cells and of subclasses of DCs, respectively, to certain types of LPS and consequently limit the recognition of certain types of Gram-negative bacteria or their LPS by intestinal cells.

Crypt cells such as Paneth cells which express TLR4 on their luminal surfaces [54] release antimicrobial molecules such as α-defensin and β-defensin into the lumen in response to stimulation of either of their luminal surfaces with LPS or of their basal surfaces with pro-inflammatory cytokines such as TNF-α and IL-1β [59]. Hence, they mediate the clearance of bacteria from the lumen rather than inducing a pro-inflammatory immune response.

2.5.1.2.3 Intestinal alkaline phosphatase and the reduction of the endotoxic potential of luminal LPS

The process of bacterial killing releases and solubilises a significant quantity of bacterial components including LPS into the lumen [62]. Enterocytes are thought to expresses intestinal alkaline phosphatase (IAP) in order to detoxify these components thus avoiding the establishment of florid pro-inflammatory reactions in the intestinal mucosal region [44, 62].

IAP is secreted by the brush border and is either membrane-bound [63] or contained within brush border membrane vesicles that are released into the lumen from the

microvilli of enterocytes [64]. Further, IAP may also be released into systemic circulation [65, 66]. Although IAP is predominantly secreted in the duodenum, in the mouse at least, it does not lose its activity as it moves distally through the small and the large intestine [67].

Treatment with enteropathogenic *E. coli* and *Shigella* induces the secretion of IAP in Caco-2_{BBE} cells [64]. Hence, the presence of luminal Gram-negative bacteria or their LPS seems to induce a signalling cascade that increases IAP activity and subsequently elevates the modification of luminal LPS [50]. The expression of IAP may be also associated with the release of sIgA and antimicrobial peptides. It has been proposed that IAP reduces the toxicity of LPS by dephosphorylation [44, 50, 62]. Hence, for the example the modification of LPS by calf-IAP (cIAP) renders it non-endotoxic to zebrafish [44]. IAP has a high affinity for LPS molecules bearing two phosphate groups but not for those with monophosphorylated lipid A moieties [68]. However, the enzymatic activity of IAP varies with substrate and pH [63, 69, 70]. Hence, it is currently unclear whether the dephosphorylation of various bacterial components that is observed *in-vitro*, occurs under physiological conditions. Nevertheless, dephosphorylation of LPS by IAP seems to be involved in the development of immune tolerance to gut-colonizing bacteria and LPS, as in zebrafish that lack IAP there is a much greater infiltration of the lamina propria by neutrophils than occurs in wild type zebrafish [44].

Interestingly, oral challenge of Iap-knockout (KO) mice that had been chronically exposed to LPS from commensal bacteria, with *Salmonella typhimurium* was able to induce an enteral tolerance of LPS but this did not occur in wild type mice. In contrast, the systemic immune response that occurred during salmonellosis was more intense in Iap-KO mice than in wild type mice [71].

Bates *et al* found that, in a germ-free zebrafish model, the expression of IAP was activated by MyD88-depedent signalling only in the presence of intact, live Gram-negative bacteria or after challenge with LPS [44]. Hence, the secretion of IAP and the subsequent reduction in the endotoxicity of LPS may be part of a TLR4-mediated delayed negative feedback loop [72] and may be a first line of defence preventing the penetration of endotoxic LPS into the GALT and the systemic circulation [50].

However, as mentioned hitherto, mature human enterocytes and Caco-2 cells only express very low levels of TLR4 receptors on their luminal surfaces and are generally hyporesponsive to the stimulation by luminal LPS [24-26, 42]. Thus it seems unlikely that IAP expression by human enterocytes *in-vivo* or in Caco-2 cells, is directly induced by the enterocyte TLR4-signalling cascade. It remains unclear how the expression of IAP is regulated in the human intestine.

Other studies have shown that IAP activity and the rates of detoxification of LPS are reduced in starved mice compared to those in fed mice [67] and that the consumption of fat appears to increase the secretion of IAP [66]. Hence, the expression of IAP could also be modulated by diet.

2.5.2 Interactions between circulating LPS and the hosts immune system

In spite of the high concentrations of LPS that exist within the intestinal lumen under normal physiological conditions, only small amounts are translocated into the systemic circulation [73]. However, LPS can also be released within immune cells during their killing of bacteria [1]. The systemic immune system responds to the presence of LPS in the systemic circulation with mechanisms that include a pro-inflammatory response, the modification of the pro-inflammatory properties of LPS and neutralisation.

2.5.2.1 Neutralisation of systemic LPS by LBP-mediated mechanisms

The site at which exposure to LPS occurs influences the manner in which LPS is cleared from that site. Within the vascular system LPS forms aggregates (micelles) that are sequestered by LPS-binding protein (LBP), an acute phase reactant produced by hepatocytes [74]. Subsequently, the LPS is transferred from LBP to mCD14 and sCD14 [28] and also to HDL and other serum lipoproteins e.g. very low density lipoprotein (VLDL) and chylomicrons [30, 75]. As described hitherto, the recognition of LPS via the TLR4/MD-2 complex in both immune and non-immune cells is mediated by CD14 [2]. The recognition process activates a broad range of host defence mechanisms. However, LPS that is bound to HDL or to other serum lipoproteins does not induce a systemic immune response and hence is said to be 'neutralised'.

It seems that the transport of LPS to CD14 is mediated mainly by LBP during the first 10 minutes after the LPS challenge whereas it is bound mainly to HDL after this [30]. When LPS is incorporated into HDL via LBP *in-vitro*, more than 50 % of the dose is neutralised within 40 min [30], a figure which corresponds with data obtained in an *in-vivo* rabbit model in which around 50 % of an intravenous LPS dose is removed from the plasma within 30 min [76]. The capability of HDL to 'neutralise' circulating LPS, and to reduce the secretion of pro-inflammatory cytokines depends on the total dose of LPS, the concentration of LBP in the serum and the relative proportions of serum lipoproteins such as HDL, VLDL and chylomicrons [30, 75, 77-80]. LPS-loaded lipoproteins are subsequently transported to the liver [76] where LPS molecules undergo modification. LPS molecules that are not rapidly neutralised and transported to the liver, may be modified by immune cells (see below). It is noteworthy that LBP production by the liver is increased by elevated circulating levels of IL-1β, IL-6 and TNF-α [74] after LPS challenge. Hence, serum LBP levels are increased by

feedback regulation to ensure adequate neutralisation of LPS. Thus, the levels of LBP in the serum are elevated in patients with sepsis compared with those of healthy control subjects [29, 81].

Whilst the system of neutralisation of LPS by HDL or other serum lipoproteins is important, it is noteworthy that more than 70 % of a dose of LPS injected into subcutaneous tissues is reported to be modified by immune cells at the putative site of infection before any of the monomers of LPS undergo transport via the lymphatic system to the liver [82].

2.5.2.2 Detoxification of LPS

On arrival in the liver, LPS is deacylated by the lipase acyloxyacyl hydrolase (AOAH) in hepatic Kupffer cells. AOAH mediates the deacylation of the two secondary acyl chains of the lipid A moiety and thus reduces the endotoxic potential of LPS molecules. However, detoxification of LPS by the liver is a relatively protracted process that takes several days [83] compared with the rapid, LBP-mediated, neutralisation of LPS in the circulation which takes only minutes to hours [30, 76].

Immune cells such as neutrophils, macrophages and DCs also express AOAH [82] and thus can reduce the endotoxic potential of LPS molecules that remain within the circulation [76] or in subcutaneous tissues [82]. This AOAH-mediated deacylation by immune cells can occur either extracellularly [84] or intracellularly following either the phagocytosis of the organism or the absorption of LPS [85]. It seems that the production of AOAH is independent of the TLR4-mediated pathway since TLR4-deficient macrophages can take up LPS and deacylate it intracellularly without inducing a pro-inflammatory immune response [86]. Nevertheless, the deacylation of LPS in TLR4 deficient mice occurs at lower rates than in wild type mice [82]. In

rabbits the intravenous injection of LPS augments plasma AOAH activity [84] indicating that LPS-induced signalling may enhance its production.

Regardless of the site at which it operates, AOAH is generally more effective in the deacylation of hexa-acylated lipid A than less acylated moieties [82]. Deacylation by AOAH appears to mitigate the immune response as AOAH-mediated modification of LPS molecules limits the production of IgM and IgG3 by B cells after LPS challenge [82, 87]. Hence, AOAH is an important host defence mechanism in reducing the endotoxic potential of LPS.

2.5.2.3 The development of temporary systemic LPS tolerance following exposure to high concentrations of endotoxic LPS

Whilst LBP-mediated neutralisation of circulating LPS molecules occurs relative rapidly and the detoxification of LPS by AOAH is an efficient process, both in the circulation and in tissues, both mechanisms do not prevent the development of an initial pro-inflammatory immune response [83, 88].

As discussed above, the dosage of LPS influences the pattern of immune response. A single high intravenous dose of highly endotoxic *E. coli* LPS (>10 ng/ml) induces a strong pro-inflammatory response with expression of TNF-α, IL-1β and IL-6 [38, 53, 89]. A subsequent similar dose induces a somewhat reduced pro-inflammatory immune response [35, 38, 40, 41, 90-93]. This phenomenon is termed systemic LPS or endotoxin tolerance. Systemic LPS tolerance is temporary and is associated with the action of elements of the innate immune system such as antigen presenting cells. Systemic tolerance to LPS during infection lasts from 5 to 10 days [88]. Whilst a single low dose of LPS (< 10 ng/ml) fails to mediate LPS tolerance [38, 39] a further

dose enhances production of the pro-inflammatory cytokines TNF-α and IL-6, an action termed LPS priming [35, 38].

2.5.2.3.1 The effect of LPS dose on the development of tolerance

The reason that different intravenous doses of LPS induce different mid-term immune responses is related to the nature of the primary immune response to the first challenge. As noted hitherto, initial high doses of LPS (> 10 ng/ml) induce the rapid TLR4-mediated expression of pro-inflammatory cytokines [52, 53, 89] as well as the production of the anti-inflammatory cytokine IL-10, which later then down-regulates the pro-inflammatory immune response [39]. Hence in humans, the immune response to high concentrations of LPS comprises an initial pro-inflammatory phase that takes up to 2 hours and a subsequent anti-inflammatory stage that lasts from 2 to 4 hours [37].

The pro-inflammatory cytokines that are produced during the initial phase of the high dose response influence the maturation of macrophages. LPS tolerant macrophages have the characteristic phenotype of IL-12^{low}, IL-23^{low} and IL-10^{high} that is typical of M1 macrophages. The pro-inflammatory immune response of these macrophages is suppressed after a subsequent dose of LPS [43].

In humans the initial high dose of LPS does not appear to influence the expression of the anti-inflammatory cytokine TGFβ but a subsequent dose leads to an increase in its expression whilst those of TNF-α, IL-6 and IL-10 are reduced [37]. Hence, it seems that IL-10 is necessary for the development of tolerance whilst TGFβ is required for its maintenance.

In contrast, the administration of low doses of endotoxic LPS (< 10 ng/ml) does not induce the expression of IL-10, presumably as it fails to induce the delayed anti-inflammatory feedback loop or to initiate the maturation of the LPS tolerant macrophages that are observed after high doses of LPS [39]. Rather, the maturation of non-LPS-tolerant M2 macrophages with the different cytokine phenotype of IL-12^{high}, IL-23^{high} and IL-10^{low} is supported [39]. These non-LPS-tolerant M2-macrophages demonstrate increased production of TNF-α after subsequent challenge with LPS [39]. Further in murine macrophages, low doses of endotoxic LPS seem to induce the translocation of TLR4 complexes from the cell membrane into the mitochondria where they enhance the production of reactive oxygen species (ROS). These ROS then stabilize the transcription factor ATF2 which supports a prolonged mild pro-inflammatory immune response [21].

In summary, systemic tolerance of LPS is induced by an IL-10-mediated negative feedback loop that commences after initial exposure to high levels of endotoxic LPS. The ambient concentration of LPS which determines whether pro-tolerant or pro-inflammatory macrophages mature, is assumed to be around 10 ng/ml [35, 43].

2.5.2.3.2 Ancillary mechanisms in the development of tolerance to LPS

A number of other mechanisms may contribute to the down-regulation of the TLR4 pathway in addition to the IL-10-mediated negative feedback loop. A reduction in the expression of the LPS receptor TLR4 on the surfaces of murine peritoneal macrophages was observed one hour after initial challenge with a high dose of LPS, and the responsiveness of these cells to subsequent LPS challenge was reduced in a pattern similar to that described hitherto [40]. However, the reduced expression of TLR4 on the surfaces of peritoneal macrophages was thought to result from the

internalisation of TLR4 complexes [94] as part of the TRIF-dependent pathway [5]. In contrast, no changes in the cell surface expression of TLR4 were seen in human monocytes following a similar high dose challenge with LPS [90]. Hence it seems likely that the genetic expression and secretion of TLR4 receptor is better able to keep pace with internalisation in human macrophages than in mouse macrophages.

As described above, the initial steps in the MyD88-dependent pathway that are involved in the response to LPS exposure, include the recruitment of IRAK-4 to MyD88 followed by the activation (phosphorylation) of IRKA-1 [32]. Initial challenge with LPS causes enhancement of activation of IRAK-1 within 5 to 10 min in human monocytes [90] and activates both NF-κB and MAPK pathways [31]. The phosphorylated IRAK-1 is immediately degraded [95] thus IRAK-1 activity returns to baseline 20 min after the LPS challenge [90] down-regulating the initial immune response.

In human monocytes, a subsequent dose of LPS does not activate IRAK-1 indicating the development of tolerance [90]. This inability to activate IRAK-1 may result from reduced recruitment of MyD88 to the TLR4 complex [90] as its smaller isoform (termed MyD88s) may compete with the larger isoform for binding with the TLR4 receptor. This spliced variant of MyD88 lacks the small intermediate domain which separates the C-terminal TIR-domain from the N-terminal death domain, so whilst MyD88s is able to link the TLR4 complex with IRAKs, it cannot then initiate the phosphorylation of IRAK-1 [96]. Hence, in isolated monocytes, the lack of the IRAKs recruitment to MyD88 [91] and the consequent inability to phosphorylate IRAK-1 [96] lead to both the inhibition of the MyD88-dependent signalling cascade and the expression of pro-inflammatory cytokines during tolerance phase [40, 90]. However, the role of MyD88s *in-vivo* is still unclear. Although the MyD88s that is activated by LPS is highly expressed *in-vitro* in THP-1 monocytes [96] and in leukocytes [36],

the levels are only slightly elevated in circulating monocytes obtained from healthy human subjects up to 2 hours after systemic challenge with LPS [36].

A further molecule that may be involved in the development of tolerance to LPS is SHIP (SH-2 containing inositol phosphatase), an inhibitor of the NF-κB signalling cascade. The expression of SHIP is increased via the MyD88-dependent activation of the PI3K pathway after an initial high dosage with LPS [41]. Since low concentrations of LPS fail to induce the negative feedback loop via this pathway [21] it is possible that the lack of SHIP-1 production is responsible for the failure to establish tolerance to endotoxin after exposure to low doses of LPS (ambient concentrations under 10 ng/ml). Further, the elevation in production of SHIP after the challenge of human subjects with high doses of LPS seems to be relevant to the development of LPS tolerance during the initial phase, since the production of SHIP protein is no longer evident 3 hours after stimulation with LPS [36]. However, the levels of SHIP production and the duration of LPS tolerance were correlated in mice after a challenge with high doses of LPS [41] indicating there may be species-specific variations in the mechanism by which tolerance to LPS is maintained.

2.5.2.3.3 The maintenance of tolerance to LPS in humans is mediated by IRAK-M

Since no IRAK-1 activity is detected in LPS tolerant monocytes [90] it seems that its activity may be modulated by other inhibitors of the response to challenge with LPS during the later phase or in maintenance of LPS tolerance [36].

IRAK-M is thought to be a negative regulator of the IRAK-1 activity as it interacts with IRAK-1 and subsequently prevents the phosphorylation of IRAK-1 by IRAK-4 [36]. Moreover, IRAK-M is exclusively expressed by LPS tolerant monocytes/ macrophages [36, 43]. Since IRAK-M production in humans is induced by high

ambient concentrations of TNF-α and the duration of the period of tolerance correlates with the level of IRAK-M *in-vivo* it seems likely that IRAK-M participates in the down-regulation of the initial pro-inflammatory phase and in the maintenance of pro-tolerant phase, as long as pro-inflammatory mediators are present initially to support the production of IRAK-M [36].

2.5.2.3.4 Other mechanisms involved in the maintenance of tolerance to LPS

An increase in the concentration of circulating LBP in septic mice prevents these animals from mounting a further pro-inflammatory immune response to a subsequent lethal dose of LPS [77]. The fact that AOAH-mediated detoxification of these high levels of circulating LPS may also be involved in the regulation of LPS tolerance is indicated by the finding that AOAH-deficient mice develop prolonged tolerance to a second dose of LPS that lasts for up to 2 months compared to that in wild type mice which remain tolerant to LPS only for 5 to 10 days [88]. Further, the establishment of prolonged tolerance of LPS in AOAH-deficient mice is associated with an increase in their mortality after a second challenge with a pathogen [88]. Together these observations suggest that neutralisation and deacylation of circulating LPS are critical to the appropriate regulation of tolerance to LPS. Hence, fine tuning of the interplay between these two processes and the pattern of immune response appear critical in the response to any LPS that is released during acute infection with Gram-negative bacteria.

2.5.2.3.5 The development of cross-tolerance

Dosage with pure LPS, to ambient levels over 10 ng/ml, can induce tolerance to other bacterial components that were not included in the material used for the challenge. This phenomenon is termed cross-tolerance. Hence, LPS tolerant THP-1 cells develop a cross-tolerance to lipoteichoic acid (LTA) [92] and to macrophage-activating lipoprotein (MALP)-2 [93], a product of Gram-positive bacteria. Both of these agents are ligands of the TLR2 receptor. Conversely, challenge with LTA is not able to induce a tolerance to LPS in THP-1 cells [92]. However, MALP-2 is able to induce tolerance to LPS in murine peritoneal macrophages and in THP-1 cells via reduced activation of IRAK-1 by the MyD88-dependent pathway [93, 97]. A similar effect occurs in murine macrophages with the ligands of TLR5, TLR7 and TLR9, whilst double stranded RNA, the ligand of TLR3, induces tolerance to LPS via inhibition of the MyD88-independent pathway [93, 98].

2.5.2.3.6 The benefits of tolerance to LPS

The increased production of TNF-α following an initial challenge with high doses of LPS induces the migration of polymorphonuclear neutrophils (PMN) to the site of challenge. It seems that in mice, bacterial clearance by PMN is more efficient during the phase of tolerance to LPS [99]. The phagocytic activity of human macrophages is also strongly increased during the phase of tolerance to LPS whereas their antigen presenting capability is reduced [43]. This suggests that the innate antimicrobial activity of these cells is increased, whilst their ability to mediate an adaptive immune response is inhibited. Hence, it is assumed that tolerance to LPS and cross-tolerance to other bacterial components are beneficial for the host as they prevent undue pro-inflammatory immune reaction and limit the tissue damage during infection [88].

Nevertheless, the duration of tolerance to systemic LPS is crucial for the "long-term" regulation of immune response as septic patients have an increased chance of mortality to a second challenge during this period [88].

2.5.2.4 The immune response to high concentrations of less endotoxic forms of LPS

As discussed above, the pattern of the host's immune response to LPS is altered, not only by its concentration, but also by its molecular structure. So far we have discussed the immune response to highly endotoxic LPS. Given that several mechanisms have developed to reduce the toxicity of LPS to avoid the overproduction of pro-inflammatory mediators in response to highly endotoxic LPS [88] it follows that the immune response to high levels of less endotoxic (modified) LPS differs from that of challenges with highly endotoxic LPS (Table 2-1).

2.5.2.4.1 The activation of macrophages depends on the structure of LPS

The maturation of murine peritoneal macrophages in response to challenge with LPS is mediated predominantly by TRIF-dependent pathways [52]. However, the less endotoxic monophosphoryl lipid A (MPL) from *E. coli* induces macrophages to produce TNF-α but not active IL-1β [53]. Given that the release of active IL-1β is concomitant with the onset of endotoxic shock [100] the lack of production of IL-1β may reflect the reduction in the endotoxicity of the LPS.

The release of active IL-1β from macrophages is regulated by two separate steps, the expression of an inactive precursor of IL-1β and the subsequent activation of this precursor. Hence, both pathways must be activated to induce the release of IL-1β [101]. Although MPL is able to induce the expression of IL-1β mRNA and its

translation into the IL-1β precursor, it fails to induce the maturation of the IL-1β precursor into its active form as it does not activate the caspase1 [53] that is necessary for the formation of an inflammasome. This last step is a prerequisite to the activation and secretion of active IL-1β [101].

In spite of the reduction in the magnitude of the pro-inflammatory response to less endotoxic LPS, the presence of suitably primed mature macrophages may permit a more prompt response to any further challenge with more endotoxic forms of LPS. All this underscores the importance of luminal dephosphorylation of LPS by IAP and production of monophosphorylated LPS in preventing an unduly robust inflammatory response.

2.5.2.4.2 The effect of LPS structure on dendritic cell function

In-vitro studies with bone-marrow derived dendritic cells (DCs) and *in-vivo* studies on splenic DCs indicate that neither MyD88- nor TRIF-dependent signalling is necessary for their maturation, but that maximal release of MyD88-dependent cytokines such as TNF-α, IL-6 and IL12p40 as well as TRIF-dependent cytokine IFN-γ occur on synergistic stimulation of both pathways. In contrast, IL-10 production by DCs is more influenced by MyD88-dependent signalling than by TRIF-dependent signalling [52]. These findings indicate that whilst maturation of DCs can be activated by structurally different LPS molecules, the TRIF bias that is observed after the exposure to the less endotoxic dephosphorylated LPS will induce only a weak production of pro-inflammatory TNF-α, IL-6 and IL12p40 and production of anti-inflammatory IL-10. However, this TRIF bias is associated with a relative increase in the production of antibacterial peptides [31] which improve bacterial clearance process. This constitutes

yet another example of how modification of the LPS structure by IAP may influence the outcome of the subsequent immune response.

2.5.2.4.3 The effect of LPS structure on regulation of the adaptive immune response

DCs have the capacity to regulate the adaptive immune response. It seems that the MyD88-mediated pathway for maturation and production of cytokines in DCs is more critical for DC-mediated presentation of antigen to T cells than is the TRIF-dependent pathway. However, the concerted activity of both signalling pathways achieves a more efficient proliferation of T cells and production of IL-12 by the T cells. Hence, the simultaneous activation of the MyD88- and TRIF-dependent pathways that occurs after challenge with endotoxic LPS, influences both DC and T cell function [52]. Conversely, TRIF bias that is mediated by the vaccine adjuvant MPL® (monophosphoryl lipid A) includes a reduction in both antigen-presentation function by DCs and production of pro-inflammatory cytokines by T cells [102]. Whilst MPL®, like endotoxic LPS, induces the differentiation of T cells into IFN-γ producing T_{h1} cells, it does not up-regulate the production of acute-phase protein SAA by the liver. Further, MPL® induces clonal expansion of CD4+ T cells but not the long-term immune response by CD4+ T cells that occurs with endotoxic LPS. The authors of the study suggest that the long-term survival of CD4+ T cells may depend on the level of expression of MyD88-dependent pro-inflammatory cytokines such as TNF-α and IL-6 [102].

Whilst both human and murine DCs mediate the maturation of naïve T cells into T_{h1} cells and also activate $CD8^+$ T cells when stimulated with tetra- and hexa-acylated LPS, stimulation with tetra-acylated LPS also induces differentiation of T_{reg} cells in humans but not in mice. This suggests that there are species-specific variations in the

immune response to tetra-acylated LPS [19]. Further, in humans, that AOAH-mediated deacylation favours the activation of a pro-tolerant immune response.

In sum, it is evident that the acylation and phosphorylation patterns of lipid A in LPS affect the response of both innate and adaptive immune cells and that their deacylation/dephosphorylation can influence the subsequent immune response.

Table 2-1. Overview of the immune response to LPS depending on its structure and dose

	initial immune response	subsequent effect	tolerance to LPS
endotoxic LPS			
high dose	- robust production of pro-inflammatory IL-6, TNF-α and IL-1β - enhanced production of IRAK-1 ➔ *strong initial pro-inflammatory immune response*	- delayed production of anti-inflammatory IL-10 - immediate degradation of IRAK-1 - expression of inhibitors of the MyD88-dependent signalling cascade i.e. MyD88s, SHIP-1, IRAK-M ➔ *down-regulation of the initial pro-inflammatory immune response* - maturation of tolerogenic macrophages ➔ *improved bacterial clearance* - increased levels of serum LBP ➔ *enhanced neutralisation of circulating LPS*	YES
low dose	- weak production of TNF-α - production of ROS	- fails to induce production of anti-inflammatory IL-10 - fails to induce the expression of inhibitors of the MyD88-dependent pathway ➔ *prolonged mild pro-inflammatory immune response* - maturation of non-tolerogenic macrophages ➔ *stronger pro-inflammatory immune response to the second LPS challenge*	NO
less endotoxic LPS			
high dose	- weaker production of TNF-α and IL-12 <u>TRIF bias induced by monoacylated LPS :</u> - fails to express active IL-1β ➔ *reduced pro-inflammatory immune response* - increased release of anti-bacterial peptides ➔ *enhanced bacterial clearance*	<u>TRIF bias induced by monoacylated LPS :</u> - reduced antigen presenting function of DCs - reduced production of pro-inflammatory cytokines by T cells ➔ *more a pro-tolerant than pro-inflammatory immune response*	NO

2.5.3 The limitations of in-vitro models

The LPS-induced production of ROS by murine macrophages seems to be TLR4-dependent [21] whereas similar ROS production by human innate immune cells seems to be TLR4-independent [103]. Again, the murine TLR4/MD-2 complex interacts differently with tetra-acylated LPS than does the human recognition complex and leads to two different immune outcomes [19, 22]. Hence, there are significant species-specific variations in the immune response to LPS.

A number of *in-vitro* models do not incorporate all of the responses to challenge with LPS of which the host is capable. Hence for example, the ambient levels of circulating HDL *in-vivo* are often not considered *in-vitro*. Thus, in human subjects low levels of circulating HDL are associated with a pro-inflammatory and oxidative bias [104] as the corresponding reduction in LBP-mediated neutralisation of circulating LPS and prolongation of its half-life lead to an elevated pro-inflammatory immune response.

As discussed hitherto, IAP is secreted into the lumen to dephosphorylate highly endotoxic LPS molecules with two phosphate groups into less endotoxic LPS molecules [44, 50, 62] before they enter the circulation and AOAH is secreted by liver cells and immune cells to deacylate circulating endotoxic hexa-acylated LPS molecules [82-85]. Hence, secretions by the host are used to modify the structure of the lipid A moiety of LPS molecules to reduce their endotoxic potential [7]. The total concentration of LPS in the circulation to some extent reflects the permeability of the gut. Given that there is detoxification of LPS both prior to and after entry into the circulation it is likely that the ratio of unmodified to modified LPS within the circulation will reflect the effectiveness of host in reducing the endotoxic potential of LPS molecules at a given level of permeability. In which case, *in-vitro* models may not

adequately indicate the effectiveness of the process of detoxification and subsequent immune response *in-vivo* at different levels of gut permeability.

As we have seen the ratios between the more and the less endotoxic LPS molecules may influence the outcome of the immune response. The ratio may also be influenced by the ratio of the various types of normal commensal microbiota within the lumen. Hence, under normal conditions, whilst *E. coli* is the predominant Gram-negative aerobic commensal species, Gram-negative bacteria of the phyla Bacteroidetes such as *B. fragilis* are generally more numerous [7]. The latter produces greater quantities of less endotoxic penta-acylated LPS molecules with one phosphate group [17] and also expresses polysaccharide A (PSA) which together reduce the consequent pro-inflammatory immune response [14]. Hence, experiments which determine the character and magnitude of the immune response to pure LPS from *E. coli* do not describe those obtained with mixtures of types of LPS obtained from the normal mixture of species of commensal bacteria.

2.6 Consequences of the LPS-induced immune response

The gut colonizing bacteria live in a symbiotic relation with their host. On-going interactions i.e. cross-talk between the host and commensal bacteria (and/or their chemical components) are essential for the development, maintenance and balance of the intestinal and systemic immune systems [7]. Hence, the GALT has to fulfil a critical task. On one hand the GALT must tolerate the luminal microbiota and avoid excessive pro-inflammatory stimulation, on the other hand it must regulate the composition and the number of commensal bacteria to prevent overgrowth and to protect the host from inappropriate translocation of commensal and pathogenic bacteria or their products into the circulation [7]. Again, the hosts immune system must react rapidly and efficiently to counteract toxicity or infection. Hence, a fine tuning of the cross-talk between commensal bacteria and the host intestinal and systemic immune systems is required.

2.6.1 Is the acute pro-inflammatory immune response to systemic endotoxic LPS from luminal commensals used as gatekeeper?

The fact that most pathogens express less endotoxic tetra-acylated LPS molecules may indicate that modification of lipid A by pathogens like *Yersinia pestis* and *Francisella tularensis* is a survival strategy [16]. As discussed in this chapter, LBP-mediated neutralisation of less endotoxic LPS molecules occurs at a much slower rate than does the neutralisation of endotoxic LPS [76]. This slower neutralisation may enable suitably adapted pathogens to survive and multiply in the host.

These findings invite the question as to why the host has developed mechanisms that are able to clear and to modify LPS from harmless commensal bacteria rather than mechanisms that provoke a pro-inflammatory immune response to pathogens? Whilst

the response to LPS is mediated by innate immune system, the response to pathogens that secrete specific toxins that institute necrosis is largely within the domain of the adaptive immunity as such complex and diverse molecules that are produced by them do not trigger an innate immune system. The innate response to LPS and other markers is mediated by archaic heritable immune receptors that can detect and respond to certain generic molecular signals that indicate the presence of microbiota. Whilst such signals may have originally been entirely defensive, subsequent co-evolution has allowed a degree of symbiosis to become established. Hence, the innate system appears to have evolved mechanism to ameliorate the pro-inflammatory response to provide a degree of tolerance to LPS derived from commensals within the gut lumen in order to reap the benefits of symbiosis with normal microbiota.

In spite of this specialisation in GALT, translocation of LPS from gut lumen into the systemic circulation promotes an innate immune response whose magnitude depends on the degree of exposure and type of antigen. Since, under normal physiological conditions only small quantities of LPS can enter the circulation and the levels of circulating residues of the endotoxic *E. coli* and its LPS are reported to be promptly and significantly elevated during the early phase of an infection [7], it is conceivable that such an increase can still provide indication of degree of infection or dysbiosis.

As discussed in this chapter, different types of Gram-negative bacteria produce different types of LPS with varying degrees of endotoxicity [19] and the host detoxifies them at different rates [44, 50, 62, 82-85]. Hence, the balance between the concentrations of the various types of LPS may reflect the ability of the host to detoxify/neutralise highly endotoxic types and ultimately determines the characteristic of the immune response.

Whilst extremely high levels of endotoxic LPS in the systemic circulation may produce an overwhelming pro-inflammatory immune response that can be fatal [9], somewhat lower levels of circulating endotoxic LPS promote a temporary systemic tolerance to LPS. The latter is associated with an increase in the clearance of bacteria by innate immune cells from the circulation [43, 99] and may limit tissue damage during infection [88]. Hence, even with exposure to high systemic levels of LPS there appear to be mechanisms that ameliorate immune response to anything but an overwhelming dose of LPS.

2.6.2 Do chronically low levels of LPS represent a long term health risk?

It is known that acute high concentrations of LPS in the circulation represent a short term health risk as it can induce septic shock [9] but little is known about the long term health risk of a persistent exposure to low concentrations of LPS in the systemic circulation.

2.6.2.1 The association of circulation levels of endotoxin with the development of metabolic diseases

During acute experimental endotoxemia in human subjects, intravenous injection of 3 ng/kg LPS caused the level of TNF-α and IL-6 in plasma to increase along with the gene expression of the two cytokines in adipose tissues. The elevation of systemic LPS during acute infections leads to a transient increase in systemic insulin resistance and a consequent inhibition of the insulin signalling pathway in adipose tissue but does not influence the pancreatic β-cell function [105].

Although the continued presence in the circulating system of low concentrations of endotoxic LPS does not immediately lead to the onset of clinical symptoms, it does induce a prolonged low grade inflammation. Hence, chronic low grade elevation of systemic LPS in a mouse model, also referred to as metabolic endotoxemia, is correlated with an increase in subclinical inflammation, the onset of obesity and insulin resistance [10]. Metabolic endotoxemia has also been correlated with the onset of diabetes in humans [12, 106]. However, the method used for the preparation of plasma or serum samples in those studies is questionable (discussed in detail in Chapter 12).

A number of conditions associated with disorders in the absorption and disposition of LPS may contribute to the susceptibility to LPS-mediated metabolic disorders. Hence, IAP-deficient mice have chronically increased gut permeability and elevated levels of systemic endotoxin compared to their wild type counterparts. These mice also exhibit glucose intolerance, insulin resistance and obesity and develop type 2 diabetes following a high fat diet [107].

2.6.2.2 The impact of nutritional factors on the levels of circulating endotoxin

Diet seems a particularly potent factor in promoting conditions that are associated with chronic subclinical elevations of LPS. Hence, the feeding of a high fat (HF) diet to wild type mice also leads to metabolic endotoxemia, increased gut permeability and the development of metabolic syndrome including glucose tolerance, insulin resistance, obesity, dyslipidaemia and fatty liver. Again, when mice that were maintained on a fat diet were given oral supplements with IAP, they did not develop these conditions. Moreover, the same work showed that oral supplementation of IAP reversed established metabolic endotoxemia induced by a HF diet as well as glucose intolerance and insulin resistance [107]. Moreover, host-mediated modification of lipid A within

the lumen of the gut has been shown to help maintain intestinal barrier function and limit the translocation of LPS from the gut lumen into the systemic circulation [107]. Hence, a diet high in fat may compromise the efficient detoxification of LPS.

In healthy humans neither the ingestion of 300 ml of water nor pure orange juice or a 300 kcal solution containing 75 g of glucose (Glucola) caused the levels of circulating endotoxin to rise [108]. Conversely, the ingestion of either a 900 kcal meal containing around 50 g of fat (which comprised around 50 % of the calories), or of a drink of plain cream (33 g), lead to a post-prandial rise in the levels of circulating endotoxin [73, 108-111]. However, no post-prandial rise in the level of circulating endotoxin was observed when a similar 900 kcal meal was ingested in combination with either a drink of orange juice, or of a muscadine grape extract containing a mixture of resveratrol (100 mg) and other polyphenols to a total of 75 mg [110, 111]. Another study showed that the levels of serum endotoxin are elevated after the consumption of a single meal where fat comprised 94 % of the calories, in healthy non-obese subject, in obese subjects and in subjects with either impaired glucose tolerance or with type 2 diabetes [112]. There were no significant elevations in levels of plasma endotoxin after the consumption of a 900 kcal meal in which the fat content had been reduced to around 27 g (equal to around 27 % of calories from fat) [109]. Other work with animals also suggests that plasma endotoxin levels depend on the amount of fat in the diet. Feeding mice a diet containing 72 % fat caused plasma endotoxin levels to increase 2.7 fold whereas a diet containing 40 % fat caused a 1.4 fold increase in endotoxin compared to those mice fed with a control diet [10]. Further, dietary oligofructose supplements have also been shown to prevent an inflammatory reaction and elevation of plasma and adipose tissue endotoxin concentrations in mice fed with a HF diet [113, 114]. These findings suggest that either co-consumption of other dietary items or a reduction in the relative proportions of fat in the meal may ameliorate any post-prandial rises in

systemic endotoxin. Hence, it seems conceivable that a fatty diet promotes the endotoxin translocation which in turn leads to endotoxin-induced post-prandial inflammatory events. In addition, that a fatty diet may be the link between persistent translocation of gut-derived LPS into the systemic circulation and the development of diet-associated metabolic disorders.

2.7 The permeability of the gut to luminal LPS

In order to assess the role of dietary-associated metabolic endotoxemia as a long term health risk factor, it is important to understand how LPS can be translocated from the gut lumen into the systemic circulation during the absorption of fat. The gastrointestinal epithelium forms a physical barrier to prevent the uncontrolled flux of nutrients and of commensal or pathogenic bacteria and their products into the systemic circulation. The permeability of the gut to luminal molecules is the sum of absorption by transcellular mechanisms through the intestinal epithelial cells and the charge and size selective uptake via the paracellular route along the intercellular space.

2.7.1 The paracellular permeability of the gut to luminal LPS

In health, the paracellular permeability of the gut is limited to selective ions and molecules with a molecular radius from less than 4.0 Å. In contrast, a leaky gut leads to an increased paracellular uptake of macromolecules, bacteria and bacterial products [115].

2.7.1.1 The formation of the intercellular space by tight junctions

The intercellular space is formed by adjacent epithelial cells that are linked with each other through apical located adherence junctions (AJs) and basal located desmosomes. However, the passive paracellular diffusion of molecules and microorganism is mediated by the permeability of tight junctions (TJs). TJs are dynamic multiprotein complexes that are located above AJs (Figure 2-7) [115]. TJs are either barrier-forming through the connection of adjacent cells with each other, or channel-forming through the building of paracellular channels between adjacent cells at the apical site along the intercellular space [115, 116]. TJ proteins of the occludin/tricellulin family

and of the junction adhesions molecule (JAM) family are involved in the signalling and regulation of TJ permeability, whereas members of the claudin family forms a selective diffusion barrier [115]. The composition of TJs and their interaction with cytosolic plaque proteins such as zonula occludens (ZO), and the actin cytoskeleton is a dynamic process that is mediated by extracellular and intracellular signals i.e. pro-inflammatory cytokines, resulting in a selectively and dynamic TJ permeability or paracellular permeability, respectively [115, 116]. Further the permeability of TJ varies along the gastrointestinal tract and between health and disease leading to intestinal surface of different tightness [116].

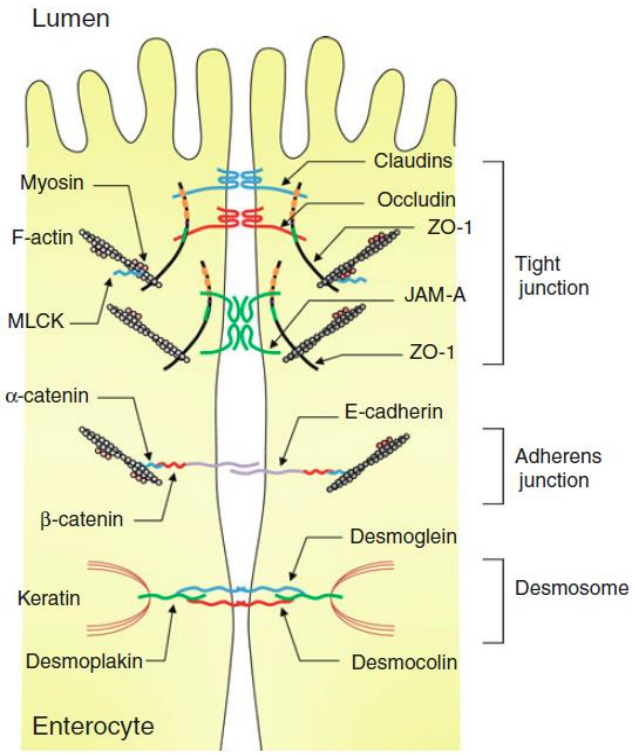

Figure 2-7. Schematic overview of connections between adjacent cells.

Tight junctions (TJ) form barrier- and pore-forming TJ strands to regulate the paracellular flux of ions, molecules, water and bacterial products. Adherence junction and desmosomes form strong adhesive bonds between adjacent epithelial cells but are not involved in the regulation of paracellular permeability (Figure 2 from Suzuki [4]).

2.7.1.2 The regulation of paracellular permeability by cytokines

Data from various intestinal cell lines and from mouse studies indicate that cytokines can influence the distribution of TJ proteins, and the organisation of both the TJ meshwork and the cytoskeleton. For instance, TNF-α increased the paracellular flux of macromolecules from the luminal to basolateral site [117, 118] and facilitated the

transport of FITC-labelled 4kDA dextran from the basolateral to the luminal site [119]. It seems that such changes in TJ permeability were due to the TNF-α induced activation of the myosin light chain kinase (MLCK) which is involved in the contraction of the actin cytoskeleton and increases the paracellular flux of macromolecules [118]. Similar, IL-1β activated the MLCK which in turn increased the TJ permeability and subsequently led to the loss of barrier function in Caco-2 cells in combination with elevated paracellular flux of inulin [120]. Further, TNF-α was shown to reduce the number of phosphorylated occludin which correlated with decreased barrier function [117], whilst IL-1β was shown to down regulate the production of occludin and also caused the irregular arrangement of occludin [121]. An increased paracellular flux of ions and larger molecules can also be mediated by the pro-inflammatory cytokine INF-γ. The latter induced the internalization of claudin 1 [119, 122], occludin [122, 123] and JAM-A from the TJ strand into cytosolic compartments which was followed by the storage of these TJ proteins in early/recycling endosomal compartments. IFN-γ also reduced the number of ZO-1-colocalised occludin [123]. Hence, the loss of TJ integrity during inflammation can lead to an enhanced passive paracellular flux of molecules and bacterial products from the lumen into the basolateral site and vice versa.

In contrast, the anti-inflammatory cytokine IL-10 was able to prevent the IFN-γ-induced increase in paracellular flux of macromolecules [124]. Furthermore, IL-10 knock-out mice developed spontaneous colitis with increased intestinal permeability and elevated levels of TNF-α, IFN-γ and IL-6 [4] indicating that a balance between pro- and anti-inflammatory immune responses is crucial in the regulation and maintenance of TJ assembly.

2.7.1.3 The hypothesis of the translocation of LPS via the paracellular route during the absorption of dietary fat

Dietary fat plays a crucial role in the diet-mediated intestinal immune response i.e. FAs can generate directly a pro- and anti-inflammatory immune response by a broad range of intestinal immune cells [125]. It is noteworthy that the function of dietary fat and its immunomodulatory effect are dependent on the composition of FAs and the total amount of dietary fat consumed [125]. For instance, butter, rich in saturated long chain fatty acids (LCFAs) such as stearic and palmitic acid, enhanced the adhesion of lymphocytes to microvessels of the small intestinal mucosa and mediated the release of TNF-α by macrophages in the mouse lamina propria [125]. Whilst olive oil, rich in monounsaturated LCFAs like oleic acid, elevated the adhesion of lymphocytes but did not induce the expression of TNF-α by macrophages [125]. In contrast, polyunsaturated LCFAs of the omega-3 family i.e. eicosapentaenoic acid and docosahexaenoic acid, induced anti-inflammatory immune responses [126-128].

Under normal physiological conditions, single LPS molecules or LPS aggregates (micelles) are too big to pass through the intestinal mucosa along the intercellular space. However, a western HF meal/diet, which is in rich in saturated LCFAs, can induce oxidative stress and inflammation and subsequently can reduce the TJ integrity. Hence, HF diets may allow the greater absorption of LPS trough the paracellular route. Arguments for this hypothesis include data showing that the consumption of a HF meal led to increased oxidative stress and increased expression of pro-inflammatory cytokines such as TNF-α and IL-1β, which both correlated with elevated post-prandial levels of systemic endotoxin [108, 109]. In contrast, the intake of an orange juice together with a HF meal prevented the HF-meal-induced oxidative stress and pro-inflammatory cytokine expression as well as the elevation of systemic endotoxin [111]. Such an effect was also shown in humans following the consumption

of an HF meal in combination with a resveratrol and phenol supplement [110]. However, given the fact that both TNF-α and IL-1β are also released in response to LPS it is unclear whether the increase in pro-inflammatory cytokines was actually due to dietary fat or due to LPS that entered the systemic circulation together with dietary fat via the transcellular route (see below).

Further, the accumulation of large numbers of chylomicrons along the basal site of enterocytes after the absorption of a high amount of fat is thought to disrupt the integrity of TJ and to lead to post-prandial injuries of the intestinal epithelium [129]. This would subsequently increase the paracellular permeability of the gut and may enable an augmented paracellular flux of bacteria and/ or their products i.e. LPS, into the lamina propria [130] at a later time point during the absorption of dietary fat.

It is noteworthy, that the presence of high concentrations of LPS in the gut lumen seems not to be able to mediate alteration of gut permeability which goes hand in hand with the observation that the TLR4 was not expressed at the apical site of enterocytes. However, the exposure to LPS at the basolateral site of Caco-2 cells reduced the barrier function and increased the flux of inulin. It seems that this observation was due to changes in the integrity of TJ proteins and elevated paracellular permeability [131]. Taken together, the paracellular translocation of LPS following the ingestion of a fatty meal may slowly increase the basolateral concentrations of LPS to a level at which LPS can mediate further alterations in the intestinal permeability. Hence, the fat-induced epithelial injuries in combination with the paracellular influx of LPS may lead to a prolonged reduced intestinal barrier function which may in turn allow the paracellular absorption of even greater amounts of LPS.

2.7.2 Transcellular mechanisms involved in the translocation of luminal LPS during the process of fat absorption

Since LPS molecules have a lipophilic part it might be possible that luminal LPS, free or incorporated into micelles, is absorbed together with dietary fat by enterocytes via transcellular mechanisms.

2.7.2.1 The digestion and absorption of dietary fat

Dietary fat in form of triglycerides (TGs) is emulsified in the stomach leading to the formation of large micelles that contain TG and phospholipids. These large micelles are split into smaller micelles by bile acid in the duodenum. Pancreatic lipase and co-lipase then split fatty acids (FAs) from TG at position 1 and 3 resulting in monoglycerides (MGs) and free FAs. MGs and free FAs then form smaller micelles that can be absorbed by enterocytes via transcellular mechanisms. Absorbed MGs and free FAs are subsequently reassembled to TGs in the cytoplasm of enterocytes. The reassembled TGs then form chylomicrons that are excreted by enterocytes at the basolateral site. Chylomicrons then travel via the lymphatic system to the systemic circulation [132].

2.7.2.2 The hypothesis of the translocation of LPS from the gut lumen into the systemic circulation via the transcellular route

LPS molecules that are either free or incorporated into LPS aggregates or dietary fat micelles, are thought to be absorbed into the cytoplasm of enterocytes in association with MGs and free FAs. In this scenario LPS is subsequently incorporated into chylomicrons in the cytoplasm of enterocytes followed by the release of LPS-containing chylomicrons into the basolateral site of enterocytes (Figure 2-8). The

LPS-loaded chylomicrons then get into the systemic circulation via the lymphatic system [133-135] and reach organs and tissues during the lipid metabolism of chylomicrons. It seems likely that gut-derived LPS incorporated into chylomicrons can avoid both the recognition by TLR4 receptor in the intestinal epithelium and clearance by the liver. However, chylomicron-bound LPS may be subsequently recognised by systemic immune cells and non-immune cells such as adipocytes where it may induce a local pro-inflammatory immune response. Hence, the digestion of dietary fat may act as a Trojan horse for LPS.

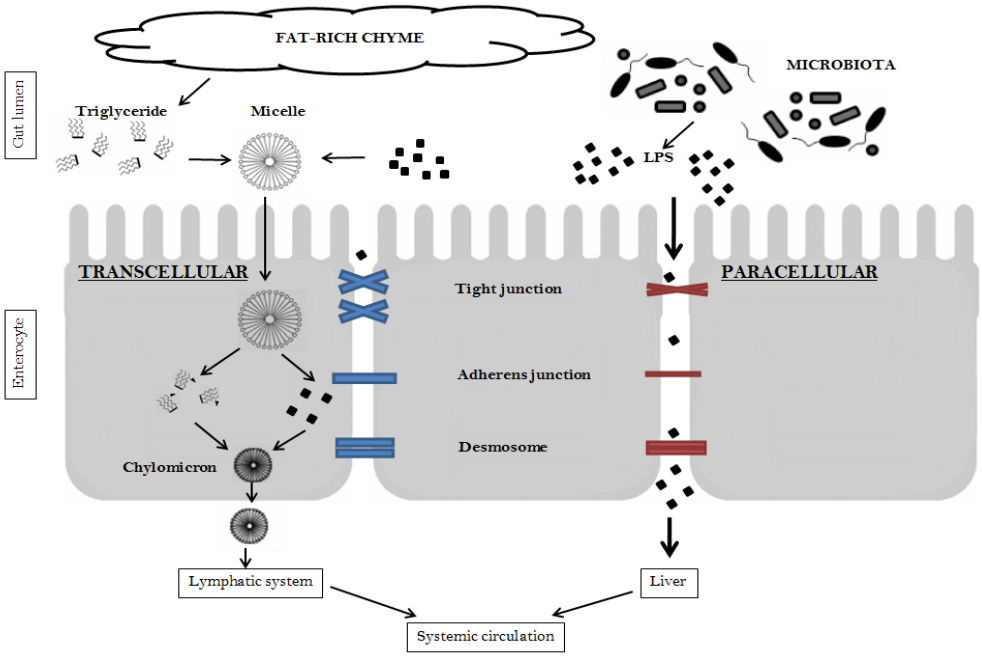

Figure 2-8. Schematic overview of the proposed absorption routes of LPS from the gut lumen into the systemic circulation.

left enterocyte, transcellular route; between the two enterocytes on the right hand side, paracellular route (modified from Figure 1 from Kelly et al. [3]).

This hypothesis is supported by findings in a mouse model i.e. the blocking of the formation of chylomicron prevented the fat-induced elevation in the levels of systemic endotoxin [133]. Moreover, data suggest that the ratios of saturated, mono-unsaturated and poly-unsaturated FAs in dietary oils and the degree of emulsification of the dietary fat can influence the fat-associated translocation of LPS from the gut lumen into the systemic circulation [135, 136].

2.7.3 The paracellular permeability of the gut to luminal LPS in subjects with Crohn's disease

Crohn's disease (CD) is a chronic and relapsing inflammatory bowel disease which can affect the gastrointestinal tract from the mouth to the anus in which the terminal ileum, colon and caecum are the most affected intestinal compartments. CD-affected areas are characterized by a thicker intestinal wall [137] which is caused by aggressive T_{h1}- and Th_{17}-mediated immune response against bacterial antigen after unknown environmental stimulation. The pathogenesis of CD is associated with complex interactions between the mucosal immune system, commensal and pathogenic intestinal bacteria and environmental factors in individuals with genetic susceptibility [138]. The course of the disease is relapsing with mild to severe disease activity and remission. CD is not curable thus the goal of the treatment is the induction and maintenance of mucosal healing (remission) and the prevention of disease flare up [137].

Subjects with CD are reported to have both high concentrations of pro-inflammatory cytokines IL-1β, IFN-γ and TNF-α, and augmented paracellular permeability to tracer probes such as lactulose [139-143]. Hence, it is thought that persistent on-going inflammation in the GALT may cause the loss of barrier function in those subjects. Given the fact that subjects with CD seem to have an persistent increased paracellular permeability they were used as a model in the last two studies, the Intralipid study (Chapter 7) and the Crohn's disease study (Chapter 11), to investigate whether changes in the paracellular permeability rather than transcellular mechanism that are involved in the absorption of dietary fat, are responsible for fat-induced elevations of systemic endotoxin levels.

2.8 References

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CHAPTER 3 OVERVIEW OF METHODS

DESCRIPTION OF THE METHODS AVAILABLE FOR THE QUANTIFICATION OF LPS/ENDOTOXIN IN HUMAN BLOOD AND FOR THE ASSESSMENT OF IN-VIVO GUT PERMEABILITY IN HUMANS

3.1 Methods of choice

In order to investigate the permeability of the gut to bacterial products such as LPS, *in-vivo* in humans methods are required that allow the concurrent determination of the two parameters. The methods that are available to address the research questions are briefly described and the methods that were selected to use, are named in this section.

3.1.1 Methods for the quantification of systemic LPS/endotoxin

3.1.1.1 Direct methods for the quantification of LPS and endotoxin

As explained previously, the term LPS refers to the whole molecule which comprise a lipid A moiety, a core oligosaccharide and an O-antigen [4], whilst the term endotoxin refers to the pro-inflammatory epitopes of LPS molecules. Again, the pro-inflammatory potential of a given endotoxin depends upon the chemical configuration of the lipid A moiety [5-9]. The endotoxic component of LPS can be detected using the Limulus Amebocyte Lysate (LAL) test. The LAL assay has been approved by the United States Food and Drug Administration (FDA) for the quantification of endotoxin in parenterally delivered drugs, in biological products and in medical devices in the late 1980ies [10, 11]. Since then this assay has been used in various research settings for the quantification of systemic endotoxin i.e. in patients with bacterial infections [12-23] and in healthy subject before and after the ingestion of a fatty meal [24-32]. However, the test has produced generally conflicting results when used with blood samples such as plasma and serum (discussed in detail in Chapter 12). Hence, there are continuing questions as to its reproducibility and accuracy.

Recently a number of ELISAs have become available from various manufacturers for the quantification of LPS in human blood samples i.e. plasma and serum. To the best of my knowledge there are no published studies that used the latter method for the

determination of LPS in plasma or serum. Hence, it is unclear whether those LPS - ELISAs are reliable tests.

3.1.1.2 Indirect methods for the assessment of LPS exposure in the systemic circulation

The Endotoxin Activity Assay (EAA) determines the endotoxin load in whole blood samples based on the level of free radicals released from neutrophils in response to endotoxin [33]. The EAA was developed nearly 15 years ago and has been approved by the FDA as a diagnostic aid for the assessment of the risk of septic shock in human subjects since 2004 [34]. The EAA has been used in this respect in clinical scenarios [35-41], and in a broad range of research settings that determined the exposure to endotoxin in the systemic circulation in healthy subjects [42] and in subjects with metabolic disorders such as obesity [43-45]. Data indicate that the assay is a reliable method for the assessment of the degree of endotoxin exposure in the systemic circulation [12]. However, the assay requires fresh blood i.e. the assay must be completed within 3 h of collection [e-mail communication with manufacturer] and is assayed by sequential reading of individual samples in a dedicated chemiluminometer [33], which may be awkward for the use in surveys with large numbers of subjects and with multiple samples per subject.

Another indirect indicator of the degree of the exposure to LPS in the systemic circulation is the level of systemic LPS-binding protein (LBP). LBP can be determined in blood samples using a (human specific) LBP - ELISA. The assay has been used in a broad range of research projects investigating the levels of systemic LBP in humans with various infections as well as in healthy or obese subjects [15, 18, 20, 22, 46-55].

3.1.1.3 Methods selected for the studies

The EAA in its current format i.e. analysis of the sample within 3 h of collection and sequential and individual reading of six subsamples for each sample in a dedicated chemiluminometer, seems to be impractical for use in my experimental settings i.e. the collection of multiple samples from a number of participants at the same time. Hence, the LAL assay was selected to determine the levels of systemic endotoxin. In addition, the commercial new available ELISA kit for the quantification of LPS was selected to investigate the relationship between levels of systemic endotoxin and those of 'total' systemic LPS. The principles and procedures of the LAL assay and of ELISAs for the quantification of LPS are explained in section 3.2 and 3.3, respectively.

Due to technical difficulties with the former two tests during the pilot study (see Chapter 5 for more details), the LBP-ELISA was used in the last two studies (see Chapter 7 and Chapter 11) as an indirect measurement of the degree of LPS exposure in the systemic circulation. The procedure of LBP – ELISAs used in those studies is described in section 3.4.

3.1.2 Methods for the assessment of gut permeability

3.1.2.1 Direct tests for the assessment of the permeability of the gut

The *in-vivo* permeability of the gut in human subjects can be assessed by a non-invasive test that determines the permeability of the gut mucosa to probe sugars such as sucrose, lactulose, mannitol, rhamnose, cellulose and sucralose, or to chemical probes such as Cr-EDTA [56]. It is noteworthy that the probe sugars are not commonly found in food. They are also degraded in different parts of the gastrointestinal tract (GIT) but they are not metabolised once absorbed, and are promptly cleared by the kidney and excreted with the urine. The sites at which the

probes are absorbed vary. Sucrose is absorbed in the stomach and in the first part of the duodenum and is then hydrolysed in the distal duodenum. It can thus be used to determine the permeability of the gastro-duodenal region. Lactulose and mannitol are metabolised by commensal bacteria in the colon and can therefore be used to determine the permeability of the small intestine [56]. Those two latter sugars were used to assess changes in the permeability of the gut in patients with inflammatory bowel diseases (IBD) such as Crohn's disease and ulcerative colitis, in their healthy relatives and in healthy control subjects [57-61]. The permeability of the entire colon can be assessed by the probes sucralose and Cr-EDTA as they are not metabolised along the GIT [56]. Hence, the relative quantity of those sugars excreted in the urine over a certain period of time after the ingestion of a known dose provides an indicator of the permeability of particular regions of the gut.

3.1.2.2 Indirect methods for determination of gut permeability

The permeability of the gut can also be assessed *in-vivo* in humans by indirectly biomarkers such as plasma levels of claudin-3 (CLDN3) that can be determined by ELISA. CLDN3 is a TJ protein that is predominantly expressed in mucosal cells of the duodenum and of the colon. The levels of systemic CLDN3 were reported to be elevated when the paracellular permeability was increased in these regions [62].

3.1.2.3 Method selected for the studies

Since I was particular interested in the permeability of the gut during the absorption of fat I needed a method that firstly can determine the permeability of the small intestine and secondly can reflect (temporary) changes in the permeability of the gut in 'real time'. Previous work by Dr Ivana Sequeira, a former PhD student at this

institute, indicated that the excretion of lactulose and mannitol in urine over a period of 3 h after the ingestion of the two tracer sugars is a sensitive and reliable method for the assessment of temporary change in the permeability of the small intestine i.e. after the intake of a single dose of aspirin [63, 64]. Hence, the lactulose-mannitol test with the collection of urine samples over a period of 3 h was used in the current studies. It is noteworthy that the larger molecule of those two sugars, lactulose, is thought to be an indicator of the paracellular permeability whilst the smaller one, mannitol, is thought to be an indicator of the transcellular permeability [63, 64]. The principle and the procedure of the lactulose-mannitol test for the assessment of the permeability of the small intestine are described in section 3.5.

3.2 Quantification of endotoxin in serum using the Limulus Amebocyte Lysate assay

In this section the history and general principle of the Limulus Amebocyte Lysate (LAL) assay are explained. The principle of the type of assay used in the experimental work, namely the chromogenic LAL assay, is then described in more detail. In total two types of the chromogenic LAL assay, manufactured by different companies, were used. The general procedure of each of the two assays is also described in this section.

The constituents in serum are reported to interfere with the LAL assay. Hence, additional preparation of samples prior to the assay is required. Various pre-treatments of plasma and serum samples so as to avoid this problem are described in the literature. Those methods include simple dilution [20, 65, 66], the treatment with perchloric acid [67-69] and a combination of dilution and heating at 68 - 85°C for 5 to 30 min [26-28, 30, 35, 37, 44, 50, 70, 71], the latter method being most commonly used. The evaluation of pre-treatment methods and further refinements are described in detail in Chapter 4, Chapter 6 and Chapter 10.

3.2.1 History and general principle of the LAL assay

In the 1960s Frederik B. Bang and Jack Levin observed that the blood of the North American horseshoe crab, *Limulus polyphemus*, clots during infection with Gram-negative bacteria [72] presumably to prevent septicaemia. The clotting results from LPS/endotoxin initiating a signalling cascade [73] that leads to the coagulation of a protein in amebocytes, a cellular component of blood of *L. polyphemus* [74] (Figure 3-1). It is noteworthy that the clotting cascade may also be activated by some β-glucans, components in the cell walls of fungi, leading to false-positive results (Figure 3-1). However, greater quantities of β-glucan than those of endotoxin are needed [75].

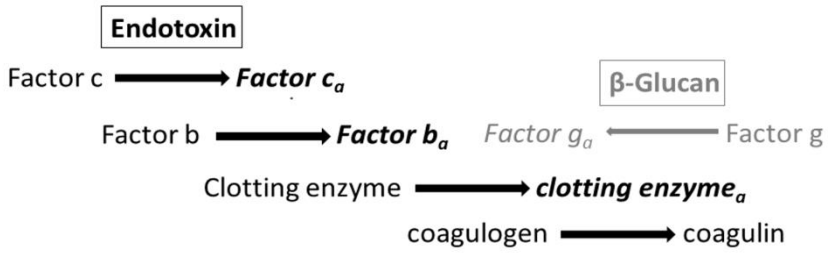

Figure 3-1. Clotting cascade generated in amebocytes of the horseshoe crab showing pint of activation by endotoxin and β-glucan.

The clotting cascade is initiated by endotoxin (black box) via Factor c and by β-glucan (grey box) via Factor g (modified from Figure 1 from Novitsky [3]).

Hence, an assay capable of detecting endotoxin was developed from a lysate of amebocytes [74]. The original gel-clot Limulus Amebocytes Lysate (LAL) assay was based on clotting of these components in the presence of endotoxin to form a rigid gel [72, 74]. Technological improvements in the isolation and purification of the enzymes and proteins, involved in this reaction, led to the development of the turbidimetric and the chromogenic LAL assays in the 1980s. The latter two quantitative assays use the same clotting cascade in an assay based either on an endpoint or on reaction kinetics. In the turbidimetric LAL assay, endotoxin levels are quantified by the turbidity that develops in the presence of endotoxin and is measured by the absorption at 360 nm [76]. The principle of the chromogenic LAL assay is explained in the section below. In both the turbidimetric and the chromogenic LAL assay, the levels of endotoxin can be determined using either an endpoint or a kinetic format. Both the chromogenic and the turbidimetric LAL assays have been approved by the US Food and Drug Administration (FDA) in the late 1980s for the quantification of endotoxin in parenterally delivered drugs, for biological products and for medical devices [10]. Recently, the chromogenic LAL assay has been predominantly used for the determination of levels of systemic endotoxin in human blood samples.

It is noteworthy that in early work endotoxin was predominantly expressed in weight units such as ng/ml. However the configuration of the lipid A moiety varies broadly

between Gram-negative bacteria species as does its endotoxicity [8, 77] i.e. the sensitivity of the test varies according to the source of LPS from pg/ml to ng/ml [6]. Further, the determined concentration of endotoxin in a given sample is influenced by the source of LPS that is used to generate the standard curve. Hence, LPS of different endotoxic potentials used for the calibration makes it difficult to compare data from different studies and laboratories with one another. The use of a control standard endotoxin (CSE) (LPS from E.coli O55:B5) avoid such problems and was adopted in the late 1990's. Since then the concentration of endotoxin is generally expressed in endotoxin unit (EU)/ml in which EU reflects the endotoxic activity of the mixture of LPS molecules within a given sample relative to that of the control standard endotoxin. The WHO International Standard for endotoxin currently defines one EU as the biological activity induced in the LAL assay by around 120 pg of LPS from *Escherichia coli* O113H10:K [78].

In the research described in this thesis two types of LAL assays were used for the determination of endotoxin levels in serum, namely the Endosafe® PTS (Portal Test System) from Charles River (Charleston, SC, USA) and the kinetic-QCL™ LAL kit from Lonza (Walkersville, MD, USA). Both are kinetic formats of the chromogenic LAL assay.

3.2.2 The principle of the chromogenic LAL assay

The chromogenic LAL assay is based on the initial part of the amebocytes clotting cascade but the coagulogen is replaced by a synthetic chromogenic substrate that is linked with p-nitroaniline (pNA) [75] such as Ac-Ile-Glu-Ala-Arg-pNA [79]. Activation of the cascade cleaves pNA from the colourless substrate creating a yellow product [80]. The change in the concentration of pNA that is released i.e. the

intensity of the yellow colour, its optical density (OD), is directly and quantitatively proportional to the concentration of endotoxin in the sample [79] and is quantified by absorption at 405 nm [80]. In the kinetic version of the LAL assay the colour change can be determined either over a set period of time [81] or by the time taken for a set change in OD [39]. In the kits manufactured by both Charles River Laboratories and Lonza, the reaction time is determined as the time taken for OD to increase by 0.2 OD from the initial OD value. The latter is used to calculate the concentration of endotoxin by comparison of the reaction time of the sample with those of standards containing known concentrations of endotoxin.

3.2.3 The Endosafe® PTS manufactured by Charles River Laboratories

3.2.3.1 Description of the Endosafe® PTS kit

The Endosafe® PTS (Portal Test System) kit manufactured by Charles River Laboratories (Charleston, SC, USA), consists of a portable optical density reading machine and is used with entirely pre-formulated, disposable, single-test cartridges that each have a 'built-in' endotoxin standard curve. In my case, the portable optical density reading machine was connected to a computer with Endosafe® PTS software (Figure 3-3).

Each pre-formulated, disposable test cartridge contains four sample analysing units, in which each sample analysing unit consists of a sample reservoir, a sample channel, an optical cell and a pump connection (Figure 3-2 on top). The sample channel is subdivided into 2 or 3 reagent stations which are formulated according to. the use of the channel i.e. for the detection of endotoxin (sample channel) or for the quantification of inhibition/enhancement of the LAL reaction (Positive Product Control; PPC (spike) channel) as determined by the recovery of a known spiked dose of

endotoxin (in percentage) (Figure 3-2 zoom) [1]. Further, there are two types of cartridges available, namely the Inhibition/Enhancement Screening (I/E Screening) cartridge and the Endosafe® PTS cartridge. The I/E Screening cartridge is intended

Figure 3-3. The Endosafe® PTS kit.

The Endosafe® PTS kit in our laboratory consists of a portable reader machine (white machine) that has been connected with a computer with installed Endosafe® PTS software. In this picture a cartridge is inserted into the reader machine.

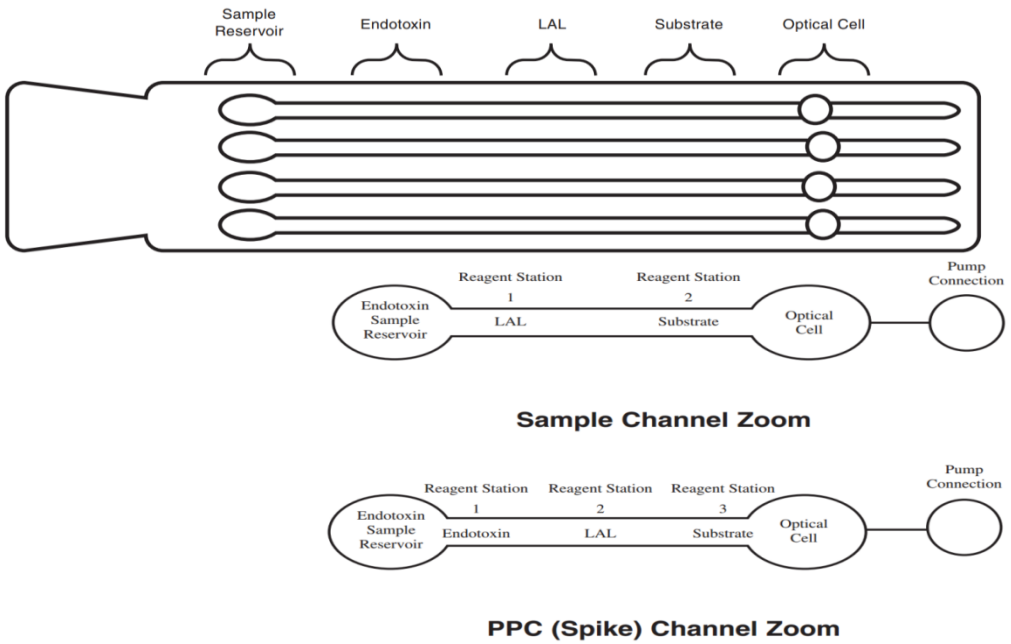

Figure 3-2. Schematic composition of the pre-formulated, disposable, single-test cartridges for use in the Endosafe® PTS reader.

On top a schematic composition of the test cartridge and below a schematic composition of the two types of test units within the cartridges, the sample channel and the PPC (spike) channel (figure from Charles River Laboratories [1]).

to be used for the concurrent quantification of inhibition/enhancement in four samples that are prepared differently. Hence, each of the four channels in the I/E Screening cartridges comprises a known concentration of endotoxin of around 0.5 EU/ml (the dose varies between batches) [82]. Although the endogenous concentration of endotoxin in the sample is not determined in any of the four samples and thus an accurate determination of the PPC for each sample is not possible, this type of cartridges is a useful tool for the evaluation of methods for the pre-treatment of blood samples with regards of trends for inhibition or enhancement of the assay by the sample matrix. The experimental work associated with the latter is described in detail in Chapter 4 and Chapter 5.

In the Endosafe® PTS cartridges two of the four channels are used to determine the endogenous concentration of endotoxin in the sample in duplicates and the remaining two channels are spiked with a known concentration of endotoxin (around 0.1 EU/ml; the dose varies between cartridges from different batches) [83] to determine the PPC of the sample in duplicates [1].

3.2.3.2 The protocol of the Endosafe® PTS assay

In order to perform the endotoxin measurement the appropriate cartridge was inserted into the Endosafe® PTS reader machine and the cartridge lot number and the calibration code were entered into the Endosafe® PTS menu. When the reader was ready for analysis a sample volume of 25 µl was pipetted into each of the four sample reservoirs. In case of Endosafe® PTS cartridges, aliquots from the same sample were pipetted into each sample reservoir, whilst four different samples were pipetted into each sample reservoir in case of I/E Screening cartridges. After adding the sample, the test was initiated concurrently in each channel by pressing the 'ENTER' button on the

portable reader. The samples in each sample reservoir were then automatically pumped into the channels where the sample was either spiked with endotoxin and then mixed with LAL reagents or directly mixed with LAL reagents depending on the type of channel (Figure 3-2 zoom). Following the initiation of the LAL reaction samples were then pumped into the optical cell where their optical density (OD) were determined at around 395 nm every second. The sequence of reading was halted once OD values in all four channels crossed the pre-determined threshold (varies between cartridges from different batches) or after around 17 min when the reaction time of the lowest standard (0.005 EU/ml) was exceeded. Data were automatically uploaded into the Endosafe® PTS software on the computer connected to the portable reader. The endotoxin concentrations in each channel and/or the PPCs were automatically calculated by the Endosafe® PTS software based on the time it taken for each sample OD to cross the pre-determined threshold and were compared to the 'built-in' endotoxin standard curve. The Endosafe® PTS cartridges that were used for my experiments were reported to have a detection range between 0.005 EU/ml and 0.5 EU/ml. The According to the manufacturer's specification the Endosafe® PTS test was valid when the intra-assay CVs of both the sample and the PPC were below 25 % and when the PPC was between 50 and 200 %.

3.2.4 The kinetic-QCL™ LAL kit manufactured by Lonza

3.2.4.1 Description of the kinetic-QCL™ LAL assay

The kinetic-QCL™ LAL assay (Lonza, Walkersville, MD, USA) is performed on a 96-well micro-plate and OD values can be recorded in any commercially available micro-plate reader with a temperature setting at 37°C. The detection range of this assay is reported by the manufacturer to be from 0.005 EU/ml to 50 EU/ml.

3.2.4.2 The protocol of the kinetic-QCL™ LAL assay

In order to perform this assay, 100 µl of each standard and sample were pipetted into the corresponding well of a 96-well micro-plate. The plate was then incubated in the micro-plate reader (ELX808IU, Bio-Tek Instruments, Inc., USA) at 37°C for 10 min. Each vial of LAL reagent was reconstituted by adding 2.6 ml LAL reagent water and reconstituted reagents were placed into a single reagent reservoir while the 96-well micro-plate was incubated at 37°C. One hundred µl of reconstituted LAL reagent was then added with a multi-channel pipette to each well and the plate was shaken for 30 s. The OD of each sample was read at 405 nm immediately after shaking and every 15 or 30 s thereafter for a period of 1.5 h to 2 h. Data were subsequently uploaded into KC4 software or into Gene5™ software (both from Bio-Tek Instruments, Inc., USA) depending on the micro-plate reader that was used. Data were then transferred into Microsoft EXCEL 2010 to determine the reaction time of each standard and sample. A standard curve was generated by plotting the reaction time of the standard against the logarithms of the corresponding known concentration of endotoxin. The concentration of endotoxin in serum samples was determined by comparing their reaction times with those of endotoxin standards. Details regarding the definition of reaction time and the pre-treatment of serum samples prior to the assay are given in Chapter 6 and Chapter 10.

3.3 Quantification of LPS in serum using ELISA assays

The human specific LPS-ELISA kits from two different manufactures, namely 'Sun Red' (Shanghai Sunred Biological Technology Co., Ltd, Shanghai, P.R. China) and 'Cusabio' (Cusabio Biotech Co., Ltd, Wuhan, P.R. China) were evaluated. The general principle of ELISA assays and the assay procedure for each of the two ELISAs are described in this section. Again, the initial evaluation of the two kits regarding the reliability to quantify LPS in serum is described Chapter 4.

3.3.1 The principle of ELISA assays

The quantification of LPS concentration in serum samples by LPS-specific enzyme-linked immunosorbent assay (ELISA) is based on interactions between the LPS antigens contained in the sample and LPS antibodies incorporated in the assay (Figure 3-4). Briefly, each well of the 96-well micro-plate provided with the kit is pre-coated with human LPS monoclonal antibodies. Hence, any LPS in the sample will bind to the LPS-antibodies held on the wall of the well to form a LPS-antibody-antigen complex. Mobile LPS-antibodies that are labelled with biotin are then added to the sample. These bind to the well-bound LPS-antibody-antigen-complex forming a double-antibody-antigen sandwich. Any remaining 'unbound' mobile LPS-antibodies are removed by washing with a buffer provided with the kit. HRP-avidin or Streptavidin is then added to the well which binds to the biotin held in the double-antibody-LPS sandwich. Any remaining 'unbound' HRP-avidin/Streptavidin is then removed by washing with a buffer provided with the kit. Finally a solution of the chromogenic TMB substrate is added which reacts with the well-bound enzyme complex which causes a blue colour to develop. This colour change is fixed by the addition of a stop solution that causes the colour to change from blue to yellow. The intensity of the

yellow colour i.e. its optical density (OD) is then determined at 450 nm and is directly proportional to the concentration of LPS in pg/ml in the sample.

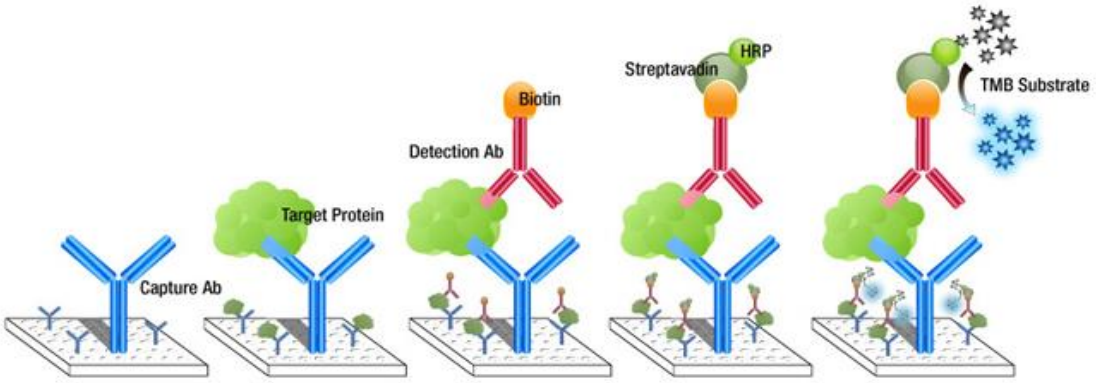

Figure 3-4. Principle of the ELISA assay.

Antigen (target protein) binds to well-bound antibodies (Ab). Mobile (detection) Ab labelled with biotin then binds to the well-bound Ab-Antigen-complex forming a double-Ab-Antigen complex. HRP-avidin/Streptavidin then binds to the double-Ab-Antigen sandwich resulting in the formation of an enzyme-complex which then reacts with TMB substrate leading to a colour change from colourless to blue (figure from Epitomics [2]).

3.3.2 The protocol of the LPS – ELISA kit manufactured by Sun Red

The human LPS - ELISA kit manufactured by 'Sun Red' (Shanghai Sunred Biological Technology Co., Ltd, Shanghai, P.R. China) came with a 96-well micro-plate pre-coated with LPS in the form of 8 strips with 12 wells per strip.

The assay was performed according to the manufacturer's instructions. Briefly, in case of LPS standards, 50 µl of each LPS standard solution was added to the corresponding wells followed by adding 50 µl of Streptavidin-HRP to the well. In case of samples, 40 µl of each tested sample was added to the corresponding wells followed by adding 10 µl of the LPS-antibody solution and 50 µl of Streptavidin-HRP. The wells for the blanks were kept empty. After this initial loading the plate was covered with an adhesive strip and incubated at 37°C for 60 min. The strip was then removed and the liquid in each well decanted. The plate was washed three times using the wash buffer provided with the kit. After washing, the remaining buffer was removed by inverting the plate and blotting it with clean paper towels. Fifty µl of each chromogen solution A

and chromogen solution B were then added to each well. The plate was again covered with a fresh adhesive strip and wrapped in aluminium foil to protect it from light. The plate was then incubated at 37°C for 10 min. The aluminium foil and the strip were removed and 50 µl of stop solution was added to each well. Finally, the OD of each well was determined at 450 nm using the ultra micro-plate reader ELx808 (Bio-Tek Instruments, Inc, USA) and data were uploaded into KC4 3.0 Rev 29 software (Bio-Tek Instruments, Inc, USA). The OD values of the standards were plotted against known concentrations of LPS in CurveExpert Professional version 2.02 and a standard curve was generated by the software. The concentration of LPS in each sample was determined by reference to the standard curve in CurveExpert Professional version 2.02.

3.3.3 The protocol of the LPS – ELISA kit manufactured by Cusabio

The LPS pre-coated 96-well micro-plate of the human LPS – ELISA kit manufactured by 'Cusabio' (Cusabio Biotech Co., Ltd, Wuhan, P.R. China), was provided in the form of 12 strips with 8 wells per strip. Further, the LPS standard and LPS-antibodies of this kit were separate.

Again, the assay was performed as described in the user manual. Briefly, 100 µl of each standard, sample and sample diluent (blank) were added to the corresponding well. The plate was then covered with an adhesive strip and incubated at 37°C for 2 h. The cover was removed and the liquid in each well then removed using a multichannel pipette and 100 µl of biotin-labelled LPS-antibodies were then added to each well. The plate was then again covered with a fresh adhesive strip and incubated at 37°C for 1 h. The strip was then removed and the plate was inserted into the auto plate washer ELx405 (Bio-Tek Instruments, Inc., USA). The liquid in each well was automatically

aspirated and 200 µl of wash buffer were immediately added to each well. The wash buffer was also automatically aspired after 2 min period of incubation and the wash step was repeated twice more. Following the last wash step, any remaining wash buffer was removed by inverting and blotting the plate against clean paper towels. One hundred µl of HRP-avidin was then added to each well. The plate was again covered with a fresh adhesive strip and incubated at 37°C for a further hour. The strip was removed and the plate was then inserted into the auto plate washer. The wash step was repeated five times as described above and 90 µl of TMB substrate were then added to each well. The plate was covered with a fresh adhesive strip and wrapped in aluminium foil to protect it from light. The plate was then incubated at 37° for 15 to 20 min depending on the intensity of the blue colour that was developed in the standard wells. The aluminium foil and adhesive strip were removed and 50 µl of stop solution was immediately added to each well. The OD of each well was then determined at 450 nm using the ultra micro-plate reader ELx808 (Bio-Tek Instruments, Inc., USA). Data were uploaded into KC4 3.0 Rev 29 software (Bio-Tek Instruments, Inc., USA). The OD values of the standards were plotted against known concentrations of LPS in CurveExpert Professional version 2.02 and a standard curve was generated by the software. The concentration of LPS in each sample was determined by reference to the standard curve in CurveExpert Professional version 2.02.

3.4 Quantification of LBP in serum using ELISA assay

The principle of the ELISA for the quantification of LBP in human blood is the same as described previously in section 3.3.1 with exception that the wells for the former were pre-coated with LBP antibodies and that mobile LBP antibodies were added rather than LPS antibodies. Hence, the principle of the assay is not repeated in this section.

Human-specific LBP – ELISAs manufactured by two different companies, namely Cusabio and Hycult, were used. The assay procedure of the ELISA manufactured by Hycult is described in this chapter. Again, the LBP - ELISA kit, manufactured by Cusabio, was supplied with separate LBP standard and mobile LBP-antibodies. Since the procedure of this LBP - ELISA is the same as those for the LPS - ELISA see 3.3.3 for details regarding the assay procedure. The evaluation of the LBP - ELISA manufactured by Cusabio, is described in Chapter 6 and those of the ELISA manufactured by Hycult is described in Chapter 10.

3.4.1 The procedure of the LBP – ELISA manufactured by Hycult

The 96-well micro-plate pre-coated with LBP was provided in the form of 12 strips with 8 wells each strip. Again, the LPS standard and LPS-antibodies were separate.

The ELISA was performed according to the manufacturer's instructions. Briefly, the two hundred µl of wash buffer was added to each well and incubated for 20 s. The plate was emptied by inverting the plate and shaking the contents out over the sink. The wash step was repeated three times. After washing, the remaining buffer was removed by blotting the inverted plate with clean paper towel. One hundred µl of each standard and sample was then pipetted to the corresponding well. The plate was covered with a fresh adhesive strip and incubated at room temperature for 1 h. The strip was removed

and the wash step repeated four times as described above. One hundred µl of mobile LBP antibodies were then added to each well. The plate was covered with a fresh adhesive strip and incubated at room temperature for another h. The strip was removed and the wash step repeated four times as described above. One hundred µl of streptavidin-peroxidase was then added to each well and the plate again covered with a fresh adhesive strip and incubated at room temperature for 1 h. The strip was then removed and the plate washed four times. One hundred µl of TMB substrate were then added to each well. The plate was covered with a fresh adhesive strip and wrapped in aluminium foil to protect it from light. The plate was then incubated at room temperature for 30 min. The aluminium foil and the strip were removed and 100 µl of stop solution added to each well. The OD of each well was then determined at 450 nm using the ultra micro-plate reader ELx808 (Bio-Tek Instruments, Inc., USA). Data were uploaded into KC4 3.0 Rev 29 software (Bio-Tek Instruments, Inc., USA). The OD values of the standards were plotted against known concentrations of LPS in CurveExpert Professional version 2.02 and a standard curve was generated by the software. The concentration of LPS in each sample was determined by reference to the standard curve in CurveExpert Professional version 2.02.

3.5 Assessment of gut permeability using the standardised lactulose-mannitol test

This section describes the principle and the procedure of the lactulose-mannitol test for the assessment of gut permeability that was developed in this laboratory by Dr Ivana Sequeira, along with the methods for the analysis of urinary mannitol and lactulose [63, 84].

3.5.1 The principle of the lactulose-mannitol test

Lactulose and mannitol are two non-toxic water soluble sugars that are thought to be absorbed across the gut mucosa via different routes. Small molecules such as mannitol are thought to be absorbed via the transcellular route whilst larger molecules such as lactulose are thought to be absorbed via the paracellular route. Both sugars are not metabolised after they have been absorbed and are thus promptly excreted by urine. Hence, they are excreted in the proportions that they are absorbed from the gut and are therefore indicators of changes in the permeability of the gut [85]. The amount of those two sugars excreted over a period of 3 h after the ingestion of the tracer sugars is reported to be an indicator of the permeability of the small intestine [63, 64].

3.5.2 The protocol for the lactulose- mannitol test

Each participant was given 100 ml of a solution containing 10 g of lactulose (duphalac or Laevolac) and 5 g of mannitol (Sigma-Aldrich, St. Louis, Mo, USA) after fasting overnight. Urine was collected on arrival of the participant at the laboratory and over a period of 3 hours after the consumption of the sugar solution. The volume of each urine sample was determined and a 15 ml subsample was centrifuged at 3500 g and

4°C for 10 min. The supernatant was then decanted and subdivided into 2 aliquots, each of 2 ml. Urine samples were stored at -80°C pending HPLC analysis.

It is noteworthy that the source of lactulose varied between studies i.e. duphalac (Abbott, Hoofddrop, the Netherlands) was used in the pilot study (Chapter 5) and in the Intralipid study (Chapter 7) whereas Laevolac (Douglas Pharmaceuticals Ltd, Auckland, New Zealand) was used in the Crohn's disease study (Chapter 11). The change in the lactulose sources was required as duphalac was no longer available in pharmacies in New Zealand. Both products are laxatives that contain 3.34 g of lactulose in 5 ml as well as minor quantities of other sugars i.e. 0.5 g of Galactose and 0.3 g of Lactose, respectively, in 5 ml of duphalac. The HPLC profile of sugars in Laevolac did not differ from those in duphalac (Figure 3-5).

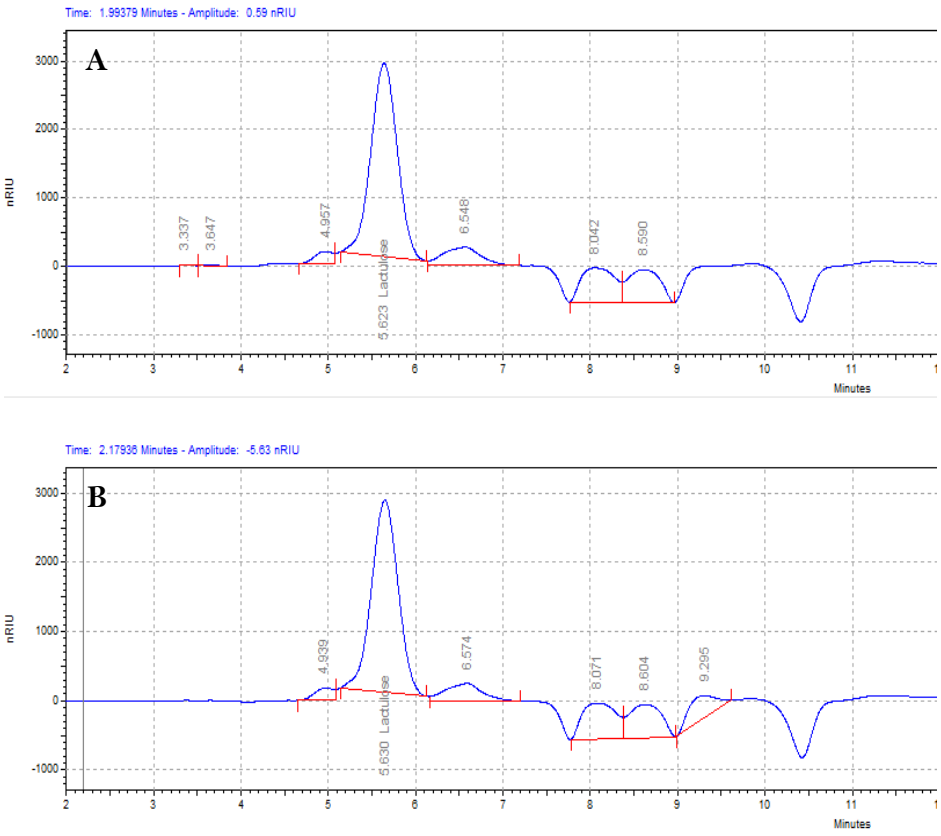

Figure 3-5. HPLC profile of duphalac (A) and Laevolac (B).

3.5.3 Quantification of lactulose and mannitol in urine samples using HPLC

A one ml aliquot of each urine sample was diluted with an equal volume of MilliQ filtered water and desalted with 1 g Amberlite® resin (Amberlite IRA-410® chloride form and Amberlite® IR120 hydrogen form; Fluka® Analytical, Sigma Aldrich, St. Louise, MO, USA) in a proportion of 1:1 (w/w). The mixture was then vortexed for 15 s and the supernatant filtered through a 0.2 µm (pore size) cellulose acetate filter (Micro-Analytix Pty Ltd, Auckland, New Zealand) into a 2 ml HPLC vial (Thermo Scientific, Thermo Fisher Scientific, UK).

HPLC analysis was performed on a 20 µl aliquot of filtrate from each in a HPLC system. The Shimadzu HPLC system (Japan) equipped with Degasser Model DGU-20A, a Pump Model LC-20AT Prominence, an Autosampler Model SIL-20AC, a Column Oven CTO-20A, a 250 x 4.0 mm cation-exchange column (Aminex HPX87C, Bio-Rad Laboratories, Richmond, CA, USA) protected by a Micro Guard Cartridge pre-column (Bio-Rad Laboratories) [86] and a Refractive Index Detector Model RID-10 A, was used for the analysis of samples of both the pilot study (Chapter 5) and the Intralipid study (Chapter 7). Samples of the Crohn's disease study were analysed in a HPLC system from Agilent Technologies (1200 Series) equipped with Degasser Model G1322A, a Quant Pump Model G1311A, an Autosampler Model ALS-G1329A, a Column Oven Model TCC-G1316A and a Refractive Index Detector Model RID-G1362A. In both systems filtered MilliQ water was used as the mobile phase at a flow rate of 0.3 ml/min at 590 psi and at 60°C over 20 min for each standard of lactulose and mannitol and over 40 min for each sample filtrate, respectively. The component sugars in each standard and each filtrate were detected with a Refractive Index Detector.

The concentrations (in µg/ml) of lactulose and mannitol in each filtrate were calculated from the areas under the curve (AUC) relative to those of a series of

concurrently run standards. The total quantity of each sugar (in mg/ml) excreted in 3 hours was then determined by multiplication of the volume of the urine sample with the dilution factor of the subsample. The quantities of lactulose and mannitol excreted by each subject and each treatment were expressed as a percentage recovery of the administered dose (10 g and 5 g, respectively). The lactulose-mannitol ratios (LMR) were determined from the percentages lactulose and mannitol excreted.

Calibration curves for lactulose were linear up to 500 µg/ml and those on for mannitol were linear up to 2,000 µg/ml. The accuracy of the method of the quantification of these sugars that were used in our laboratory by HPLC analysis has been described previously by Dr Ivana Sequeira [63, 84, 86]. The intra-assay coefficient of variation was in the range 0.2 - 7.7 % for lactulose and 0.1 - 4.6 % for mannitol when samples were analysed with the Shimadzu HPLC system and was in the range 0.1 – 7.7 % for lactulose and 0.1 – 1.6 % for mannitol when samples were analysed with the Agilent Technologies system.

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CHAPTER 4 METHOD EVALUATION – PILOT STUDY

THE EVALUATION OF THE METHODS FOR THE QUANTIFICATION OF ENDOTOXIN AND LPS IN HUMAN BLOOD

The part of the evaluation of the Endosafe PTS kit, manufactured by Charles River Laboratories, for the quantification of endotoxin in human serum is published in part as:

Gnauck A, Lentle RG, Kruger MC. The Limulus Amebocyte Lysate assay may be unsuitable for detecting endotoxin in blood of healthy female subjects. J Immunolog Methods. 2015. 416:146-56. doi:10.1016/j.jim.2014.11.010.

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STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Anne Gnauck

Name/Title of Principal Supervisor: Prof Roger G. Lentle

Name of Published Research Output and full reference:
Gnauck A, Lentle RG, Kruger MC. The Limulus Amebocyte Lysate assay may be unsuitable for detecting endotoxin in blood of healthy female subjects. J Immunolog Methods. 2015. 416: 146-56. doi: 10.1016/j.jim.2014.11.010.

In which Chapter is the Published Work: Chapter 4

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

  and / or

- Describe the contribution that the candidate has made to the Published Work:
  - Obtaining approval from the Massey University Human Ethics Commitee
  - Recruitment of subjects
  - Preparation of blood samples i.e. plasma and serum
  - Planing and conducting of the experimental work
  - Statistical data analysis and presentation of data
  - Literature search and writing the draft of the paper

A. Gnauck
Candidate's Signature

10/12/2015
Date

Principal Supervisor's signature

10/12/15
Date

GRS Version 3– 16 September 2011

Abstract

The aim of the experimental work described in this chapter was the evaluation of the methods selected for the quantification of endotoxin and LPS, respectively, in human blood samples. Data indicate that a pre-treatment of serum samples by a combination of dilution and heating is sufficient to reduce interactions with the chromogenic LAL assay manufactured by Charles River Laboratories, and allows the quantification of endotoxin in serum. Further, that the ELISA manufactured by Sun Red, is not an appropriate assay for the quantification of LPS in any kind of sample. However, the ELISA manufactured by Cusabio, seems to be a robust assay for the quantification of LPS, at least when samples were appropriately prepared i.e. blood samples were allowed to clot for 2 hours prior the separation of serum.

4.1 Introduction

The exposure to LPS in the systemic circulation was assessed in research settings by the quantification of endotoxin using the LAL assay in the last three decades. Hence, I selected a kinetic chromogenic format of the LAL assay, namely the Endosafe® PTS kit manufacture by Charles River Laboratories, to produce data that can be compared with those previously published. Since the LAL assay has been reported to be interfered by constituents of (human) blood [1, 2] the aim of the initial experimental work was to find the best method for the pre-treatment of serum samples prior to the LAL assay. As mentioned previously, endotoxin reflects the pro-inflammatory potential of LPS rather than the total amount of circulating levels of LPS. Hence, I chose two newly commercial available LPS - ELISA kits from different manufacturers, namely Sun Red and Cusabio. These two assays were evaluated based on their reliability to quantify LPS in human blood samples. The experimental work related to the latter is also described in this chapter.

4.2 Materials and methods

4.2.1 Subjects and sampling of blood samples

In total four sets of blood samples taken from a total of 18 female volunteers were used for the experimental work described in this chapter. For the first set fasted venous blood samples were drawn into plastic 'plasma' EDTA vacutainers (K2E 7.2 mg, 4 ml, Ref. 367839, Becton, Dickson and Company (BD), UK). Samples were then centrifuged at 3,500 g at 4°C for 15 min (Megafuge 1.0R, Heraus Instruments GmbH, Hanau, Germany) within 30 min of sample collection. Plasma was decanted into micro-centrifuge tubes. The remaining three sets of fasted venous blood samples were drawn into plastic 'serum' vacutainers (6.0 ml, Ref. 367837; BD, UK). Samples were allowed to clot at room temperature prior to centrifugation at 3,500 g at 4°C for 15 min (Megafuge 1.0R, Heraus Instruments GmbH, Hanau, Germany). Serum was then decanted into micro-centrifuge tubes. Aliquots of serum were analysed either on the same day or stored at -80°C pending further analysis.

The project was approved by Massey University Human Ethic Committee (MUHEC): Southern A (application number 12/61) (Appendix I - i) and informed written consent was given by all volunteers (Appendix I - iii).

4.2.2 Determination of endotoxin levels in serum samples using the Endosafe® PTS kit manufactured by Charles River Laboratories

Ten I/E Screen cartridges (lot 2135827) and five Endosafe® PTS cartridges (Charles River Laboratories, Charleston, SC, USA) with a stated detection range from 0.005 to 0.5 EU/ml (lot 3120564) were used for the evaluation of methods for the pre-treatment of serum samples prior to the quantification of endotoxin in serum samples.

The assay was performed according to the manufactures instruction (see 3.2.3.2 for more details).

4.2.2.1 Determination of the effect of sample diluent on the recovery of spiked endotoxin

Aliquots of five serum samples from healthy female postgraduate students collected into plastic 'serum' vacutainers, were thawed and pooled. Aliquots of the pooled serum were then diluted 5, 10, 20, 40 and 100 fold with Endosafe® LAL reagent water (LRW). Aliquots of pooled serum were similarly diluted with Endosafe® Endotoxin-specific Buffer (ESB) and with Endosafe® Dispersing Agent (DA) (all from Charles River Laboratories, Charleston, SC, USA). In addition, one aliquot of the pooled sample was also diluted 30 fold with DA. The diluted samples were subsequently loaded onto I/E Screening cartridges for the determination of recoveries of spiked endotoxin to assess their relative inhibition and enhancement.

4.2.2.2 Determination of the effect of combined dilution and heating of raw serum samples on the recoveries of spiked endotoxin

Aliquots of the pooled sample similarly diluted with similar diluents were then each transferred into endotoxin-free glass tubes (depyrogenated pack of Endosafe® 10x75mm Borosilicate Glass Tubes, Charles River Laboratories, Charleston, SC, USA) and heated for 10 min at 80°C in a heat block (Thermomixer®, Eppendorf AG, Hamburg, Germany). Heated samples were then allowed to cool before being decanted into micro-centrifuge tubes and loaded onto I/E Screening cartridges for the determination of recoveries of spiked endotoxin. The recoveries obtained with the diluted and heated sera were compared with those obtained with diluted but unheated serum to determine their relative inhibition and enhancement. The results were

interpolated to find the optimum sample diluent and dilution factor i.e. recovery of spiked endotoxin that was closest to 100 % with a minimum of dilution, for use in the subsequent determination of endogenous endotoxin in serum samples using Endosafe® PTS cartridges.

4.2.2.3 Determination of endogenous endotoxin in serum samples

Four thawed and one fresh serum sample were each diluted 1:30 with DA and heated for 10 min at 80°C in a heating block. Heated samples were allowed to cool before being loaded onto Endosafe® PTS cartridges to assay for endogenous endotoxin levels and the recoveries of spiked endotoxin.

4.2.3 Determination of circulating levels of LPS using the LPS-ELISA manufactured by Sun Red

The human-specific LPS - ELISA kit manufactured by Sun Red (Shanghai Sunred Biological Technology Co., Ltd, Shanghai, P.R. China), was used according to the manufacturer's instructions (see 3.3.2 for more details). The reported detection range of this LPS - ELISA was 12 to 4000 EU/l.

4.2.3.1 Calibration of the assay

The standard curve in the assay range was expected to be linear according to the manufacturer's instructions. However, my initial test found that the entire LPS standard curve in the range between 12 to 4000 EU/l (Figure 4-1A) was non-linear and fitted a sixth-order polynomial function. The early part of the standard curve (between 12 to 2400 EU/l) was best fitted by a second-order polynomial function

(Figure 4-1B). Hence, further work was based on LPS standard solution in the range from 12 to 2400 EU/l.

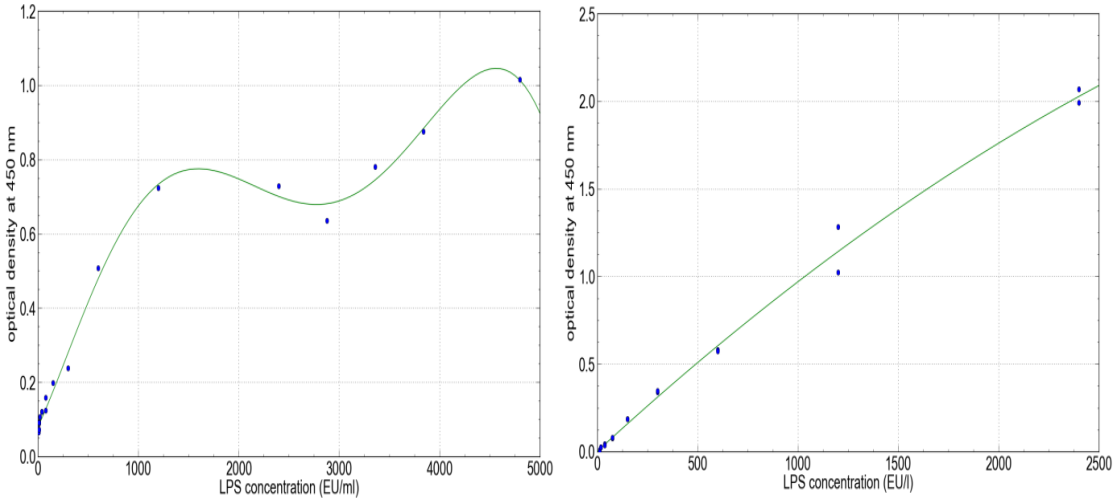

Figure 4-1. LPS standard curves obtained with the ELISA kit manufactured by Sun Red.

A) The LPS standard curve up to 4800 EU/l was sixth-order polynomial. B) The LPS standard curve up to 2400 EU/ml was second-order polynomial.

4.2.3.2 Determination of the effect of sample pre-treatment prior to assay

MilliQ water, endotoxin-free water (Sigma Aldrich, Sigma® Life Science, Switzerland) and the standard diluent provided with the kit were each tested for its suitability as diluent. Hence, each plasma sample was diluted with each of the three diluents at 2, 4 and 8 fold.

4.2.4 Determination of circulating levels of LPS using the LPS-ELISA from Cusabio

Again, the human-specific LPS - ELISA manufactured by Cusabio (Cusabio® Biotech Co, Ltd, Wuhan, Hubei Province, P.R. China), was used according to the manufacturer's instruction (see 3.3.3 for more details). The range of detection of this assay was reported by the manufacturer to be from 6.25 to 400 pg/ml.

4.2.4.1 *Calibration of the assay*

The standard curve of LPS was best fitted by a second order polynomial function (Figure 4-2). The correlation coefficients of the standard curves all exceeded 0.970.

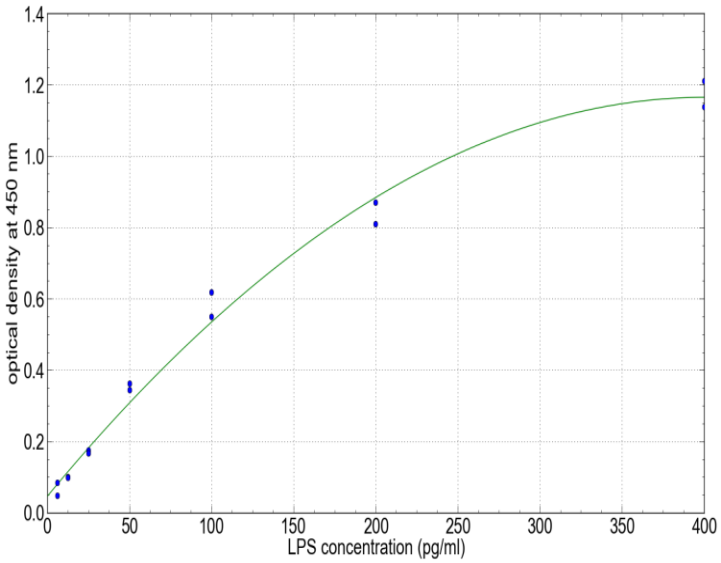

Figure 4-2. LPS standard curve obtained with LPS - ELISA manufactured by Cusabio.

The standard curve was second order polynomial in the detection range from 6.25 to 400 pg/ml.

4.2.4.2 *Testing for interference of the assay by constituents of serum*

To test whether constituent of serum interfere with the assay, a series of LPS standard solutions were spiked with either pooled serum (prepared from blood that was allowed to clot for 30 min) or a solution of 40 µg/ml albumin at a ratio of 1:10 (v/v) prior to assay.

4.2.4.3 *Determination of the effect of sample pre-treatment prior to the assay*

A series of LPS standard solutions spiked with either serum (prepared from blood that was allowed to clot for 30 min) or of solution of 40 µg/ml albumin at a ratio of 1:10 (v/v) were heated at 70°C for 10 min prior to the assay.

4.2.4.4 Determination of the effect of changes in the clotting time prior to the separation of serum

Typical plastic serum separation tubes (SST) were used for the blood collection. In the laboratory practice blood samples were allowed to clot for 30 min prior to separating serum by centrifugation. However, the manufacturer of the LPS - ELISA kit that was used in this experimental work, recommended to allow the blood to clot for 2 hours prior to the separation of serum by centrifugation. I therefore then tested whether allowing blood to clot for 2 hours prior to the separation of serum had an effect on the performance of the LPS - ELISA. In this work, a series of LPS standard solutions were spiked with serum, prepared from blood that was allowed to clot 2 hours, at a ratio of 1:10 (v/v) prior to the assay.

4.2.4.5 Determination of recoveries of LPS spiked into raw serum samples

A series of serum samples that were allowed to clot for 2 hours prior to separation of serum, were each spiked with a known concentration of LPS at a ratio of 1:10 (v/v) in order to determine the recoveries of LPS in serum samples.

4.2.4.6 Testing for effect of components in blood collection tubes on the levels of LPS detected in serum

Since the LAL assay for endotoxin was shown to be affected by constituents within blood collection tubes (discussed later in Chapter 6), I then determined whether there were differences between the quantities of LPS detected in serum prepared from plain glass blood collection tubes and those in serum prepared from plastic blood collection tube. The latter one contained clot activator. Eight blood samples were simultaneously collected into glass and into plastic vacutainers for this purpose. Again, samples were allowed to clot for 2 hours prior to the separation of serum.

4.3 Results

4.3.1 Endosafe® PTS method

4.3.1.1 The effect of sample diluents on the recovery of endotoxin spiked into diluted serum

The recoveries of spiked endotoxin ranged between 85 to 114 % in pooled serum samples that had been diluted either 1:30, 1:40 or 1:100 with DA. In contrast, the recoveries of endotoxin spiked into serum samples that had been similarly diluted with LRW were below 55 % and below 25 % when ESB was used as a sample diluent (Table 4-1).

Table 4-1. Recoveries of spiked endotoxin from diluted serum samples and from diluted and heated serum samples

Dilution Factor	Recovery (%) of endotoxin spiked after dilution			Recovery (%) of endotoxin spiked after dilution and heating		
	LRW	ESB	DA	LRW	ESB	DA
5	19	< 11	22	37	26	20
10	55	< 11	33	24	21	33
20	26	18	49	30	23	60
30	NA	NA	89	NA	NA	90
40	35	22	114	46	28	122
100	34	22	85	54	27	108

4.3.1.2 The effect of a combination of dilution and heating on the recovery of endotoxin spiked into denatured serum

Similarly to the previous set of experiment, only pooled serum samples that had been diluted either 1:30, 1:40 or 1:100 with DA prior to heating step, showed recoveries of spiked endotoxin between 90 % and 122 %, whereas samples that had been diluted either with LRW or ESB prior to heating step had recoveries below 55 % (Table 4-1).

4.3.1.3 Endogenous levels of endotoxin in serum samples

No endotoxin was detectable in serum samples that have been stored at -80°C whereas endotoxin was detectable in the fresh serum sample.

4.3.2 LPS-ELISA manufactured by Sun Red

In three out of five analysed plasma samples the levels of LPS, that were detected, were above the standard curve.

4.3.2.1 The effect of sample pre-treatment prior to the assay

Whilst dilution with each diluent caused a slight decrease in the levels of OD (Figure 4-3) the corresponding levels of LPS were two to five-fold higher than expected, in three out of five samples (data not shown). Further, the LPS-ELISA, manufactured by Sun Red, detected similar levels of LPS in all diluents that were tested (data not shown).

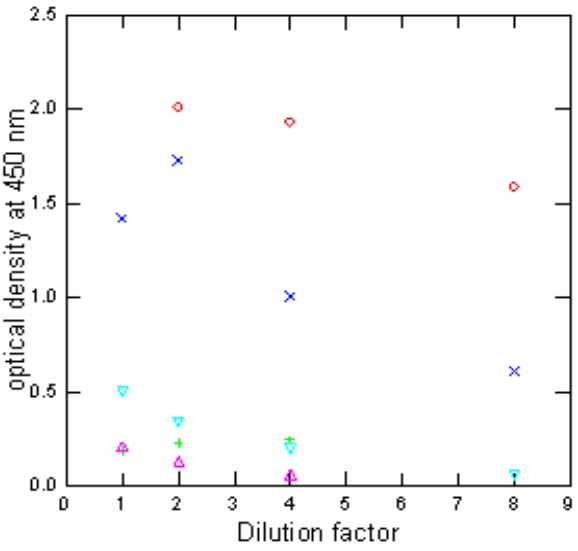

Figure 4-3. Dilution series of serum samples with MilliQ water as sample diluent using LPS - ELISA kit from Sun Red.

Sample 1, o; 2, x; 3,+; 4, ▽and 5, △

4.3.3 LPS-ELISA manufactured by Cusabio

The levels of LPS in five serum samples that were used for the evaluation of this assay, were within the lower half of the detection range of the assay (data not shown).

4.3.3.1 Interference by constituents of serum with the assay

The slopes of the LPS standard curves that were obtained were shallower when either pooled serum (prepared from blood that was allowed to clot for 30 min) or albumin were added to the series of LPS standards (Figure 4-4A).

4.3.3.2 *The effect of sample pre-treatment prior to assay*

The slopes of the standard curves for spiked and heated LPS standard solutions were shallower than those of unheated but spiked LPS standard solutions (Figure 4-4B).

4.3.3.3 *The effect of changes in the clotting time prior to the separation of serum*

The OD values obtained with the LPS standard solutions and those with the serum-spiked LPS standard solutions were similar when LPS standard solutions were spiked with sera prepared from blood that was clotted for 2 hours (Figure 4-4C).

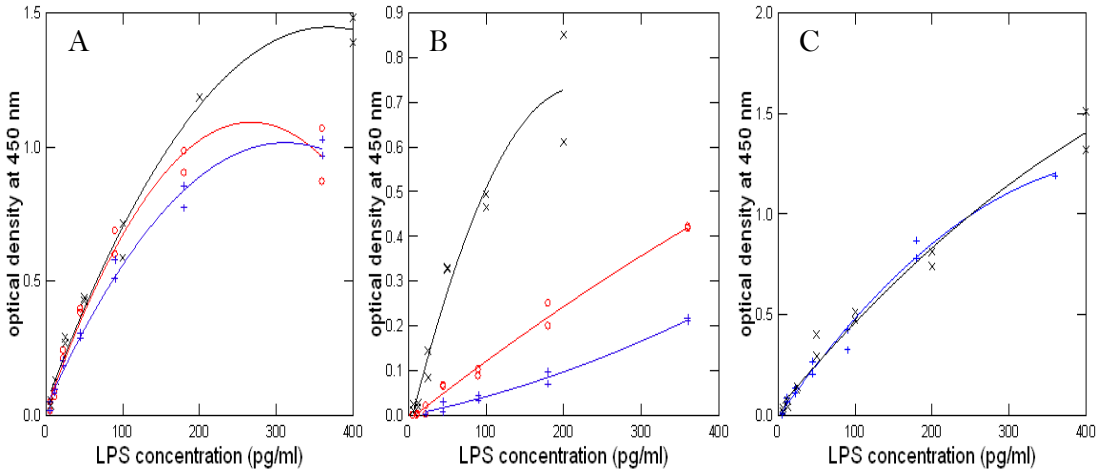

Figure 4-4. LPS standards compared to LPS standards spiked with serum or albumin.

A) LPS standards spiked with serum (from blood that was allowed to clot for 30 min) or albumin (40 µg/ml) at a ratio of 1:10. B) LPS standards that were spiked with serum (from blood that was allowed to clot for 30 min) or albumin (40 µg/ml) at a ratio of 1:10 (v/v) and then heated at 70°C for 10 min. C) LPS standards that were spiked with serum that was prepared from blood which was allowed to clot for 2 hours prior to separation of serum. LPS standards, black x; LPS standards spiked with serum, blue +; and LPS standards spiked with albumin, red o.

4.3.3.4 *Recoveries of LPS spiked into raw serum samples*

Analyses of 26 serum samples spiked with LPS indicated that between 85 and 126 % (median 97 %) of the spiked dose of LPS was recovered.

4.3.3.5 The effect of components in blood collection tubes on the levels of LPS detected in serum

There were no significant differences on paired t-test in the quantities of LPS detected in serum from blood collected into glass vacutainers from those collected into plastic vacutainers. Hence, plastic blood collection tubes were used for all subsequent work for safety reasons when blood was collected for the quantification of LPS in serum.

4.4 Discussion

Comparison of PPC values of pooled serum samples that were either simply diluted or diluted and heated prior to the LAL assay, indicated that a combination of dilution and heating was sufficient to reduce interference of the LAL assay by constituents of sera. However, data also indicated that at least with the Endosafe® PTS assay manufactured by Charles River Laboratories, no endotoxin seemed to be detectable in serum samples that were stored at -80°C whereas endotoxin was detectable in the fresh serum sample. Hence in further work, levels of systemic endotoxin were determined in fresh serum samples.

The LPS - ELISA, manufactured by Sun Red, detected significant levels of LPS in all diluents that were tested. Since the levels of LPS detected in endotoxin-free labelled water was similar to those in MilliQ water and the standard diluent provided with the kit, it seems unlikely that all three diluents were contaminated with LPS. Hence, it is likely that the tested diluents interfered with agents of the ELISA kit from Sun Red. Indeed, the manufacturer subsequently confirmed by e-mail that the kit was only intended for use with undiluted plasma samples.

A further problem with the kit manufactured by Sun red was that the LPS standard provided had mobile LPS-antibodies included with it so that no additional mobile LPS-antibodies were added during the performance of the assay. Hence, mobile LPS-antigen-antibody complexes within the standard solution reacted with the well-bound LPS-antibodies. Further, it appears that the quantity of mobile LPS-antibodies contained in the LPS standard solutions decreased from the highest to the lowest LPS standard. Hence, the LPS standards included with the kit seemed in effect to be a dilution series in which the OD values of standards decreased from the highest to the lowest concentration of LPS as the amount of mobile biotin-labelled LPS-antibodies in

LPS standard solutions decreased. This contrasted with the sample methodology in which a standard concentration of mobile biotin-labelled LPS-antibodies was added to each sample. Hence, the quantity of mobile LPS-antibodies that were added differed between the standards and the samples. Further the source of mobile LPS-antibodies varied between the two. Taken together, the assay procedure differed between the standard solutions and the samples. It is therefore questionable whether the OD values of LPS standards and those of samples were comparable and the calculation of the concentration of LPS in plasma samples were correspondingly suspect. Given the manner in which the LPS - ELISA manufactured by Sun Red is calibrated it seems unlikely that this kit is appropriate for the quantification of LPS in any sample.

In contrast, LPS standards and samples are processed in the same manner using the LPS - ELISA manufactured by Cusabio. Hence, OD values obtained in those two were comparable with one another. Data indicated that the quantification of LPS by this ELISA was inhibited by constituents of the sera e.g. albumin when blood samples were allowed to clot for 30 min prior to separation of serum. However, the inhibitory effect of serum was prevented when blood samples were allowed to clot for 2 h prior to separation of serum. Again, around 100 % of the known concentration of LPS spiked into serum was recovered when serum was prepared from blood that was allowed to clot for 2 h. Hence, it seems that a prolonged clotting interval of 2 h is required either to enable all fibrogen and associated proteins that may interfere with the LPS assay, to get bound in the clot or to remove constituents of the serum that may sequester LPS. It is noteworthy that the quantification of LPS by the ELISA from Cusabio was strongly inhibited when LPS standards spiked with serum were heated prior to assay. Since LPS is heat stable i.e. denatures only after heating at 250°C for 30 min, it seems likely that either the heating procedure denatured the serum proteins i.e. albumin, which then interfered with the assay in a greater degree than non-denatured serum

proteins do. Further, it is possible that LPS was sequestered by serum proteins during the process of denaturation which reduced the amount of LPS detected by the assay. In sum, the LPS - ELISA manufactured by Cusabio might be an appropriate assay for the quantification of LPS in serum at least when serum samples are allowed to clot for 2 h prior to assay.

4.5 References

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CHAPTER 5 PILOT STUDY

ASPIRIN-INDUCED INCREASE IN INTESTINAL PARACELLULAR PERMEABILITY DOES NOT AFFECT THE LEVELS OF LIPOPOLYSACCHARIDE IN VENOUS BLOOD OF HEALTHY WOMEN

The chapter is published as:

Gnauck A, Lentle RG, Kruger MC. Aspirin-induced increase in intestinal paracellular permeability does not affect the levels of LPS in venous blood of healthy women. J Innate Immun. 2015 Jul. 21(5):537-545.doi:10.1177/1753425914557101

Note: Unpublished work with the LAL assay manufactured by Charles River Laboratories, is described in section 5.6 after the references of the original research article. See also Appendix VI for the published paper.

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STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Anne Gnauck

Name/Title of Principal Supervisor: Prof Roger G. Lentle

Name of Published Research Output and full reference:
Gnauck A, Lentle RG, Kruger MC. Aspirin-induced increase in intestinal paracellular permeability does not affect the levels of LPS in venous blood of healthy women. 2015.21 (5): 537-45. doi:10.1177/1753425914557101.

In which Chapter is the Published Work: Chapter 5

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

  and / or

- Describe the contribution that the candidate has made to the Published Work:
  - Obtaining approval from the Massey University Human Ethics Commitee
  - Recruitment of subjects
  - Planing and conducting the study
  - Preparation of blood samples i.e. serum and urine, and sample analysis
  - Statistical data analysis and presentation of data
  - Literature search and writing the draft of the paper

A. Gnauck — Candidate's Signature

10/12/2015 — Date

Principal Supervisor's signature

10/12/15 — Date

GRS Version 3– 16 September 2011

Abstract

The presence of subclinical levels of lipopolysaccharide (LPS) from Gram-negative bacteria, also referred to as endotoxin, in the circulation may induce a pro-inflammatory immune response that leads to the development of obesity and insulin resistance. Recent data indicate that high-fat meals may elevate circulating levels of LPS. However, it is currently unclear how the LPS transits from the gut lumen to the general circulation. We determined whether aspirin-induced damage of the small intestinal mucosa, evidenced by an increase in the paracellular permeability, allows greater transit of LPS into the systemic circulation. The 3 hour cumulative excretion of lactulose was significantly increased after the consumption of aspirin solution relative to that after the consumption of an equal volume of water in 15 healthy female human subjects (median after aspirin 0.09% of dose vs. median after water 0.03% of dose, $p = 0.004$). Dosage with aspirin also significantly increased the lactulose-mannitol ratio (median after aspirin 0.014 vs. median after water 0.005, $p= 0.017$). However, serum LPS levels after the consumption of the aspirin solution were not significantly different from those after consumption of the control (plain water). Further, there was no correlation between body fat content and circulating levels of LPS.

5.1 Introduction

A number of publications suggest that chronically elevated levels of circulating endotoxin, also referred to as lipopolysaccharide (LPS), may be responsible for the pro-inflammatory status associated with obesity and insulin resistance in mice [1, 2] and humans [3]. Endotoxin is the immunoadjuvant fraction of the outer cell membrane of Gram-negative bacteria that is released on their lysis in the intestinal lumen. Plasma endotoxin levels are reported to increase post-prandially after the consumption of a high fat meal (HF) in both healthy [4-7] and morbidly obese human subjects [8]. Since in a mouse model, low levels of exogenous LPS are reported to have a similar effect in promoting the development of obesity, diabetes and insulin resistance as a HF-diet it has been assumed that elevated levels of circulating LPS are the link between diet-induced inflammation and the development of metabolic syndrome [1]. Hence, it seems that the rate of translocation of bacterial endotoxin from the gut lumen into the circulation may be influenced by diet. However, the mechanism by which a HF meal could facilitate the transit of endotoxin across the intestinal barrier is unclear. One hypothesis is that the ingestion of a HF meal induces oxidative stress in mucosal cells which leads to local inflammation and subsequent reduction in the integrity of the mucosal barrier, allowing greater quantities of endotoxin to traverse paracellular junctions [6, 9]. Published data showing that both oxidative stress and the expression of pro-inflammatory cytokines are increased in circulating monocytes after the ingestion of a HF meal and that both of these effects are accompanied by elevations in the levels of plasma endotoxin [5, 6] support such a hypothesis. Further, the observation that the consumption of orange juice with the HF meal mitigates the cellular oxidative stress, the rise in pro-inflammatory cytokine expression and the rise in plasma endotoxin level [9] suggests that the immune response may be influenced by the relative proportions of macronutrients. A similar effect is reported in human

subjects when HF meals are supplemented with a mixture of resveratrol and other polyphenols [10] suggesting that secondary plant metabolites may be instrumental in this mitigation.

An alternative hypothesis for the transit of endotoxin is that either free or micelle-bound luminal endotoxins are able to cross the mucosal barrier as a result of dietary absorption by enterocytes. The transiting endotoxin may then be incorporated into chylomicrons and released from the basolateral site of enterocytes into the systemic circulation or solubilised and released into the portal venous system. The former hypothesis is supported by evidence indicating that concentrations of endotoxin in the fatty (chylomicron) fraction of plasma from human subjects are higher than those in the aqueous phase [7, 8, 11]. This mechanism is further supported by the finding that blocking the formation of chylomicrons in a mouse model prevents the dietary fat-induced increase of circulating endotoxin [11]. Note, it is also conceivable that the accumulation of chylomicrons at the basolateral membrane could subsequently increase paracellular permeability, as it is thought to influence tight junction integrity [12], and thus could lead to LPS entry via the aqueous and not the fatty phase.

Given that the circulating component that carries the endotoxin varies according to the route by which it is absorbed, fat associated endotoxin that is absorbed transcellularly would be found in the chylomicron fraction whereas water soluble endotoxin that is absorbed via the paracellular route would be found in the aqueous phase of the serum.

A further hypothesis, based on findings in obese mice, is that the consumption of a HF diet leads to shifts in the composition of the enteral microbiota and a change in the characteristics of the 'cross-talk' between microbiota and mucosa that leads in turn to a reduction in tight junction integrity with consequent augmentation of paracellular

absorption of endotoxin [13]. However, it seems unlikely that a single HF meal would promptly alter the composition of intestinal microbiota.

The aims of the current study were firstly, to investigate whether an augmentation in paracellular permeability is accompanied by an increase in the translocation of LPS/endotoxin from the lumen of the gut into aqueous components of serum of healthy subjects. The increased permeability of the gut to larger probes i.e. lactulose (a molecule with a hydrated molecular volume of 0.262 nm^3) [14] following a single 600 mg dose of aspirin is well described in healthy female subjects [15, 16]. This may result from disruption of the mechanical integrity of the enterocyte apex [17-19]. We therefore examined whether, in healthy female subjects, the consumption of this dose of aspirin augmented circulating levels of LPS in the serum from systemic venous blood, using a human LPS-specific ELISA. This LPS - ELISA, rather than the Limulus Amebocyte Lysate (LAL) assay for endotoxin, was used as the LAL assay may be inconsistent i.e. dose equivalence between LPS (pg/ml) and endotoxin (endotoxin units/ml) may vary between bacterial species [20] and hence not consistently reflect the total quantity of circulating LPS. We also examined whether there was any correlation between percentage body fat and LPS levels in the systemic circulation.

5.2 Subjects and Methods

5.2.1 Subjects

15 healthy female non-smokers of mixed ethnicity, aged between 20 and 37 years (median age 28 years), of body height between 1.58 to 1.73 m, and body mass between 55 and 75 kg, were recruited from a fit and healthy population of female postgraduate students and university staffs in Palmerston North, New Zealand. Female subjects were used so as to avoid confounding of the result by variation of gastric emptying times [21, 22] and body fat content with gender that could influence the recoveries of lactulose and mannitol.

Given prior work linking obesity determined by waist and abdominal circumference and intestinal permeability to lactulose [23] particular attention was paid to eliminating obese subjects and quantifying any effect from variations in percentage body fat. Hence, subjects with a BMI above 30 were excluded from the study. The percentage of body fat of each subject was determined by whole body densitometry using air-displacement plethysmography in a Bod Pod [24]. Further, each subject completed a health screening questionnaire (Appendix II - iii) to exclude those with a family or personal history of gastrointestinal disorders, any endocrine or metabolic disorders including diabetes; and hepatic disorders or with a history of recent abdominal pain, nausea, vomiting, diarrhoea or of passage of blood and mucus in stool. Subjects who had a history of current urinary tract infections, vaginal discharge, history of aspirin sensitivity or who had recently consumed prebiotic or probiotic products and who consumed more than one alcoholic drink per day were also excluded from the study.

5.2.2 Study protocol and design

Participants were instructed to refrain from consuming any antibiotics for at least two weeks and from any NSAIDs for at least one week prior to each experimental session. In addition, participants were instructed not to consume any alcohol for at least three days prior to each experimental session nor to eat any fried or fatty food and to avoid exercise on the day prior to attending each experimental session. Participants attended the Human Nutrition Lab at Massey University Palmerston North campus after an overnight fast. On arrival participants were instructed to empty their bladder into a container and subsequently a 4 ml sample of venous blood was drawn for baseline measurements. Each participant then ingested either 100 ml of water (negative control) or a single dose of 600 mg soluble aspirin (Dispirin®; Reckitt Benkinser Healthcare, UK) dissolved in 100 ml of water.

Each of the two treatments was administered to each subject in randomized order with a 1-week interval between treatments. One hour after each treatment had been administered, each participant consumed a drink containing 10 g lactulose (duphalac®; Abbott, Netherlands) and 5 g mannitol (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 100 ml of water followed immediately by further 300 ml of water. Subjects were instructed to urinate into a single container during the subsequent 3 hour period so as to bulk their urine over this period. At 3 hours after the administration of the lactulose-mannitol solution, each subject was instructed to empty their bladder into the same container. A further venous blood sample was then drawn. The study protocol was approved by the Massey University Human Ethics Committee (MUHEC): Southern A 13/31 (Appendix II - i) and informed written consent was given by all volunteers (Appendix II - iv).

5.2.3 Determination of urinary lactulose and mannitol

The volume of the initial urine sample and the total three hour volume of urine were determined for each subject. A 15 ml aliquot of each sample was centrifuged at 3500 *g* at 4°C for 10 min, and the supernatant stored at -80°C pending HPLC analysis.

Each urine sample was pre-treated with 1 g Amberlite® resin (Fluka® Analytical, Sigma Aldrich, St. Louise, MO, USA) and a 20 µl aliquot of filtrate from each sample or standard then analysed in a Shimadzu HPLC system as described previously (3.5.3). The concentrations (in µg/ml) of lactulose and mannitol in each filtrate were calculated from the areas under the curve (AUC) relative to those of a series of concurrently run standards with lactulose and mannitol at a concentration ranged from 62.5 to 500 µg/ml. Calibration curves for the two sugars were linear up to 500 µg/ml. The intra-assay coefficient of variation was in the range 0.2 - 7.7 % for lactulose and 0.1 - 4.6 % for mannitol.

5.2.4 Quantification of levels of LPS in human blood

Venous blood was collected into BD vacutainer® serum (6.0 ml; Becton, Dickinson and Company, UK) and allowed to clot at room temperature over a period of 2 hours before centrifuging for 15 min at 3.500 *g* and 4°C. The serum was decanted into endotoxin-free micro-centrifuge tubes (Biopur® Safe-Lock micro test tubes; Eppendorf, Germany). Serum samples were further centrifuged at 12,000 rpm and 10°C for up to 4 hours. Aliquots of each sample were stored at -20°C pending LPS analysis.

Serum LPS concentrations were determined by LPS - ELISA (Human Lipopolysaccharides (LPS) ELISA kit; Cusabio® Biotech Co., Ltd, Wuhan, Hubei Province, P.R. China) according to the manufacturer's instructions. Only endotoxin-free certified tubes (Biopur® Safe-Lock micro test tubes; Eppendorf, Germany) and

pipette tips (Endosafe® tips, Charles River, France) were used for the performance of the LPS - ELISA. Standard curves with known concentrations of LPS in the range between 6.25 and 400 pg/ml were run concurrently with the samples. The results were plotted in CurveExpert Professional version 2.02. The LPS concentration in each sample was determined by comparison with the standard curve. The intra- and inter-assay precisions for the LPS - ELISA determined in our laboratories were < 10 %.

5.2.5 Statistical analysis

Statistical analyses were performed in the SYSTAT statistical software package version 13 (Systat Software Inc., Chicago, IL, USA). Differences between treatments in baseline LPS levels were assessed by paired Student's t-test. Differences between baseline and post-treatment levels of LPS after the consumption of water (negative control) and after the consumption of aspirin were also each assessed by paired Student's t-test. Differences between baseline and post-treatment levels of LPS with the negative control and with the treatment with aspirin were assessed by doubly repeated measures ANOVA. The effects of aspirin and the negative control on the excretion of lactulose and mannitol and on LMR were each compared by paired Student's t-test. Differences were considered significant at the $p < 0.05$ level.

The correlation between the baseline LPS levels taken prior to control and those before the treatment with aspirin was assessed by linear regression and Pearson correlation coefficient and the Bartlett Chi-square statistic. Correlations between baseline LPS levels and the excretions of lactulose following treatment with water and following aspirin, respectively, were similarly assessed by linear regression, Pearson correlation coefficient and Bartlett Chi-square statistic. Correlation was considered as significant at the $p < 0.05$.

Bland-Altman plots of LPS levels at baseline and post-treatment following consumption of water and following consumption of the solution of aspirin were each assessed for correlation. Bland-Altman plots depict the variations in differences in values obtained with two treatments with the mean of the values obtained with the two treatments and hence are considered to better indicate the consistency of correlation between two parameters than does simple linear regression [25].

5.3 Results

5.3.1 Body composition of subjects

The 15 female subjects were of similar body height (median 1.66 m; range 1.58 – 1.73 m) but their body mass (median 62.6 kg; range 59.8 – 74.4 kg), quantity of body fat in kg (median 17.5 kg; range 8.3 – 27.8 kg), percentage of body fat (median 28.1 %; range 13.5 – 40.7 %) and Body Mass Index (BMI) (median 23.1; range 21.1 – 27.5) varied considerably.

5.3.2 Variations in serum baseline LPS levels in healthy human subjects

The serum LPS concentrations required Asinh conversion by Johnson Transformation in Minitab version 16 to render them amenable to parametric statistical analysis.

The baseline levels of serum LPS varied widely between subjects. There were no significant differences on paired Student's t-test in the baseline levels of LPS taken prior to the two treatments. There was a significant linear regression (df = 1, 13; F= 19.920; $p = 0.001$) and the correlation coefficient between the two was significant (df = 1; Bartlett Chi-Square = 10.420; $p = 0.001$; $r^2 = 0.752$) (Figure 5-1). Hence, baseline LPS levels were relatively invariant within subjects.

Given the considerable range in baseline serum LPS levels between subjects, the subjects were grouped on a basis of their magnitude (Table 5-1). Hence, group 1 (n = 2) comprised subjects with no detectable baseline LPS. Group 2 (n = 8; 33.03 ± 13.37 pg/ml) comprised subjects with baseline levels of LPS between 0.10 and 0.70 pg/ml and group 3 subjects (n = 3; 99.05 ± 12.03 pg/ml) with baseline LPS concentration over 0.70 pg/ml. Two subjects were excluded from this classification as their baseline values fell across groups.

The baseline serum LPS levels were not significantly correlated, either with body weight (kg), body fat (kg and %) or with BMI (data not shown). Similarly control lactulose-mannitol ratios were not correlated with either percentage of body fat or BMI (data not shown).

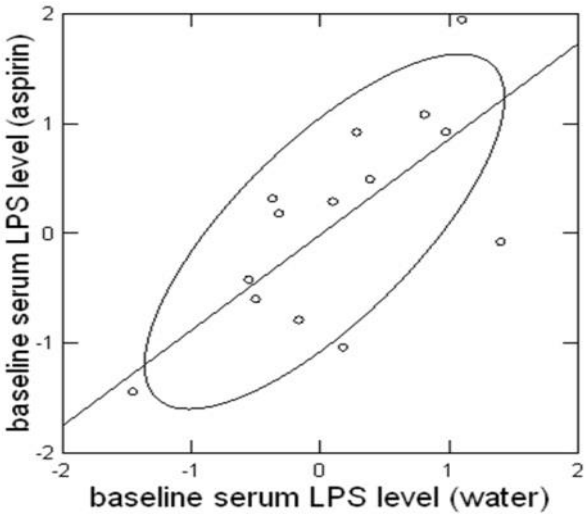

Figure 5-1. Correlation between successive serum baseline LPS levels from 15 subjects.

There was a strong correlation between the two baseline LPS levels within subjects (r = 0.752; p = 0.001; confidence ellipse of p = 0.6827).

Six of 15 subjects reported that they did not consume any alcohol, seven of 15 subjects drank less than three alcoholic drinks a week and two of 15 subjects drank between three and seven alcoholic drinks a week. However, there were no significant differences in LPS levels and lactulose - mannitol ratios between these three groups of subjects (data not shown). No lipid fraction was visible after the centrifugation of serum samples taken from any of the subjects with either of the two treatments. Hence, the quantity of LPS contained within it could not be quantified.

Table 5-1. Mean LPS levels at baseline and 4 hours after taking water

	water		aspirin	
	baseline	after 4 h	baseline	after 4 h
group 1 ***(n = 2)***	< 6.25 pg/ml	< 6.25 pg/ml	< 6.25 pg/ml	< 6.25 pg/ml
group 2 ***(n =8)***	33.03 ± 13.37 pg/ml	35.43 ± 15.89 pg/ml	37.34 ± 21.25 pg/ml	39.67 ± 20.02 pg/ml
group 3 ***(n = 3)***	99.05 ± 12.03 pg/ml	109.21 ± 10.18 pg/ml	141.26 ± 69.40 pg/ml	125.12 ± 79.48 pg/ml

group 1: no LPS detectable; group 2: baseline LPS range 0.10 - 0.70 pg/ml; group 3: baseline LPS level > 0.70 pg/ml

5.3.3 The effect of aspirin on serum LPS levels

There were no significant differences on paired Student's t-test between pooled baseline and post-treatment LPS levels on results after dosage with water. Similarly there were no significant difference between pooled baseline and post-treatment LPS levels after dosage with aspirin solution. Again, there were no significant differences between the three groups in the change in LPS levels from baseline values to those after consumption of water or in the change in LPS levels from baseline values to those after consumption of aspirin solution (Table 5-1). Similarly, there were no significant differences between treatments on doubly repeated ANOVA of baseline levels of LPS and post-treatment levels of LPS after consumption of water and after the consumption of aspirin solution. Neither were there differences when baseline values were subtracted from the corresponding post-treatment level for aspirin and water treatment and compared by one way ANOVA (data not shown).

Bland-Altman plots of baseline LPS levels and those after the consumption of water (Figure 5-2A) showed that there were no consistent differences between the two sets of readings. There was also no consistent relationship between the magnitude of the results and the difference between them. Hence, with the exception of the two very high readings, the water treatment (negative control) had no discernible effect on LPS levels.

Similarly, Bland-Altman plots of baseline LPS levels and those after the consumption of aspirin solution (Figure 5-2B) showed no consistent differences between the two readings. Again, scatters of the differences between baseline LPS levels and those after treatment did not differ in any consistent manner between the water and the aspirin treatment.

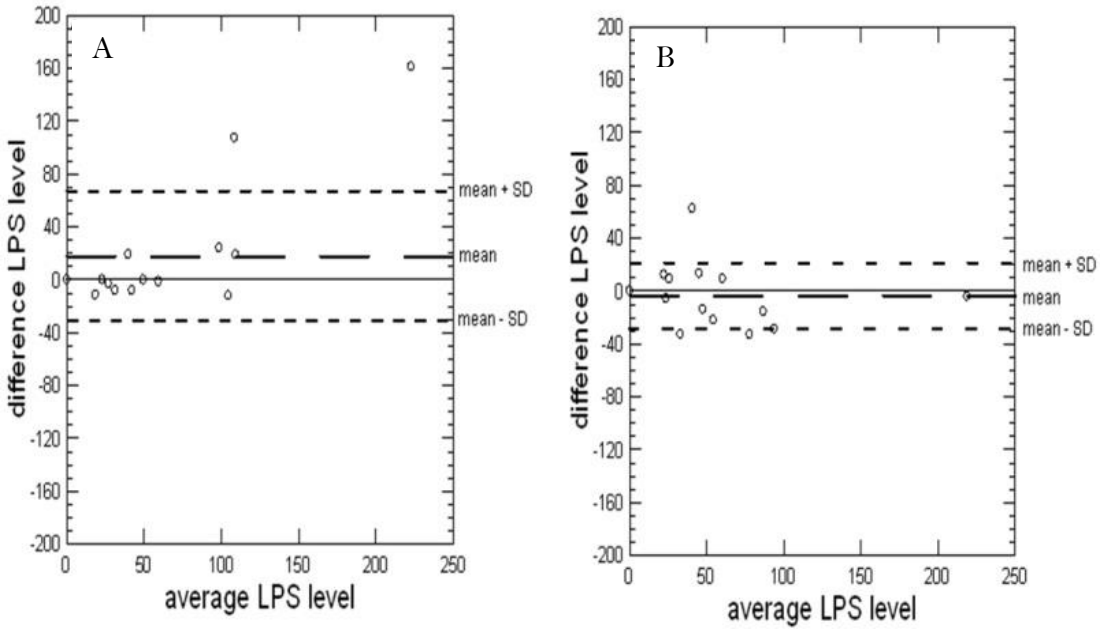

Figure 5-2. Bland-Altman plots of LPS levels after consumption of water (A) and of aspirin solution (B) treatment.

Neither plot shows a significant tend or shift from the mean with increase in magnitude of the mean. Larger (central) dashed line shows mean; smaller dashed lines are at one standard deviation from the mean.

5.3.4 The effect of aspirin on the excretion of lactulose and mannitol

The percentage of recoveries of the administered dose of mannitol were normally distributed whereas these for lactulose and the ratio of lactulose to mannitol (LMR) required Asinh and logarithmic transformation, respectively (Johnson Transformation in Minitab version 16), to render them amenable to parametric statistical analysis.

The 3 hour excretion of lactulose was significantly increased after the consumption of aspirin on paired student t-test (df = 14; t = -3.454; p = 0.004) compared to that after the consumption of water (median $_{aspirin}$ 0.090 % of dose, 25-75 % interquartile range (IQR) 0.065 – 0.135 % of dose; median $_{water}$ 0.030 % of dose, IQR 0.010 – 0.045 % of dose) (Figure 5-3 A). Conversely, the 3 hour excretion of mannitol was not significantly different after the consumption of aspirin from that after the consumption of water (median $_{aspirin}$ 6.294 % of dose, IQR 5.178 – 7.385 % of dose vs. median $_{water}$ 6.229 % of dose, IQR 4.553 – 10.036 % of dose) (Figure 5-3 B). Concomitantly, the 3

hour lactulose-mannitol ratio (LMR) was significantly greater (df = 14; t = -2.716; p = 0.017) after the consumption of aspirin treatment compared to that after the consumption of water (median $_{aspirin}$ 0.014, IQR 0.009 – 0.037 vs. median $_{water}$ 0.005, IQR 0.002 – 0.009) (Figure 5-3 C). The elevated LMR of one subject in the water treatment group was due to a greatly reduced excretion of mannitol.

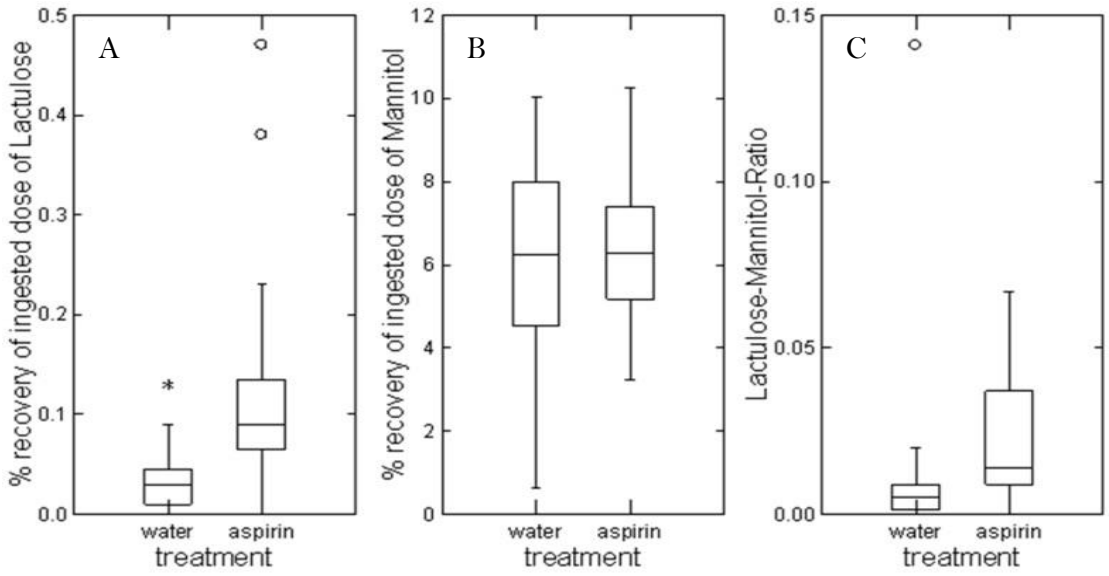

Figure 5-3. Box plots of % 3 hr recovery of total dose of lactulose (A) and of mannitol (B) and change in lactulose mannitol ratio (C) after the consumption of water or of aspirin solution.

The excretion of lactulose significantly increased (p = 0.004) after the consumption of aspirin treatment. The excretion of mannitol after the consumption of water did not differ significantly from that after the consumption of aspirin. The lactulose-mannitol ratio was significantly higher (p = 0.017) after the consumption of aspirin compared with that after the consumption of water.

5.3.5 Correlation between lactulose excretion and serum LPS concentration

There was no significant correlation within subjects between baseline serum LPS levels and the percentage of lactulose excretion after the consumption of water or after the consumption of aspirin solution (data not shown). There was no significant correlation between the differences between baseline and post-treatment levels of LPS with those of lactulose excretion either after the consumption of water or after the consumption of aspirin solution (Figure 5-4A). There was also no significant correlation between baseline and post-treatment values for LMR and those of LPS

(Figure 5-4B). Whilst there were three subjects with elevated recovery of lactulose after the consumption of water, the same subject did not have elevated baseline levels of LPS (above 70 µg/ml) before the consumption of water (although 3 other subject did). Together these results indicate that serum baseline LPS levels are not influenced by paracellular permeability of the small intestine.

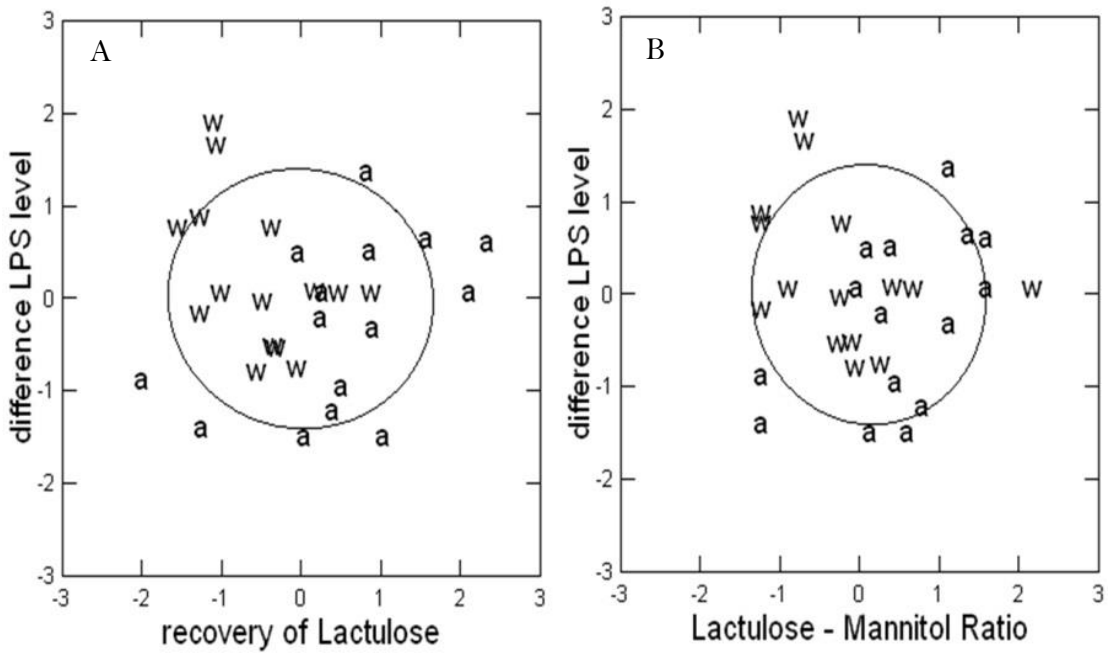

Figure 5-4. Correlations between % recovery of Lactulose (A) or LMR value (B) with differences in LPS levels prior to and after the consumption of aspirin solution (a symbol) or water (w symbol).

There was no correlation between the recovery of lactulose and the difference in LPS level over the two treatments. Similarly, the lactulose-mannitol ratio (LMR) did not correlate with the difference in LPS level over the two treatment (confidence ellipse of p = 0.6827).

5.4 Discussion

This is the first study to determine the relationship between paracellular intestinal permeability, as measured by lactulose permeability, and circulating levels of LPS.

Since the levels of serum LPS that were determined after the consumption of plain water did not differ from those determined prior to consumption of water it is evident that the consumption of the lactulose and mannitol solution had no effect on circulating levels of LPS. This result is similar to that reported with actively absorbed sugars such as glucose [6]. Hence, our method is suitable for the evaluation of any correlation between the levels of LPS in the systemic circulation and the permeability of the gut wall.

The high degree of variation in the baseline levels of serum LPS in our sample of 15 apparently healthy female subjects fits with reports of similar levels of variation in plasma endotoxin in 201 men between 45 and 64 years [26]. Our results, showing no correlations of serum LPS levels with either percentage of body fat or BMI, are in agreement with those of other studies [26, 27]. However it is noteworthy, that the range of percentage of body fat in our subjects (13.5 to 40.7 %) did not include morbidly obese subjects i.e. these with BMI > 30 whilst other studies investigating endotoxin levels have included morbidly obese subjects with an BMI > 30 [8].

The finding that the consumption of 600 mg aspirin increases gut permeability, as determined by elevation of the urinary excretion of lactulose, is in line with previous reports [15, 16]. Hence local, aspirin-induced inflammation of the intestinal mucosa [17-19] increase mucosal paracellular permeability [15-17, 28]. However, the fact that the levels of LPS in the systemic circulation were not significantly elevated after the consumption of aspirin indicates that such mucosal damage does not influence the ability of LPS to transit from the lumen to the systemic circulation. It is conceivable

that an aspirin-induced increase in paracellular permeability did allow greater amounts of LPS to enter the portal circulation via the paracellular route but that this LPS was removed by the liver and hence did not enter the systemic circulation. The latter hypothesis is supported by the finding that significant levels of endotoxin were found in the portal venous blood but not in the systemic blood of human subjects at laparotomy [29]. Similarly by the finding that the levels of endotoxin in portal venous blood were elevated in genetically obese mice with increased intestinal permeability [30]. The systemic venous blood of human subjects with hepatic cirrhosis contains significant levels of endotoxin [31] whilst those of subjects with a healthy liver do not [29]. In addition, the concentration of endotoxin in systemic venous blood is reported to be significantly correlated with the level of liver damage in human subjects with non-alcoholic liver disease [32]. Therefore, it seems likely that, in healthy subjects, any LPS in the portal venous blood will be detoxified or removed in the liver before it enters the systemic circulation [29, 30].

It is also possible that LPS is absorbed transcellularly from the gut lumen within fat micelles and incorporated into the chylomicrons [7, 11]. Hence, LPS may transit with the chylomicron fraction, via the lymphatic system, to the systemic circulation and avoid detoxification in the liver [33, 34]. Since our subjects had been fasted overnight their venous blood did not contain a significant chylomicron fraction or LPS within the fatty phase. Hence, further work is required to determine whether the paracellular transit of lipid associated LPS into the lymphatic and systemic circulation is elevated after the consumption of aspirin.

It is noteworthy that this study was conducted on non-smoking young healthy females and the results may not necessarily apply to males or to persons with BMIs > 30. Hence for example the magnitude of the effect of a single dose of aspirin may differ in

smokers and gut permeability may change in early pregnancy [35]. Again as the level of body fat differ in males the relation of this with LPS and with LMR may differ.

5.5 References

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5.6 Unpublished work with the LAL assay manufactured by Charles River

As mentioned previously, published studies determined levels of systemic endotoxin using the LAL assay to assess the degree of LPS translocation from the gut into the systemic circulation. Hence, another objective of the pilot study was to investigate whether a temporal augmentation in paracellular permeability, as induced by a single dose of 600 mg of aspirin, is accompanied by an increase in the level of systemic endotoxin. Levels of systemic endotoxin were therefore determined in aliquots of serum samples of the pilot study using the Endosafe® PTS kit manufactured by Charles River Laboratories (Charleston, SC, USA). Data obtained with this LAL assay were thought to allow comparison of my data with those published by others as well as allow description of the correlation between (total) levels of systemic LPS, as determined by ELISA, and those of systemic endotoxin, as determined by LAL assay. However, data indicated that the assay was not reliable. Hence, data obtained with the LAL assay were not published in the original research article but are now presented in this section of the chapter.

5.6.1 Quantification of levels of systemic endotoxin using the Endosafe® PTS kit

5.6.1.1 Assay procedure

Levels of endotoxin in serum samples in 10 out of 15 subjects were determined using the Endosafe® PTS (Charles River Laboratories, Charleston, SC, USA). Subjects were randomly chosen for the quantification of systemic endotoxin prior to the start of the study. The analysis was performed on aliquots of fresh serum samples prepared from venous blood that was collected into plastic serum BD vacutainer® (6.0 ml; BD, Oxford, UK). Fresh serum samples were diluted by 30-fold with Endosafe® Dispersing

Agent (Charles River Laboratories, Charleston, SC, USA) in endotoxin-free certified glass tubes (borosilicate glass tubes, Charles River Laboratories, Charleston, SC,USA) and vortexed for 30 s. Diluted samples were then heated at 80°C for 10 min. Samples were allowed to cool down before beeing transferred into endotoxin-free certificated micro-centrifuge tubes (Biopur® Safe-Lock micro test tubes; Eppendorf, Hamburg, Germany). Levels of serum endotoxin were determined by Endosafe® PTS as described previously in 3.2.3.2. Briefly, 25 µl of fresh serum was added to each of the four channels of the Endosafe® PTS cartridges. Levels of endotoxin in serum samples and the values for the PPC (recovery of known concentration of endotoxin spiked into the sample) were automatically generated by the Endosafe® PTS software and displayed on the result report.

In total forty fresh serum samples of the pilot study were analysed. Eight of those samples were analysed twice and two out of those eight samples were analysed a third time (10 repeated tests in total). Two out of the 10 repeated tests were performed because the detected levels of endotoxin in the first test were extremely high. In sum, 50 Endosafe® PTS cartridges with a stated detection range from 0.005 to 0.5 EU/ml (5 from lot 3163540 and 45 from lot 3120564) were used.

5.6.1.2 Comment on the method used for the calculation of endotoxin levels in samples

It is noteworthy that the Endosafe® PTS software calculated the mean endotoxin concentration of the sample based on the mean of the reaction time of the two measurements for each sample and subsequently calculated the CV value based on the reaction time of the two measurements. Similarly, the mean endotoxin concentration of the PPC was calculated that way. However, I would first determine the endotoxin concentration for each measurement and then use those values to determine both the

mean endotoxin concentration of the sample and the CV. Similarly, I would use this method to calculate the mean level of endotoxin of the PPC and the corresponding CV. There were no significant differences in the mean levels of systemic endotoxin between the two calculation methods. However, the CV values calculated with the Endosafe® PTS software were significantly different ($df = 40$, $t = 7.407$, $P < 0.001$) to those based on 'my' calculation method on paired Student's t-test. For example, 30 out of 50 samples had CV values below 10 %, 12 out of 50 samples had CV values between 10.1 and 25 % and 8 out of 50 samples had CV values above 25 % when the Endosafe® PTS software was used for the calculation. This data indicate a somewhat satisfactory accuracy of the assay according to the manufacturer's specification that defines tests with CV values below 25 % as valid tests. In contrast, 9 out of 50 samples had CV below 10 %, 13 out of 50 samples had CV values between 10.1 and 25 % and 28 out of 50 samples had CV values above 25 % when 'my' calculation method was used. Similarly, the intra-assay CVs of the PPC were above 25 % in 22 out of 50 endotoxin-spiked samples using 'my' calculation method. In sum, the intra-assay CVs of both sample and PPC were above 25 % in 19 out of 50 performed tests. Three out of 7 samples with repeated measurements had inter-assay CVs above 25 % and 6 out of 8 PPC had inter-assay CVs above 25 %.

5.6.2 Results

Data presented in this section were calculated based on 'my' calculation method. The levels of endotoxin determined were not normally distributed. The median level of endotoxin and the range of determined endotoxin levels in serum samples taken prior to and after the treatment with either water or aspirin are listed in Table 5-2. Data indicated that the consumption of aspirin had no effect on the levels of systemic

endotoxin in healthy women. However, since the reliability of the data was questionable no statistical data analysis was performed i.e. comparison between levels of systemic endotoxin between treatment and correlation analysis between levels of systemic endotoxin and those of systemic LPS.

Table 5-2. Median serum endotoxin levels prior to and after the treatment with either water or aspirin.

treatment	Endotoxin levels (EU/ml)	
	Prior to treatment	After treatment
Water	0.38 (0.21 - 0.96)	0.41 (0.17 - 1.04)
Aspirin	0.45 (0.26 - 0.95)	0.44 (0.21 - 0.87)

5.6.3 Conclusion

The discrepancy in CV values between the two calculation methods seems to be due to the fact that values of the reaction time (in s) in serum samples were high and the corresponding levels of endotoxin (in EU/ml) relative low. For example, the reaction time of 596 s and 718 s resulted in a CV of 13.1 % whereas the corresponding endotoxin levels of 0.041 EU/ml and 0.022 EU/ml resulted in a CV of 42.7 %. Hence, the calculation of CV values based on the reaction time overestimates the accuracy of the assay. In general, both the reliability of the Endosafe® PTS assay as determined by intra-assay CVs, and the reproducibility of the assay as determined by inter-assay CVs, were poor when 'my' calculation method was applied. This finding further indicated that the LAL reagents in this particular LAL kit were highly sensitive to interferences by constituents of serum samples.

Moreover, it is noteworthy that the recovery of spiked endotoxin (PPC) in Endosafe® PTS cartridges (and in I/E Screening cartridges) was determined based on the known concentration of endotoxin in the spiked channel and the 'endogenous' concentration of endotoxin in the sample neglected. Hence, the PPC automatically generated by the Endosafe® PTS software either underestimated the inhibition (when determined

endotoxin concentration was below the spiked endotoxin concentration) or overestimated the enhancement (when determined endotoxin concentration was above the spiked endotoxin concentration) of the assay by the sample. The PPC varied broadly between 35.2 and 220.1 % with a mean of 103 % when the calculation of PPCs included the 'endogenous' concentration of endotoxin in the sample. Again, this data indicated that the Endosafe® PTS assay was highly sensitive to interferences by pre-treated fresh serum samples. Given the fact that the accuracy of the Endosafe PTS kit was questionable no correlation analysis between serum levels of LPS determined with the ELISA and those of endotoxin determined with the Endosafe PTS was conducted.

Taken together, the accuracy and reliability of endotoxin levels obtained with the Endosafe® PTS were questionable and the Endosafe® PTS kit seemed not to be an appropriate method for the assessment of the levels of systemic endotoxin in humans.

CHAPTER 6 METHOD EVALUATION – INTRALIPID STUDY

ADAPTATION OF THE KINETIC-QCL™ LAL ASSAY FOR USE WITH SERUM SAMPLES AND EVALUATION OF AN ELISA FOR THE QUANTIFICATION OF LBP IN SERUM

The parts of the adaptation of the kinetic-QCL™ LAL assay, manufactured by Lonza, for the quantification of endotoxin in human serum are published as:

Gnauck A, Lentle RG, Kruger MC. The Limulus Amebocyte Lysate assay may be unsuitable for detecting endotoxin in blood of healthy female subjects. J Immunolog Methods. 2015. 416:146-56. doi:10.1016/j.jim.2014.11.010.

See Appendix VII for the original published paper.

DRC 16

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STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Anne Gnauck

Name/Title of Principal Supervisor: Prof Roger G. Lentle

Name of Published Research Output and full reference:
Gnauck A, Lentle RG, Kruger MC. The Limulus Amebocyte Lysate assay may be unsuitable for detecting endotoxin in blood of healthy female subjects. J Immunolog Methods. 2015. 416: 146-56. doi: 10.1016/j.jim.2014.11.010.

In which Chapter is the Published Work: Chapter 6

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

  and / or

- Describe the contribution that the candidate has made to the Published Work:
  - Obtaining approval from the Massey University Human Ethics Commitee
  - Recruitment of subjects
  - Preparation of blood samples i.e. plasma and serum
  - Planing and conducting of the experimental work
  - Statistical data analysis and presentation of data
  - Literature search and writing the draft of the paper

A. Gnauck
Candidate's Signature

10/12/2015
Date

Principal Supervisor's signature

10/12/15
Date

GRS Version 3– 16 September 2011

6.1 Abstract

Previous data indicated that the Limulus Amebocyte Lysate (LAL) assay is sensitive to interferences by constituents of serum. In this chapter the factors that may influence the performance of the LAL assay are examined using a method that directly displays the reaction curve. Data obtained with this assay indicated that a number of types of proprietary plastic vacutainers appeared to contain significant amounts of endotoxin. Further that serum interferes with the LAL assay which can be prevented by the pre-treatment of the sample i.e. a combination of dilution and heating, prior to the assay. However, the method for the calculation of endotoxin levels recommended by the manufacturers, based on the reaction time, was inaccurate with pre-treated serum samples due to the slowing of initial phase of the reaction. An alternative calculation algorithm that is more accurate for the use with pre-treated serum samples is therefore described in this chapter. Further, I found that components of fresh serum irreversibly sequester endotoxin but that this action may be largely prevented by dilution and heating, but only if this occurs prior to the addition of endotoxin. Hence, even when appropriate blood collection containers and calculation methods were used, the levels of endotoxin in serum samples detected by the LAL assay were unlikely to reflect the total quantities of endotoxin in that sample and were more likely to reflect the capacity of a given serum sample to sequester endotoxin. Given the difficulties with the LAL assay an ELISA for the quantification of LBP in human serum was evaluated. Data obtained with the LBP - ELISA manufactured by Cusabio, suggested that the performance of this assay was satisfactory and may allow therefore the indirect assessment of the degree of LPS/endotoxin exposure in the systemic circulation.

6.2 Introduction

The intra-assay CVs of endotoxin results obtained in serum samples of the pilot study (Chapter 5) indicated that the LAL assay, manufactured by Charles River Laboratories, was not a reliable method for quantifying endotoxin in human serum. Further, Positive Product Control (PPC) (recovery of endotoxin spiked into pre-treated serum) varied widely from 35.2 to 220.1 % (mean 103 %) indicting that at least this type of LAL assay is sensitive to interferences by constituents of serum. Since the Endosafe PTS® assay did not allow the reaction curve to be visualised it prevented a more detailed investigation of the effects of constituents of serum on the LAL reaction. The work in this chapter used the 'transparent' kinetic-QCL™ LAL assay manufactured by Lonza that is conducted on a micro-plate reader. This assay allowed the reaction curves to be visualised and the factors that influenced the outcome of the LAL reaction to be investigated.

Given the fact that LPS is rapidly sequestered by constituents of the blood and has therefore a short half-life in the systemic circulation [1] the levels of detectable 'free' LPS/endotoxin in serum are likely to be low and not reflect the 'true' level of exposure to LPS. Hence, the levels of LPS-binding protein (LBP) in sera are likely to be more reliable in this respect. The evaluation of the LBP - ELISA, manufactured by Cusabio, is also described in this chapter.

6.3 Methods

6.3.1 Subject and sampling of blood samples

For the experiments using the kinetic-QCL™ LAL kit, venous blood samples were drawn from three healthy, non-obese, non-smoking female postgraduate students aged between 27 and 32 years. Samples were each drawn from each volunteer into a 'plastic' 'serum' (6.0 ml, Ref. 367837; BD, UK) and a glass 'serum' (10ml, Ref. 366430; Becton, Dickinson and Company (BD), USA) vacutainer after fasting. In addition blood samples were drawn into plastic 'serum' and into glass 'serum' vacutainers from the second volunteer 1.5 and 3 hours after breakfast. Also four samples of post-prandial venous blood were drawn from the third volunteer into both plastic and glass 'serum' vacutainers on different occasions.

All samples were allowed to clot for 2 h at room temperature and subsequently centrifuged at 3,500 g at 4°C for 15 min (Megafuge 1.0R, Heraus Insturments GmbH, Hanau, Germany). Raw serum was decanted into endotoxin-free micro-centrifuge tubes. Aliquots of raw serum for the analysis of endotoxin were stored at either +4°C or -80°C pending further analysis.

Aliquots of raw serum samples that were prepared from blood that was drawn into both plastic and glass 'serum' vacutainers, and subsequently stored at -80°C, were also used for the experiments with the LBP - ELISA manufactured by Cusabio.

Both projects were approved by Massey University Human Ethic Committee Southern A (application number 12/61 (letter from 26 May 2014) (Appendix I - i) and 13/31 (Appendix II - i)) and informed written consent was given by all volunteers (Appendix I - iii and Appendix II - iv).

6.3.2 Adaptation of the kinetic-QCL™ LAL assay for the quantification of endotoxin levels in human serum

The kinetic-QCL™ LAL assay (Lonza, Walkersville, MD, USA) was performed with a micro-plate reader according to the manufacturer's instructions (see 3.2.4.2 for more details) with exception that the plate was shaken for 30 seconds and the optical density (OD) of each sample was read at 405 nm immediately after shaking and every 30 seconds thereafter for a period of 98 min. Data was subsequently uploaded into Gene5™ software (BioTek Instruments, Inc., USA).

6.3.2.1 Calibration of the kinetic-QCL™ LAL assay

The detection range of this LAL assay was stated by the manufacturer to lie between 0.005 to 50 EU/ml. Standard curves were generated in CurveExpert Professional version 2.0.2 based on the reaction times in seconds of a series of known endotoxin concentrations between 0.00625 and 0.4 EU/ml in LRW. The reaction time was defined, as recommended by the manufacturer, as the time taken for OD to increase by 0.2 units from the initial (baseline) OD value of either the standards or the samples [2]. The standard curve was subsequently used to determine the endotoxin concentration in each sample in Microsoft EXCEL 2010. The same procedure was used for all experiments with exception of the experiment to determine the effect of dilution of raw serum samples and the experiment to determine the effect of combinations of dilution and heating of raw serum samples on the temporal profile of the reaction (see 6.3.2.3 and 6.3.2.4 below). In these cases OD values were determined over a shorter period and data processed in a different software package. Hence, OD values were read every 30 seconds for a period of 70 min and 90 min, respectively, and data subsequently uploaded into the KC4 software (BioTek Instruments, Inc., USA).

The KC4 software allowed the maximum velocity of the reaction (V_{MAX}) to be calculated from the linear part of the reaction curves of each standard and that of each sample [personal communication with Douwes J, see [3] for more details]. Standard curves based on the V_{MAX} values were generated for each of the two experiments for standards of known endotoxin concentrations in the range between 0.003 EU/ml and 0.2 EU/ml and the curves subsequently used to determine the endotoxin concentration in each sample.

In addition to the quantification based on V_{MAX}, I also generated endotoxin standard curves using the same reaction time method that was used for the other LAL assays. The levels of endotoxin in serum samples were subsequently also determined based on the reaction time. The endotoxin values obtained using the reaction time and V_{MAX} methods were compared and both were also compared with those obtained using a novel method that compared the time for OD to increase from 0.5 OD above the baseline OD to 1.0 OD above the baseline OD (described in details in section 6.4.1.4).

Kinetic-QCL™ LAL kits from two different batches were used in the study. The kit from one batch (lot 0000369831) was used for the two experiments that investigated the effect of dilution and the combination of dilution and heating of raw serum samples on assay performance. The remaining experiments used four kits all from batch 0000373909.

6.3.2.2 Assessment of interference by vacutainer additives

Four types of vacutainer, all sourced from Becton, Dickinson and Company (BD) were trialled: a plastic 'serum' vacutainer (6.0 ml, Ref. 367837; BD, UK), a glass 'serum' vacutainer (10ml, Ref. 366430; BD, Franklin Lakes, NJ, USA), a plastic 'EDTA-plasma' vacutainer (K2E 7.2mg, 4ml, Ref. 367839; BD, UK) and a plastic 'heparin-

plasma' vacutainer (LH 102 I.U., 6ml, Ref. 367885; BD, UK). LAL reagent water (LRW) provided with the LAL assay, and endotoxin-free water (ETF-water) (Sigma-Aldrich, Sigma® Life Science, Switzerland), respectively, were pipetted directly into appropriate vacutainers after their rubber stoppers had been removed. This procedure averted potential contamination by syringes or their lubricants. The assays were performed after these diluents had been kept overnight in the vacutainers at room temperature.

6.3.2.3 *Effect of dilution of raw serum samples on reaction kinetics*

The first experiment used raw serum samples from blood that had been collected in plastic vacutainers and stored at -80°C. The temporal profiles of the LAL reactions obtained using undiluted raw serum were then compared with those using raw serum samples that had been diluted 1:5, 1:10 and 1:20 with LRW or similarly diluted with Endosafe® Dispersing Agent (DA) (Charles River Laboratories, Charleston, SC, USA).

6.3.2.4 *Effect of a combination of dilution and heating of raw serum samples on reaction kinetics and recovery of spiked endotoxin*

The second experiment determined whether the denaturation of serum proteins by heating improved the reaction kinetics of the assay as evidenced by the temporal profile. We used aliquots of the serum samples from the previous experiment that had been diluted 1:5 with LRW and DA, respectively. All diluted serum samples were first transferred into endotoxin-free glass tubes (pyrogen free, USP type 1 flint glass, 13x100mm, Lonza, Walkersville, MD, USA) and then heated in a water bath for 10 min at either 70°C, 75°C or 80°C. The heated samples were allowed to cool before being decanted into micro-centrifuge tubes for quantification by LAL assay.

Aliquots of heated serum samples were also spiked with 0.5 EU/ml at a ratio of 1:10 (v/v) immediately prior to the LAL assay as a Positive Product Control (PPC)). The PPCs were used as an indicator of whether the heated serum inhibited or enhanced the detection of endotoxin. A PPC in the range between 50 and 200 % recovery of the spiked dose was considered acceptable according to the instructions provided by the manufacturer.

The pH values of heated samples that had been diluted with DA were between 7.5 and 8.0 whereas the pH values of heated samples that had been diluted with LRW were between 8.0 and 8.5. Hence, only the pH values of the samples that had been diluted with DA lay in the range recommended by the manufacturer (between 6.0 and 8.0).

6.3.2.5 Effect of the type of vacutainer on endogenous endotoxin levels in serum samples

Sera from blood of three healthy females that had been collected in plastic and glass 'serum' vacutainers, respectively, were used to determine whether the type of vacutainer influenced the concentration of endogenous endotoxin detected in serum. Serum samples prepared from bloods taken into plastic and glass vacutainers were each decanted into endotoxin-free micro-centrifuge tubes and stored at -80°C prior to LAL assay. Raw serum samples were in each case diluted 1:10 and 1:30 with LRW and DA, respectively. The diluted samples were each then transferred into endotoxin-free glass tubes and heated in a water bath for 10 min at 70°C. The heated samples were allowed to cool before being decanted into endotoxin-free micro-centrifuge tubes. An aliquot of each heated sample was then centrifuged at 1100 g for 15 min (Biofuge pico, Heraeus, Kendro Laboratory Products GmbH, Hanau, Germany). The supernatant of the centrifuged heated sample was again decanted into an endotoxin-free micro-centrifuge tube. Aliquots of centrifuged and non-centrifuged heated serum samples

were each spiked with 0.5 EU/ml at a ratio of 1:10 (v/v) immediately prior to the LAL assay for the analysis of a PPC.

6.3.2.6 *Effect of storage temperature and time of spiking on the recoveries of spiked endotoxin from serum samples*

In this experiment serum from the blood of one healthy female volunteer that had been collected into glass vacutainers was used to determine the effect of storage temperature at +4°C and at -80°C on the recovery of endotoxin spiked into raw serum samples. Aliquots of raw serum were each spiked with 5 EU/ml at a ratio of 1:10 (v/v) prior to overnight storage at either +4°C or -80°C. All samples were subsequently diluted at 1:10 and at 1:20 with DA. The diluted samples were then transferred into endotoxin-free glass tubes and heated in a water bath for 10 min at either 70°C, 75°C or 80°C. The heated samples were allowed to cool before they were again decanted into endotoxin-free micro-centrifuge tubes.

I determined whether the recovery of endotoxin spiked into raw serum samples prior to storage differed from the recovery of endotoxin spiked into heated serum samples immediately prior to LAL assay (PPC) by comparing results of this procedure with those spiked immediately prior to assay. Hence for the PPCs, aliquots of the heated serum samples that had not been spiked with endotoxin prior to storage, were spiked with 0.5 EU/ml at a ratio of 1:10 (v/v) immediately prior to assay.

6.3.2.7 *Effect of heating temperature and duration of heating period on the recovery of spiked endotoxin in raw serum samples*

I determined whether an increase in the duration of the heating period from 10 min to 4 h at either 75°C or 80°C, improved the recoveries of endotoxin spiked prior to storage into raw serum samples from blood collected in glass vacutainers. Aliquots of raw serum were each spiked with 5 EU/ml at a ratio of 1:10 (v/v) prior to storage at -80°C. The raw and spiked serum samples were thawed and then diluted 1:10 with DA. The diluted samples were then transferred into endotoxin-free glass tubes and subsequently heated in a water bath at either 75°C or 80°C for either 10 min, 30 min, 60 min, 120 min or 240 min. The heated samples were allowed to cool before being decanted into endotoxin-free micro-centrifuge tubes and assayed.

I then tested whether a short heating period of samples for 5 to 15 min at either 70°C or 75°C improved the recoveries of endotoxin spiked into raw serum sample. Again aliquots of raw serum were each spiked with 5 EU/ml at a ratio of 1:10 (v/v) prior to storage at -80°C. The raw and spiked serum samples were thawed and then diluted 1:10 with DA. The diluted samples were then transferred into endotoxin-free glass tubes and heated in a water bath at either 70°C or 75°C for either 5 min, 10 min or 15 min. Again, the heated samples were allowed to cool before being decanted into endotoxin-free micro-centrifuge tubes and assayed.

6.3.2.8 *Reliability of the kinetic-QCL™ LAL assay*

Two sets of standard curves were generated the first based on the time it took for the initial OD to increase by 0.2 units and the second on the time taken for the OD to increase from 0.5 OD above the initial OD to 1.0 OD above the initial OD. Standard curves generated by the first method were used to determine whether the assay was

valid according to the manufacturer's instruction i.e. had a correlation coefficient from -1.00 to -0.980, slope from -0.400 to -0.100 and y-intercept from 2.500 to 3.500. All parameters of each of the standard curves generated on a basis of reaction time were within the recommendations defined by the manufacturer.

6.3.3 The evaluation of an ELISA assay for the quantification of LBP in serum

6.3.3.1 Calibration of the LBP – ELISA manufactured by Cusabio

The human-specific LBP - ELISA, manufactured by Cusabio® Biotech Co, Ltd, (Wuhan, Hubei Province, P.R. China), was used according to the manufacturer's instruction (3.3.3). The reported detection range of the ELISA was from 0.625 to 40 µg LBP/ml. The standard curve for LBP was non-linear but was fitted by second order polynomial function over this range ($r^2 > 0.980$).

6.3.3.2 Testing for interference between constituents of serum with the assay

Serum prepared from blood that was allowed to clot for 2 h, was used in this set of experiments. This clotting period was recommended by the manufacturer of the LBP – ELISA kit. Previous experiments with the LPS - ELISA kit from the same manufacturer, indicated that this clotting time minimised interference from serum constituents with this ELISA kit (see 4.3.3.3 for more details). Twenty serum samples were used for the assessment of interactions between constituents of serum and the LBP - ELISA by adding a known concentration of LBP at a ratio of 1:10 (v/v) to each sample.

6.3.3.3 *Determining the effects of constituents of blood collection tubes on detected levels of LBP in serum*

It was determined whether the blood collection tube that was used for the collection of blood, influenced the endogenous concentration of LBP in serum. Eight blood samples that were simultaneously collected into plain glass 'serum' vacutainers and into plastic 'serum' vacutainers were analysed in that respect. The latter vacutainer contained serum clot activator.

6.3.4 Statistical analysis

Statistical analyses were performed in the SYSTAT version 13 (Systat Software Inc., Chicago, IL, USA). Variations between the rates of the reactions of the two batches of the LAL kit were determined from the standard curves. Hence, the slopes of standard curves obtained by plotting the log of the endotoxin concentration against time from 0.5 to 1.0 OD above the initial OD, were compared by Student's two-sample t-test and overall differences in the y-intercept of these standard curves were assessed by ANCOVA.

6.4 Results

6.4.1 Adaption of the kinetic-QCL™ LAL assay manufactured by Lonza

6.4.1.1 Reliability of the assay and inter-batch variation

The intra- and inter-assay levels of precision for spiked and unspiked heated serum samples were below 10%. There were no significant differences between the two batches on the two-sample Student's t-test in the rates of reaction as assessed by the slopes of the standard curves (plots of log endotoxin concentration against time difference). There were however significant overall differences in the y-intercepts of the reaction curves from the two batches as assessed by ANCOVA (df = 3.180, F = 208.1, p = 0.002).

6.4.1.2 Levels of endotoxin detected in different vacutainers

No endotoxin was detected in either LRW or ETF-water after overnight storage in glass 'serum' type vacutainers at room temperature. In contrast, endotoxin was detected in quantities ranging between 0.3 EU/ml and 0.8 EU/ml both in LRW and ETF-water that had been kept overnight in plastic 'serum' type vacutainers. Conversely, no endotoxin was detected in either LRW or ETF-water had been kept overnight in plastic 'EDTA-plasma' type vacutainers. Endotoxin levels between 0.16 EU/ml and 0.20 EU/ml were detected in both LRW and ETF-water when they had been stored overnight in plastic 'heparin-plasma' type vacutainers.

6.4.1.3 *Effect of dilution, and of a combination of dilution and heating of unspiked serum samples on reaction kinetics*

The yellow colour of undiluted raw serum caused the initial OD readings of undiluted serum samples to be higher than those obtained from the endotoxin standards, which were dissolved in LRW. However, this effect was reduced by diluting the samples. The shapes of the reaction curves obtained with raw and diluted sera differed from those of the endotoxin standards (Figure 6-1A) indicating that dilution alone was not sufficient to reduce interference with the reaction kinetics. However, the shapes of reaction curves obtained with denatured sera were more similar to those of the standard curves indicating that a combination of dilution and heating was sufficient to reduce this interference (Figure 6-1B).

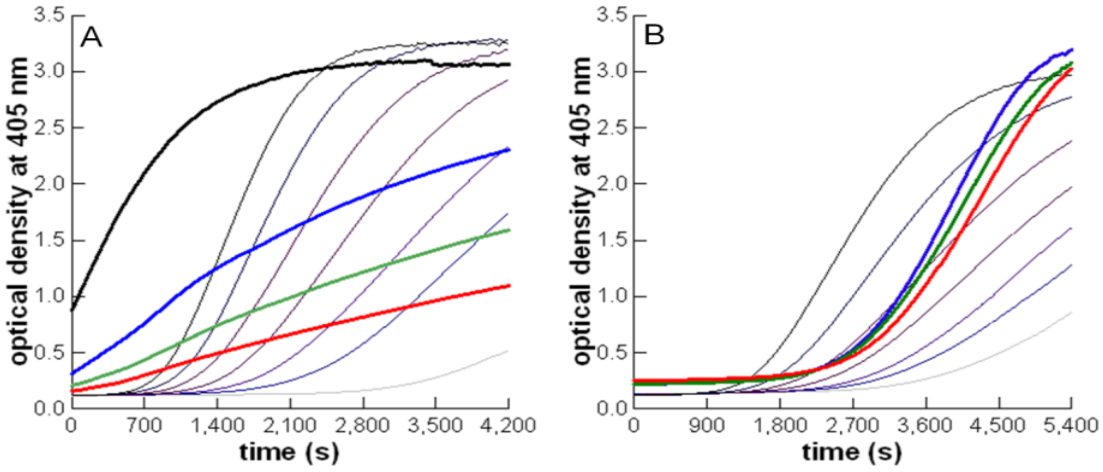

Figure 6-1. Effect of dilution and a combination of dilution and heating of unspiked raw serum samples on their reaction curves.

A) Curve for raw (heavy black) and serum samples diluted with LRW (1:5 – heavy blue line, 1:10 - heavy green line and 1:20 - heavy red line) compared to curves for endotoxin standards and plain LRW (light lines). B) Curve for serum sample that have been diluted 1:5 with LRW prior to heating at either 70°C (blue line), 75°C (green line) or 80°C (red line) in comparison to those of the endotoxin standards.

6.4.1.4 Comparison of various methods for calculating endotoxin levels in heated spiked and unspiked serum samples

The levels of endotoxin in unspiked heated serum samples calculated using the method based on V_{MAX} were 10 times greater than those using the method based on the reaction time that was recommended by the manufacturer (Table 6-1). The PPCs of endotoxin spiked into heated serum samples calculated on the basis of reaction time, ranged between 20 % with LRW diluent and 30 % with DA diluent. Correspondingly, the range of PPCs calculated on the basis of V_{MAX} lay between 77 to 88 % with LRW diluent and between 88 to 103 % with DA diluent (Table 6-1).

Table 6-1. Endotoxin levels in heated serum samples and percentage recoveries of spiked endotoxin in heated serum samples calculated on a basis of reaction time as recommended by the manufacturer, by V_{MAX} and by delta time.

sample preparation		calculation based on the reaction time		calculation based on V_{MAX}		calculation based on the delta time	
sample diluent	**heating temperature**	**Endotoxin concentration in EU/ml**	**PPC in %**	**Endotoxin concentration in EU/ml**	**PPC in %**	**Endotoxin concentration in EU/ml**	**PPC in %**
LRW	70°C	0.073	22.5	0.847	88.4	0.446	64.1
LRW	75°C	0.069	19.9	0.610	77.1	0.366	59.4
LRW	80°C	0.056	19.0	0.575	NA	0.300	54.6
DA	70°C	0.092	29.7	0.921	102.8	0.446	78.1
DA	75°C	0.076	26.8	0.778	87.6	0.366	88.3
DA	80°C	0.045	31.1	0.556	91.4	0.446	52.6

Serum samples were diluted 1:5 either with LRW or DA and heated for 10 min at 70°C, 75°C and 80°C, respectively.

The reaction curves obtained using the endotoxin standards differed from those of endotoxin spiked, heated serum samples (PPCs) in that the initial phase of the reaction was more prolonged in the latter and the time to the development of a linear colorimetric response was correspondingly longer (Figure 6-2A+B). Hence, the delay in the commencement of the reaction caused the times taken for the OD to increase by 0.2 units above the initial value, to be longer when samples contained serum than when they contained LRW (endotoxin standards). However, since the PPCs were between 77 and 103 % of the expected endotoxin concentration when the V_{MAX} method was used (Table 6-1) it was evident that the prolongation of the initial phase

did not influence the slope of the subsequent linear portion of the reaction curve. Correspondingly, concentrations of endogenous endotoxin in heated serum samples and PPCs that were calculated using the reaction time method, were lower than those calculated using the V_{MAX} method. It is noteworthy that the reaction curves obtained with standards of lower concentrations of endotoxin did not plateau during the period of the assay. Hence, V_{MAX} could not be accurately determined in those standards.

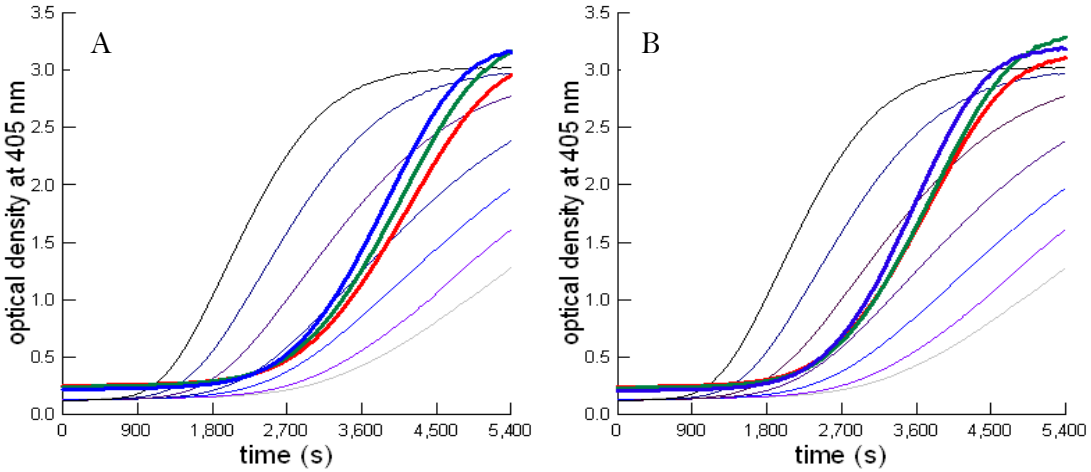

Figure 6-2. Comparison of curves of endotoxin standards (light lines) and endotoxin-spiked serum samples diluted 1:5 with LRW (A) and DA (B), respectively, that were subsequently heated to 70°C (heavy blue line), 75°C (heavy green line) or to 80°C (heavy red line) prior to spiking.

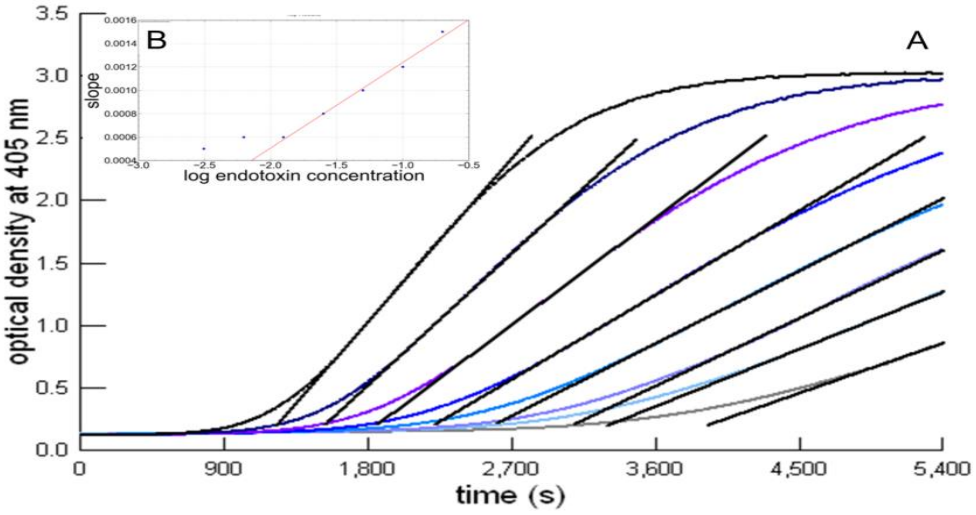

Figure 6-3. Standardisation curves for various dose of endotoxin (0.2 to0.0031 EU/ml) and plain LRW (0.0 EU/ml) showing the slopes of the linear portion of the curves (A) and the standard curve of log endotoxin concentration vs. calculated slope of the linear part of the reaction curve (B).

B) The standard curve generated from the linear portion of the plot between 0.0125 EU/ml and 0.2 EU/ml ($y = 0.0007x + 0.0020$; $r^2 = 0.9918$).

Comparisons of the reaction curves obtained from standards diluted with LRW with those obtained from PPCs and unspiked heated serum samples showed that most became linear between the point at which the OD was 0.5 units to 1.0 units above the initial value (Figure 6-3A). The logarithms of the concentrations of endotoxin in standards were linearly related to the slopes of the reaction curves when the endotoxin concentrations were between 0.0125 and 0.2 EU/ml (Figure 6-3B). Thus, I was able to calculate the concentration of endotoxin in a heated serum sample based on the time difference (in seconds) between the point at which the OD increased by 0.5 from the initial value and the point at which the OD increased by 1.0 from the initial OD value (Figure 6-4A) which is a surrogate variable of the rate of the reaction (Figure 6-4B).

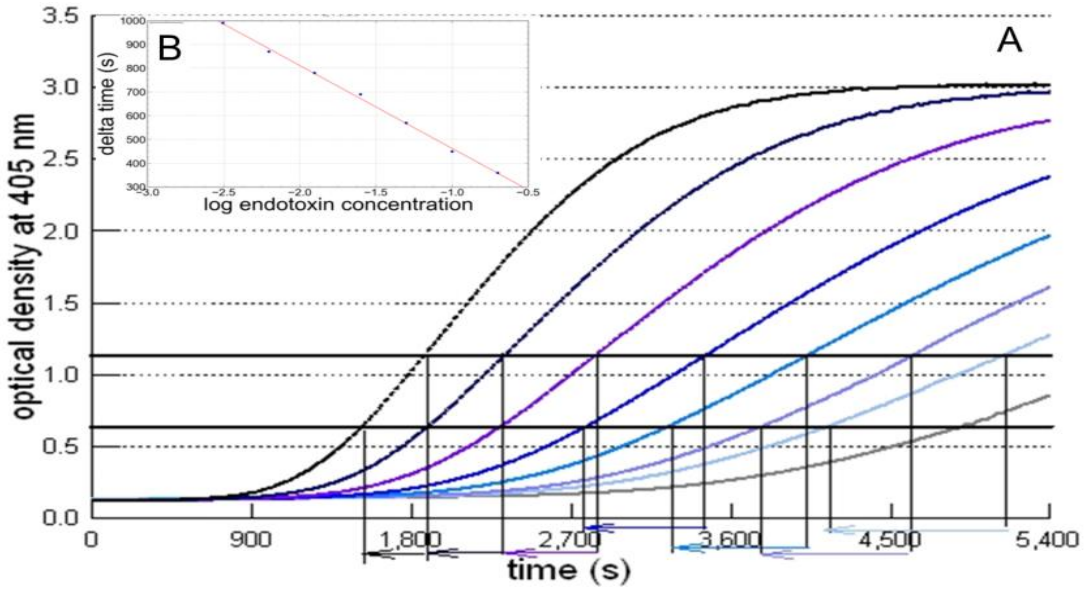

Figure 6-4. Reaction curves of endotoxin standards showing the graphical principles underlying the determination of delta time between the time point at which OD reaches 0.5 unit above the initial value and that at which OD rises one unit above the initial value (A) and the corresponding standard curve of log endotoxin concentration vs. delta time (B).

The standard curve of delta time vs. endotoxin dose rise between 0.0031 EU/ml to 0.2 EU/ml ($y = -348.80x + 114.05$; $r^2 = 0.9979$).

The levels of endotoxin detected in unspiked heated serum samples that were calculated using the new method were five times greater than those calculated with the reaction time method recommended by the manufacturer, and were half those calculated on a basis of V_{MAX}. The values of the PPCs calculated using the new method

were two to three times greater than those calculated by the reaction time but were generally lower than those calculated on a basis of V_{MAX} (Table 6-1). However when serum samples were diluted with DA and heated for 10 min at 75°C the PPCs calculated using the new method were similar to those obtained with the V_{MAX} method.

6.4.1.5 The influence of the type of vacutainer on endotoxin concentrations in unspiked serum samples

No endotoxin was detected when heated unspiked serum samples were diluted either with LRW or with DA when the serum had been prepared from blood that was collected into glass vacutainers. Conversely, endotoxin levels around 1 EU/ml were found in unspiked heated serum from blood from the same subject when the blood was collected into plastic vacutainers.

A white sediment was occasionally observed at the bottom of the micro-centrifuge tubes after heated serum samples had been centrifuged. In these cases the supernatant was again decanted into micro-centrifuge tubes before being assayed. However, the levels of endotoxin were found to be identical to those in the non-centrifuged samples. Further, the PPCs of heated serum samples that had been centrifuged did not differ from those that had not been centrifuged (data not shown).

6.4.1.6 The effect of sample diluent, storage temperature, heating temperature and duration of heating on recovery of endotoxin spiked into heated serum samples

The PPCs of heated serum samples from blood collected in glass vacutainers and diluted with DA, were around 100 %. Conversely the PPCs of heated serum from blood collected in glass vacutainers but diluted with LRW were below 50 %.

The endotoxin concentrations in unspiked heated serum samples and the recoveries of endotoxin spiked into raw serum samples prior to storage were all significant higher when samples were stored at -80°C than when they were stored at +4°C (Table 6-2). The levels of endotoxin in most spiked serum samples that were subsequently diluted 1:10 and 1:20 with DA and heated for 10 min at either 70°C, 75°C or 80°C, were close to the spiked endotoxin concentration of 0.5 EU/ml. However, in all cases these levels were below the calculated total concentration of endotoxin i.e. the sum of the spike plus the endogenous level detected in the unspiked serum. In contrast, the PPCs were between 88 and 118 % of the total calculated concentration of endotoxin in serum samples that had been stored overnight at +4°C or at -80°C, followed by dilution and heating immediately prior to spiking with endotoxin (Table 6-2).

Table 6-2. Endotoxin concentrations in heated serum samples that were spiked with 5 EU/ml at a ratio 1:10 (v/v) prior storage overnight at either +4°C or -80°C with DA as diluent.

sample preparation		calculated endotoxin concentration in EU/ml		calculated PPC (%) of denatured serum samples	
Heating temperature	**Dilution factor**	**-80°C Freezer**	**+4°C Fridge**	**-80°C Freezer**	**+4°C Fridge**
70°C	10	0.337	0.161	92.1	96.3
70°C	20	0.460	0.352	115.5	117.9
75°C	10	0.461	0.126	88.2	75.1
75°C	20	0.555	0.352	118.8	107.8
80°C	10	1.048	0.092	NA	NA
80°C	20	0.504	0.252	NA	NA

The recovery of endotoxin spiked into raw serum samples prior to their storage, subsequent dilution and heating, was influenced by the duration of the period of heating and the temperature to which it was heated. Hence the quantities of endotoxin detected in heated serum samples were significantly lower when the heating period was extended from 10 min to 4 h at either 75°C or 80°C (Figure 6-5A+B). Again, the recoveries of spiked endotoxin were lower when diluted spiked serum samples were heated to 80°C than that when they were heated to 75°C.

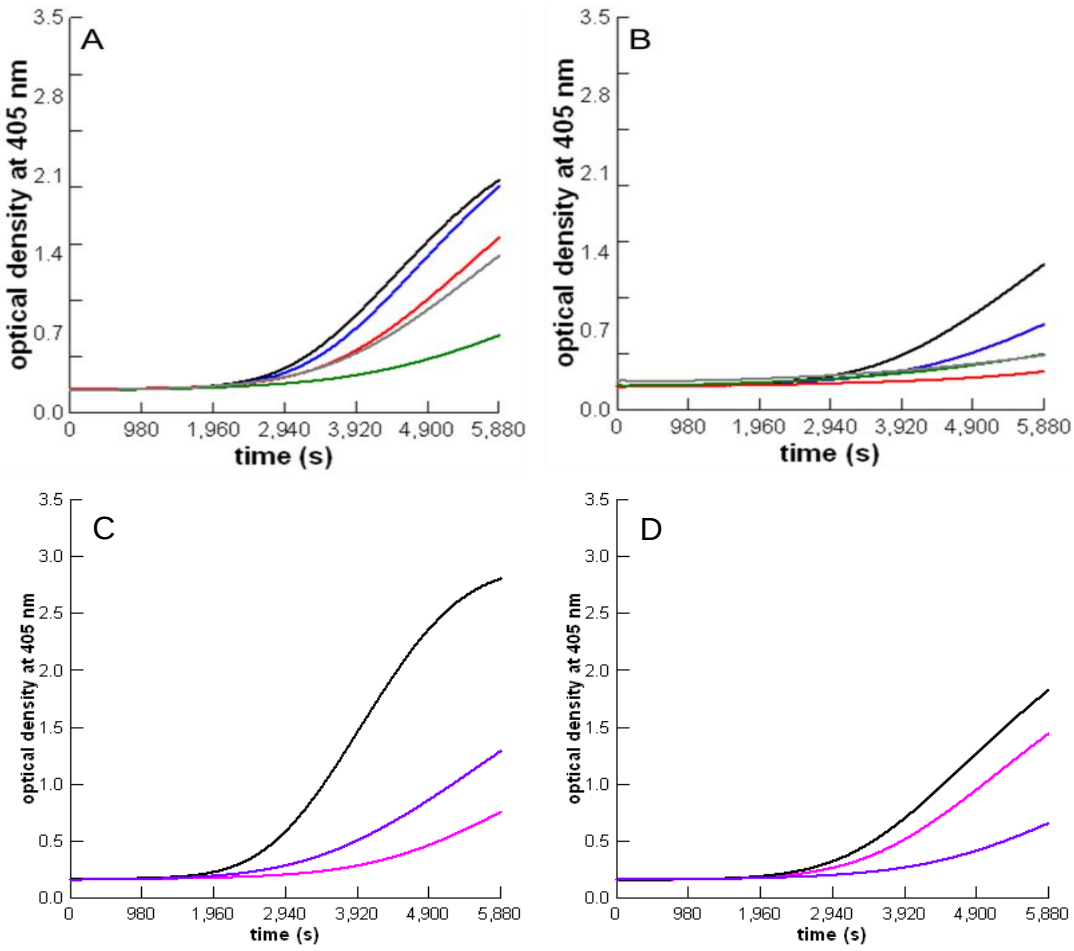

Figure 6-5. Reaction curves of serum samples that were diluted with DA at a ratio of 1:10 and heated at 75°C (A) and 80°C (B), respectively, for 10 min to 240 min or heated at 70°C (C) and 75°C (D), respectively, for 5 to 15 min.

A+B: heating times: 10 min - black line, 30 min - blue line, 60 min - red line, 120 min - green line, 240 min – grey line. C+D: heating times: 5 min – pink line, 10 min – black line, 15 min – violet line.

Similar, the recoveries of endotoxin spiked into raw serum samples prior to the storage, subsequent dilution and heating, were influenced by the period of heating and the heating temperature. Hence, heating at 75°C for 10 min resulted in more endotoxin being detected than heating for 5 or 15 min (Figure 6-5 C). The recoveries of endotoxin spiked into raw serum were also higher when diluted samples were heated at 75°C for 10 min than when they were heated at 70°C for 5 to 15 min (Figure 6-5D). Taken together, it seemed that a combination of dilution of serum samples with DA by factor 10 followed by heating at 75°C for 10 min was the best method for the pre-treatment of serum samples prior to LAL assay.

6.4.2 Evaluation of the LBP - ELISA from Cusabio

6.4.2.1 Interference of the assay by constituents of serum samples

Assay of raw serum samples spiked with a known concentration of the LBP supplied with the kit, gave levels of LBP that corresponded to between 58 and 113 % (median 87 %) of the spiked dose of LBP.

6.4.2.2 The effect of constituents of blood collection tubes on detected levels of LBP in serum

There were no significant differences on paired t-test in the quantities of LBP detected in serum from blood drawn into glass vacutainers from those drawn into plastic vacutainers. It is noteworthy that plastic vacutainers were used for all subsequent assays of LBP when blood was collected to determine concurrently the levels of LBP and LPS in serum i.e. in the Intralipid study (Chapter 7). Glass vacutainers were used when levels of LBP levels in serum were concurrently determined with those of endotoxin i.e. in the Crohn's disease study (Chapter 11).

6.5 Discussion

I have shown that when spiked heated serum samples were being assayed by the micro-plate LAL assay, the prolongation of the initial phase of the LAL reaction curve caused the time to the development of a linear colorimetric response to be longer than that in the reaction curves obtained when pure endotoxin was dissolved in LRW. Thus it seems likely that soluble components in heated serum influenced the initiation of the colorimetric reaction. Hence the calculation of endotoxin concentration based on the method described by the manufacturer as the time during which OD increases by 0.2 units above its initial value, is not appropriate for the determination of endotoxin even in heated serum samples. However, it appeared that presence of heated serum did not influence the rate of reaction once it had commenced i.e. the linear portion of the reaction curve. Since the reaction curves of standards with low concentrations of endotoxin failed to plateau during the assay period, V_{MAX} could not be determined accurately. Hence, the calculation method based on the V_{MAX} would also be unsuitable for any samples that contained low concentrations of endotoxin. Plots of the log of endotoxin concentration vs. the slope of the linear portion of the reaction curve indicated that the relationship became non-linear between 0.0031 EU/ml and 0.0125 EU/ml. This departure from linearity was likely due to the difficulty in identifying the linear portion of the reaction slope when the concentration of endotoxin was low. Hence, the accuracy of quantification of endotoxin based on the calculated slope was no better than with the method based on V_{MAX}. Conversely, the method developed in the current work, that uses the time interval between two OD values, taken at a time where the reaction generally became linear, produces the best correlation between the quantities of endotoxin that were spiked into heated serum samples and the quantities that were detected in those spiked heated serum samples. Further, the standard curve generated by the rate curve of the reaction, i.e. plotting the log of the endotoxin

concentration against the time difference between the two designated OD values on the linear portion of the reaction curve, were linear over the entire range 0.003 EU/ml to 0.2 EU/ml. This new method was independent of the time it took to initiate the colorimetric reaction and avoided any effect of serum proteins on its initiation. Further, there was no significant difference between batches in the rates of the reactions as assessed by the slopes of the standard curves when log endotoxin concentration was plotted against time difference. However, there was a significant overall difference between batches in the position of the curves on the y-intercept. This could be due to differences in shelf life between batches. The latter difference would not affect the comparisons conducted in this study as they were all based on differences in rates or surrogate variables of rates within batches. Hence, the new protocol is useful for the assessment of endotoxin levels in samples of heated human serum or plasma although it is difficult to see the circumstances in which this would be useful in studies of endogenous levels of endotoxin in blood (see below).

The detection of significant levels of endotoxin in LRW and ETF water that had been stored overnight in plastic 'serum' vacutainers or in plastic 'lithium heparin-plasma' vacutainers, indicates that these type of vacutainers should not be used for collecting blood for LAL endotoxin assays. Conversely, the fact that no endotoxin was detected in either LRW or ETF water after similar storage in glass 'serum' vacutainers or in plastic 'EDTA-plasma' vacutainers, indicates these could be used for such collections. However, it is noteworthy that EDTA is known to sequester divalent cations in the blood fraction [4] which can also affect the performance of the LAL assay [5].

Whether the endotoxin that was detected in plastic 'serum' vacutainers or plastic 'lithium heparin-plasma' vacutainers is the result of contamination of heparin by endotoxin (which is of animal origin), or the result of direct or indirect interactions of vacutainer additives (such as the clot activator in the plastic 'serum' vacutainers and

the heparin in the plastic 'heparin-plasma' vacutainers), with the agents that produces the LAL reaction is unclear. Other workers have reported that the quantity of endotoxin in plastic sodium heparin BD vacutainers varies between lots and that no endotoxin is detectable in glass sodium heparin BD vacutainers [6]. This suggests that the effect does not result from endotoxin contamination of heparin but perhaps from contamination by plasticizers either in the wall or the bung of the vacutainer. However, whilst endotoxin was detected by other workers in commercial heparin vacutainers, no endotoxin was detected when blood collection tubes that were prepared with endotoxin-free heparin [7]. Thus the LAL test conducted with commercial vacutainers of this type may have been detecting contamination by endotoxin in the added heparin. Regarding the differences in the levels of endotoxin detected in the plastic and the glass types of 'serum' vacutainers, it should be noted that no clot activators are added to glass 'serum' vacutainers but that they are added to plastic 'serum' vacutainers. Since this clot activator interacts directly with the clotting proteins in human blood it is conceivable that it also reacts with clotting proteins in the LAL reagent which are derived from the blood of the horseshoe crab. The likelihood of a false positive from interference with the test components of the LAL assay is supported by data from other work conducted in this laboratory using LPS - ELISA (see Chapter 4 for more details). This test detected no endotoxin when the sample diluent provided with the kit was kept overnight in plastic or glass 'serum' vacutainers at room temperature. Moreover, this test detected no differences between the amounts of LPS detected in serum from blood collected in glass 'serum' vacutainers and those in serum from blood collected from the same subject at the same time into plastic 'serum' vacutainers (Chapter 4).

Since little or no endotoxin was detected when a known concentration of endotoxin was spiked into raw serum it seems likely that it was sequestered by serum proteins.

The fact that heating serum before spiking with endotoxin leads to recoveries of around 100 % indicates that sequestration of endotoxin is inhibited by denaturation. This finding fits in with the prompt clearance of endotoxin by components such as LPS-binding protein (LBP) and anti-endotoxin antibodies from the bloodstream to reduce its pro-inflammatory potential [1, 8-10]. It is conceivable that variations in an individual's capacity to produce such agents and in their potency [11, 12] will influence the quantities of 'free' endotoxin that remain in the circulation and are detected by the LAL kit.

The experimental work conducted with the human-specific LBP - ELISA manufactured by Cusabio indicated that constituents of the serum variably interfered with the assay.

6.6 Conclusion

The quantification of endotoxin in blood fractions by LAL assay is confounded firstly by the type of blood vacutainer that is used, secondly by the sequestration of endotoxin by serum proteins and thirdly by the method that is used to calculate endotoxin content. Further, my data indicate that combinations of dilution and heating are not sufficient to recover significant amounts of spiked endotoxin from raw serum samples. It may therefore be necessary to use tests that indirectly determine whether there has been long term elevation endotoxin or LPS in the systemic circulation. Hence, for example, the elevation of circulating levels of LBP may be more useful in determining the link between fat and circulating levels of endotoxin [13, 14].

6.7 Reference

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CHAPTER 7 INTRALIPID STUDY

GUT PERMEABILITY AND CIRCULATING LEVELS OF LPS ARE NOT INFLUENCED BY FAT INTAKE EITHER IN HEALTHY WOMEN OR IN WOMEN WITH QUIESCENT CROHN'S DISEASE

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This chapter is based on a manuscript of a paper that has not been submitted for publication yet. Work related to the Intralipid study but which is not intended for publication, is described after references of the manuscript.

Abstract

The elevated permeability of the inflamed gut mucosa of patients with Crohn's disease (CD) is thought to allow the absorption of greater amounts of lipopolysaccharides (LPS) from enteral Gram-negative bacteria augmenting pro-inflammatory status. Recent reports suggest that the absorption and transit of LPS from the gut lumen into the systemic circulation is increased following consumption of a high-fat (HF) meal. We determined whether subjects with inactive CD had greater serum levels of LPS and LPS-binding protein (LBP) than did healthy subjects and whether the ingestion of a HF drink augmented those levels in either subjects with inactive CD or in healthy subjects. The 3-h cumulative excretion of lactulose was significantly increased in subjects with inactive CD after the consumption of either water or a HF drink compared to those in healthy (H) subjects (median $CD_{after\ water}$ 0.095 % of dose vs. median $H_{after\ water}$ 0.058 % of dose and median $CD_{after\ lipid}$ 0.102 % of dose vs. median $H_{after\ lipid}$ 0.056 % of dose; $P = 0.033$). However, the 3-h cumulative excretions of lactulose after the consumption of water did not differ from those after the consumption of HF drink either in healthy subjects or in those with inactive CD. Further, serum levels of either LPS or LBP did not differ between subjects with inactive CD and healthy controls at each time point.

7.1 Introduction

Paracellular permeability is increased when the gut mucosa is chronically inflamed i.e. in subjects with Crohn's disease (CD) [1-5]. It is thought that this enables the translocation of bacterial products such as lipopolysaccharides (LPS) and the contained endotoxin from the lumen into the systemic circulation [6, 7]. LPS can act as a pro-inflammatory stimulus when concentrations are sufficient to produce a direct toxic effect such as septic shock [8-10]. Further, the presence of low concentrations of bacterial endotoxin in the circulation has been postulated to be involved in the development of other conditions including obesity and metabolic disorders such as insulin resistance and type 2 diabetes [11-14] that are associated with chronic low grade inflammation [15, 16]. Hence, LPS may augment the pro-inflammatory status in the mucosa of subjects with CD and increase their risk of relapse [17, 18]. One group reported the levels of systemic endotoxin to be elevated in subjects with either active or inactive CD [6]. However, this is controversial given that other research reported that endotoxin levels in the systemic circulation did not differ significantly between healthy subjects and those with CD [19].

The route by which LPS traverses the mucosal barrier of the intestine is still unclear. A number of studies suggest that in healthy subjects dietary fat may augment the transport of endotoxins across the mucosa into the systemic circulation [20-23]. Whether such fat-mediated absorption of LPS takes place via the transcellular or the paracellular route is not clear. Data of my previous study showed that an aspirin-induced temporary increase in paracellular permeability was not accompanied by increased absorption and circulation of LPS in the systemic circulation [24]. However, LPS is rapidly cleared from the systemic circulation therefore its half-life is generally short [10, 25]. The level of LPS in the systemic circulation may therefore also depend on the capacity of the subject to clear LPS from the systemic circulation [26]. Hence,

other substances such as LPS-binding protein (LBP) were used as indirect markers of the degree of LPS/endotoxin exposure in the systemic circulation. The levels of systemic LBP, an acute phase protein secreted by the liver, are augmented after the challenge with LPS. Elevation of serum levels of LBP was reported in subjects with both inactive and active CD [6,19, 27]. Further, plasma LBP levels were reported to increase in healthy human subjects after the consumption of a HF meal [22]. Hence, levels of systemic LBP may be a better indicator of elevated levels of LPS/endotoxin in the systemic circulation.

The aim of the Intralipid study was firstly, to investigate whether gut permeability was augmented, and the levels of circulating LPS and LBP correspondingly elevated, in women with inactive CD compared with those in healthy women, secondly, whether the consumption of fat augmented the permeability of the gut and serum levels of LPS and LBP in healthy subjects and those with inactive CD.

7.2 Materials and methods

7.2.1 Subjects

Six fit and healthy female non-smoking postgraduate students, aged between 21 and 33 years (median age 31 years), with body heights between 1.62 and 1.72 m, and body masses between 60 and 75 kg, and four women with a history of Crohn's disease (CD), aged between 21 and 35 years (median 33 years), with body height between 1.62 and 1.72 m, and body mass between 62 and 109 kg, were recruited. All cases of CD had been diagnosed at least 1 year prior to study and had been in remission for at least 6 months prior to study. The clinical details of participants with CD are given in Table 7-1.

Table 7-1. History of Crohn's disease

Participant	Smoker	affected Area	Diagnosis	last Relapse	intestinal Surgery	Medication
1	N	unknown	2013	2014	N	Pentasa Azathioprine Tilcotil Omeprazole
2	Y	unknown	2003	2010	N	Pentasa Azathioprine Omeprazole Humira Injection
3	N	terminal ileum	2010	2010	Y	Azathioprine B12 injection
4	N	junction between small intestine and colon	1994	2002	Y	Pentasa Azathioprine colestipol

Each subject completed a health screening questionnaire (Appendix III - iv or Appendix III - v) to determine the clinical details of their CD, and in the case of healthy females, to exclude those with a family or personal history of gastrointestinal disorder. Similarly, to exclude subjects in both groups with any history of endocrine or metabolic disorder, including diabetes; and hepatic disorder or recent abdominal pain, nausea, vomiting, diarrhoea, the passage of blood or mucus or urinary or vaginal

discharge, inability to digest fat, and the consumption of more than one alcoholic drink per day.

7.2.2 Study protocol and design

Participants were instructed to refrain from taking any antibiotics for at least 2 weeks prior to each experimental session, not to consume any alcohol for at least 3 days prior to each experimental session and not to eat any fried or fatty food, and to avoid exercise on the day prior to attending each experimental session. Each participant was instructed to record her food and beverage intake using the provided Food Record Protocol (Appendix III - vi) on the day prior to each experimental session. Each participant attended the Human Nutrition Lab at Massey University, Palmerston North Campus after an overnight fast. On arrival, participants were asked to empty their bladder into a container; subsequently, a 18 GA cannula (BD Insyte™ i.v. Catheter, Ref. 381244; Sandy, Utah, USA) was inserted into the antecubital vein in the non-dominant arm and 15 ml venous blood drawn into a 20 ml syringe (BD Luer-Lok™ Syringe, Ref. 302830; Franklin Lakes, NJ, USA). Aliquots of each venous blood sample were immediately drawn into three different types of vacutainer, namely a glass 'serum' vacutainer (10.0 ml, Ref. 366430; BD, Franklin Lakes, NJ, USA), a plastic 'serum' vacutainer (6.0 ml, Ref. 367837; BD, UK), and a plastic 'serum' SST™ II Advance vacutainer (5.0 ml, Ref. 367954; BD, UK). The intravenous cannula was flushed with 3 ml of 0.9 % sodium chloride (Demo SA. Pharmaceutical Industry, Athen, Greece) immediately after drawing of samples to avoid clotting. Each participant was then given either 250 ml of caramel-flavoured water (negative control) or 250 ml of cappuccino-flavoured Intralipid® 20% (high-fat (HF) drink) (Fresenius Kabi, Australia) to drink. Each of the two treatments was administered to each subject

in randomised order with a 1-week interval between treatments. Two hours after each treatment had been administered, each participant was instructed to again empty their bladder into a container and a further 15 ml sample of venous blood was drawn and processed as described above. Each participant was then asked to drink a solution containing 10 g lactulose (duphalac; Abbott, Hoofddorp, the Netherlands) and 5 g mannitol (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 100 ml of water. This was followed immediately by a further 300 ml of plain water. Further 15 ml blood samples were drawn at 3, 4 and 5 hours after time of ingestion of either the control or the HF drink. Subjects were asked to urinate into a single container as required during a subsequent 3-h period i.e. between 2 and 5 hours after ingestion of either control or HF drink. At 5 hours after the administration of each treatment, each subject was asked to empty their bladder into the same container to bulk the urine sample with that which was collected during the 3-h period i.e. between 2 and 5 hours after ingestion of each treatment. The study protocol was approved by MUHEC: Southern A (application number 14/11) (Appendix III - i) and informed written consent was given (Appendix III - vii).

7.2.3 Determination of urinary lactulose and mannitol

The volumes of the initial urine samples, the urine samples taken 2 hours after the ingestion of either control or HF drink and the total volumes of urine collected between 2 and 5 hours after the ingestion of the treatment were each determined. A 15 ml aliquot of each sample was then centrifuged at 3,500 g at 4°C for 10 min, and the supernatant stored at -80°C pending HPLC analysis.

A 1 ml aliquot of each supernatant of urine sample was processed as described previously [28] (see also 3.5.3). HPLC analysis was performed on a 20 µl aliquot of

filtrate of each sample in duplicates in a Shimadzu HPLC system (Shimadzu, Kyoto, Japan). The quantities of lactulose and mannitol excreted were determined as described in detail previously [28] (see also 3.5.3). The percentages of ingested dose (10 g and 5 g, respectively) recovered were then calculated and the ratios of lactulose to mannitol (LMR) determined.

7.2.4 Determination of levels of LPS in serum

The samples of venous blood that were collected into plastic 'serum' vacutainers (6.0 ml, Ref. 367837; BD, UK) were allowed to clot at room temperature over a period of 2 h before being centrifuged for 15 min at 3,500 g at 4°C. The serum was then decanted into endotoxin-free micro-centrifuge tubes (Biopur® Safe-Lock micro test tubes; Eppendorf, Hamburg, Germany). A 2 ml aliquot of each serum sample was further centrifuged at 13,201 g at 10°C for 30 min. Where applicable, samples of the chylomicron fraction (CM) (the white supernatant layer) and of the aqueous phase (Aq) of the twice centrifuged serum sample were each carefully removed and transferred into fresh endotoxin-free micro-centrifuge tubes. Aliquots of each of the whole serum samples and the CM and the Aq fraction were stored at -80°C pending LPS and LBP analysis.

The levels of LPS in whole serum, CM and Aq were determined using a human-specific LPS - ELISA kit (Cusabio® Biotech Co, Ltd, Wuhan, Hubei Province, P.R. China), according to the manufacturer's instruction. Given that the detection limits of LPS in the assay was reported to be between 6.25 and 400 pg/ml, standards with known concentrations of LPS between 6.25 and 200 pg/ml in sample diluent provided were run concurrently with the assaying samples to provide standard curves. The OD reading of each LPS standard was plotted against the known concentration of LPS in

CurveExpert Professional version 2.02. The level of LPS in each sample was then determined by comparison with the appropriate concurrent standard curves. The forms of the LPS standard curves were second-order polynomial. The intra- and inter-assay precisions for the LPS - ELISA were < 10 %.

7.2.5 Determination of levels of LBP in serum

Serum levels of LBP were determined by the human-specific LBP - ELISA, manufactured by Cusabio® Biotech Co, Ltd, (Wuhan, Hubei Province, P.R. China), according to the manufacturer's instruction. The levels of LBP and those of LPS in whole serum were determined from the same aliquot (serum prepared from blood drawn into plastic vacutainers). Similarly to the LPS - ELISA, standard curves with known concentrations of LBP between 0.625 and 20 µg/ml in sample diluent were run concurrently with the assaying samples and the standard curve generated in CurveExpert Professional version 2.02. The intra- and inter-assay precision for the quantification of LBP in serum in our laboratory were < 10 %.

7.2.6 Determination of lipid profile

Samples of venous blood were collected into plastic SST™ II Advance vacutainers (5.0 ml, Ref. 367954; BD, UK) and allowed to clot for 30 min at room temperature. Samples were centrifuged for 10 min at 1,800 g at room temperature and refrigerated at +4°C pending delivery to the laboratory. Total cholesterol, triglyceride, HDL and LDL cholesterol and the total/HDL cholesterol ratio were determined in an accredited medical laboratory (Medlab Central in Palmerston North, New Zealand).

7.2.7 Statistical analysis

Test of normality of distribution were conducted in Minitab version 16.0 (Minitab Inc., PA, USA) prior to statistical analysis. Statistical analyses were performed in SYSTAT version 13 (Systat Software Inc., Chicago, IL, USA). Non-parametric Mann-Whitney U analyses were conducted when data were not normally distributed and not amenable to transformation using the Johnson algorithm (Minitab version 16) whilst doubly repeated measure ANOVA was used when data were found to be normally distributed. Differences were considered significant at the $P < 0.05$ level. The results are presented as median values with 25 - 75 % interquartile range (IQR) when non-normally distribution of data was found, unless otherwise stated.

The correlation between serum LPS and LBP levels was assessed by Pearson correlation coefficient and the Bartlett Chi-Square statistic obtained on linear regression. The correlation between levels of systemic LPS with the excretions of lactulose and mannitol, respectively, and the correlation between levels of systemic LBP with the excretions of lactulose and mannitol, respectively, were similarly assessed by Pearson correlation coefficient and Bartlett Chi-Square statistic. The correlation between baseline levels of LPS in serum with body mass and BMI, respectively, and the correlation between baseline levels of LBP in serum and body mass and BMI, respectively, were also assessed by Pearson correlation coefficient and Bartlett Chi-Square statistic. Correlation was considered as significant when P was < 0.05.

7.3 Results

7.3.1 Body composition of subjects

Four women with inactive CD of similar body height (median 1.64 m; range 1.62 - 1.74 m) but of diverse body mass (median 82.3 kg; range 62 - 109 kg) and BMI (median 29.6; range 23.3 - 36.8) were recruited. Six healthy women of similar body height (median 1.68 m; range 1.63 - 1.73 m) and of less diverse body mass (median 65.2 kg; range 60 - 74 kg) and BMI (median 23.1; range 20.3 - 26.9) were recruited.

7.3.2 Serum LPS and LBP levels in healthy women and those with Crohn's disease

The serum levels of LPS and LBP were non-normally distributed and not amenable to transformation using the Johnson algorithm (Minitab version 16). Hence non-parametric methods were used for comparison.

The baseline levels of LPS in sera of subjects with inactive CD did not differ from those of healthy subjects taken prior to consumption of water (median CD_{water1} 16.220 pg/ml; 25-75% interquartile range (IQR) 4.879 - 36.219 pg/ml vs. median H_{water1} 9.784 pg/ml; IQR 7.741 - 27.583 pg/ml) or taken prior to consumption of HF drink (median CD_{lipid1} 13.160 pg/ml; IQR 8.733 - 28.176 pg/ml vs. median H_{lipid1} 13.277 pg/ml; IQR 10.667 - 22.590 pg/ml). Similarly, no significant differences were observed between the baseline levels of systemic LBP in those from women with inactive CD and in those of healthy women taken prior to ingestion of water (median CD_{water1} 2.112 µg/ml; IQR 1.423 - 3.031 µg/ml vs. median H_{water1} 1.136 µg/ml; IQR 0.710 - 2.924 µg/ml) or taken prior to ingestion of HF drink (median CD_{lipid1} 1.515 µg/ml; IQR 1.084 - 3.040 µg/ml vs. median H_{lipid1} 1.131 µg/ml; IQR 1.034 - 1.438 µg/ml). The baseline levels of systemic LPS or LBP were not significantly correlated, either with body mass (kg) or with BMI (data not shown).

7.3.3 The effect of Intralipid on serum LPS and LBP levels

There were no significant differences between the levels of LPS in whole sera taken after the consumption of water and those taken before, either in subjects with inactive CD or in healthy controls. There were also no significant differences between the levels of systemic LPS taken after the ingestion of HF drink compared with those taken prior to the drink, either in healthy women or in those with inactive CD. Further, the levels of systemic LPS taken over five time points did not differ significantly between healthy subjects and those with inactive CD in both treatment groups on Whitney U test (Figure 7-1). No differences were observed between LPS levels in whole sera after the ingestion of HF drink and the corresponding levels of

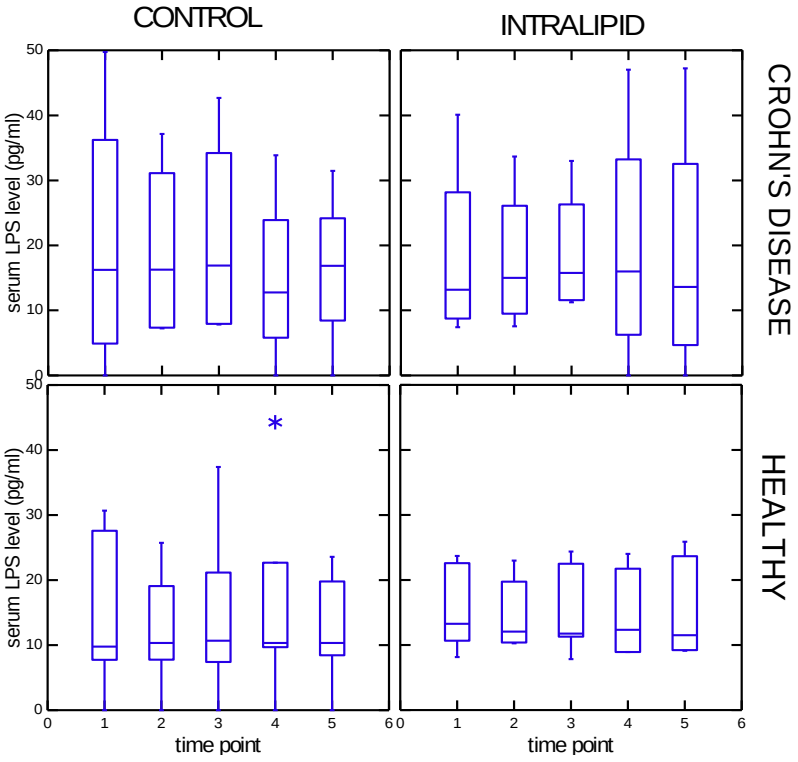

Figure 7-1. **Serum LPS level of women with CD and of healthy women after the ingestion of water (control) and Intralipid.**

No overall difference in serum LPS level was observed after the ingestion of either water or Intralipid in both women with CD and healthy women. There was no overall difference in the serum LPS level between women with CD and healthy women.
* - value above 1 SD

LPS in the chylomicron fraction or in the aqueous phase of the sera (data not shown).

There were no overall differences between the 4 levels of LBP in sera following either the consumption of water or HF drink with those taken prior to treatments in healthy women or women with inactive CD (Figure 7-2).

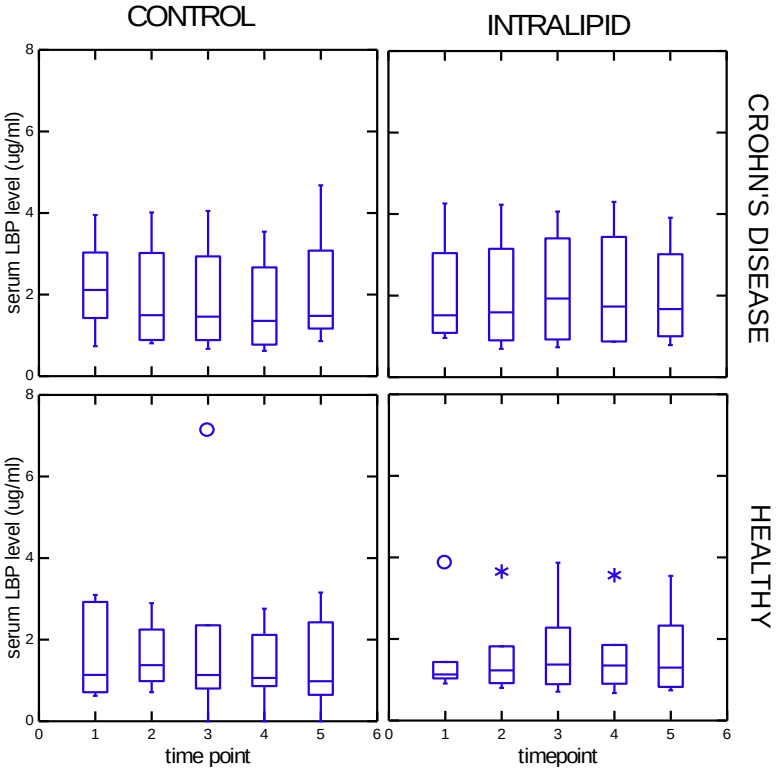

Figure 7-2. Serum LBP level of women with CD and healthy women after the consumption of either water (control) or Intralipid.

The serum LBP level did not significantly changed after the ingestion of water or Intralipid in both women with CD and healthy women. There was no overall difference in the LBP levels between women with CD and healthy women.
* - value above 1 SD ° - value above 2 SD

However, there was a significant correlation between the serum LPS concentrations and the corresponding serum LBP concentrations observed in healthy subjects and in those with inactive CD (df = 1; Bartlett Chi-Square = 130.093; $P < 0.001$; $r = 0.860$). The relationship between levels of LPS and LBP was significantly linear (df = 1; $F = 967.539$; $P < 0.001$) (Figure 7-3). Hence, the levels of LBP increased in direct proportion to those of LPS.

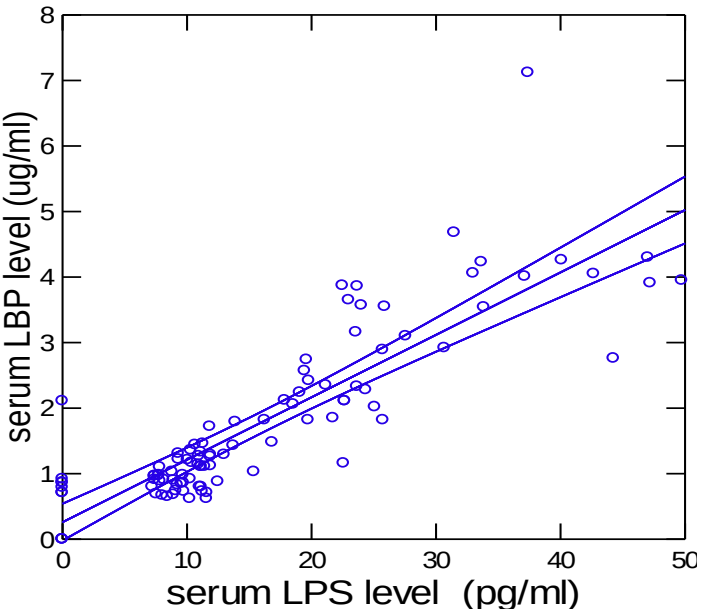

Figure 7-3. Correlation between LPS and LBP levels in serum.

There was a strong correlation between the serum LPS level and the corresponding serum LBP level ($r = 0.860$; $P < 0.001$; 0.95 confidence interval) observed in women with CD and healthy women.

7.3.4 The effect of Intralipid on lipid profile

The values for total cholesterol, LDL and HDL cholesterol levels and the ratio of total cholesterol to HDL cholesterol were all normally distributed whereas the triglyceride levels required Asinh transformation (Johnson transformation in Minitab version 16) to render them amenable to parametric statistical analysis.

Serum triglyceride (TG) levels were significantly higher ($df = 1$; $F = 9.287$; $P = 0.019$) after the ingestion of the HF drink than after ingestion of water. The levels of serum TG were significantly augmented over time following the consumption of the HF drink ($df = 4$; $F = 4.927$; $P= 0.004$). The pattern of increase in TG levels after the consumption of HF drink did not differ between healthy women and those with inactive CD (Figure 7-4). In general, the serum TG levels peaked at 2 h after the ingestion of Intralipid drink.

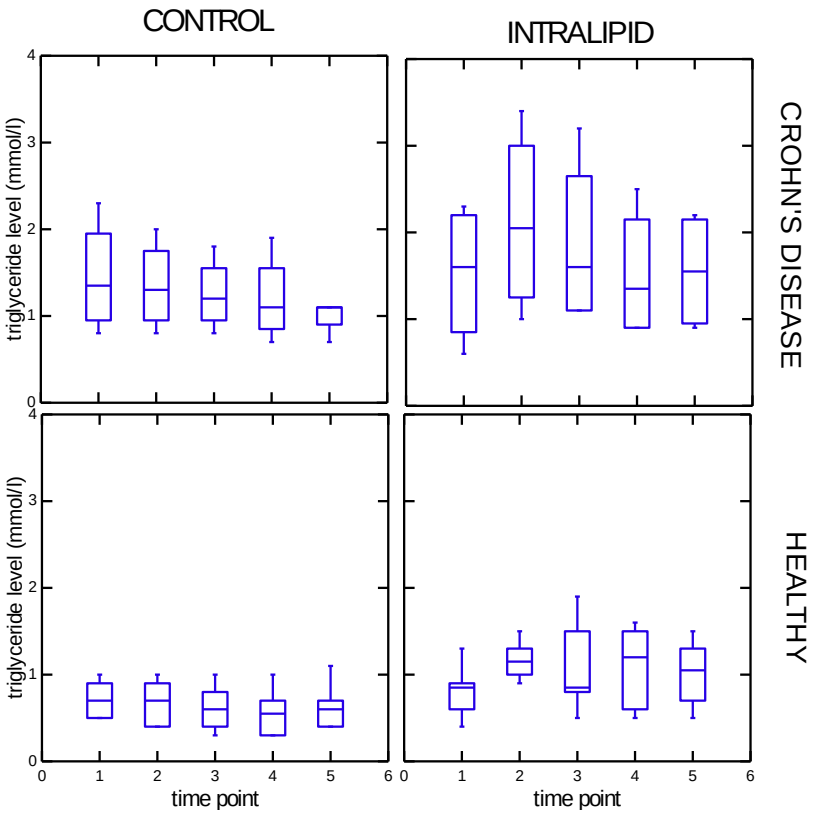

Figure 7-4. Serum triglyceride (TG) level in women with CD and healthy women after the ingestion of either water (control) or Intralipid.

There was both a significant overall difference in TG levels between the two treatments (df = 1; F = 9.287; P = 0.019) and a significant overall difference in TG levels over time (df = 4; F = 4.927; P = 0.004). The TG levels were significantly different between the two treatments over time in both women with CD and healthy women (df = 4; F = 3.585; P = 0.018).

Total serum cholesterol levels after the consumption of water and those after the consumption of HF drink did not differ overall from those taken prior either in healthy women or in those with inactive CD. However, there were significant differences over time (df = 4; F = 4.739; P = 0.005) in the total cholesterol levels of both subjects with inactive CD and healthy subjects both after the consumption of water and after the consumption of the HF drink. Total cholesterol levels in healthy subjects were unchanged either after the ingestion of water or of HF drink whilst the total cholesterol levels in women with inactive CD tended to decrease after the consumption of water and to increase after the consumption of HF drink (data not shown). Similarly, there were no significant overall differences in serum LDL cholesterol levels

before and after the ingestion of water or before and after the ingestion of HF drink in both women with inactive CD and those who were healthy. However, the temporal profiles of LDL cholesterol levels after the consumption of water and after consumption of HF drink in healthy women differed from those with inactive CD on doubly repeated measure ANOVA (df = 4; F = 4.473; P = 0.006). The temporal profiles of LDL cholesterol in healthy women did not change following ingestion of either water or of the HF drink. However, although the profiles of LDL cholesterol in women with inactive CD did not change after the consumption of water they tended to increase 4 h after the consumption of HF drink (data not shown). The serum HDL cholesterol levels following ingestion of either water or HF drink did not vary between healthy subjects and those with inactive CD. The temporal profiles of the total cholesterol to HDL cholesterol ratios following ingestion of either water or HF drink in women with inactive CD differed significantly from those in healthy women on doubly repeated measure ANOVA (df = 4; F = 3.705; P = 0.015). This was due to a decrease in the ratio of total to HDL cholesterol over time after the consumption of water and an increase in total to HDL cholesterol ratio over time after the consumption of HF drink in subjects with inactive CD (peaked at 3 h after the ingestion of HF drink) whilst there was not such a change in healthy subjects (data not shown). There were no correlations between the levels of systemic LPS and any of the serum lipid parameters. Similarly, there were no correlations between levels of systemic LBP and any of the serum lipid parameters (data not shown).

7.3.5 Variation in the excretion of lactulose and mannitol between healthy women and those with a history of Crohn's disease

The percentage recoveries of the ingested dose of mannitol were normally distributed whereas those for lactulose and the LMR both required Asinh transformation (Johnson Tranformation in Minitab version 16) to render them amenable to parametric statistical analysis.

The 3-h excretions of lactulose were significantly greater on doubly repeated ANOVA (df = 1; F = 5.410; P = 0.033) in women with inactive CD after the consumption of either water or of HF drink compared with those in healthy women (Figure 7-5A). There were no significant differences between the 3-h excretions of lactulose after the consumption of water and those after the consumption of HF drink either in healthy women or in those with inactive CD. The 3-h excretions of mannitol did not differ between healthy subjects and those with inactive CD (Figure 7-5B). Again, there were no significant differences between the 3-h excretions of mannitol after the consumption of water and those after the consumption of HF drink, either in women with inactive CD or in healthy women. The LMRs based on 3-h excreted values did not differ between healthy women and those with inactive CD nor did the LMRs after the consumption of water differ from those after the consumption of HF drink (Figure 7-5C).

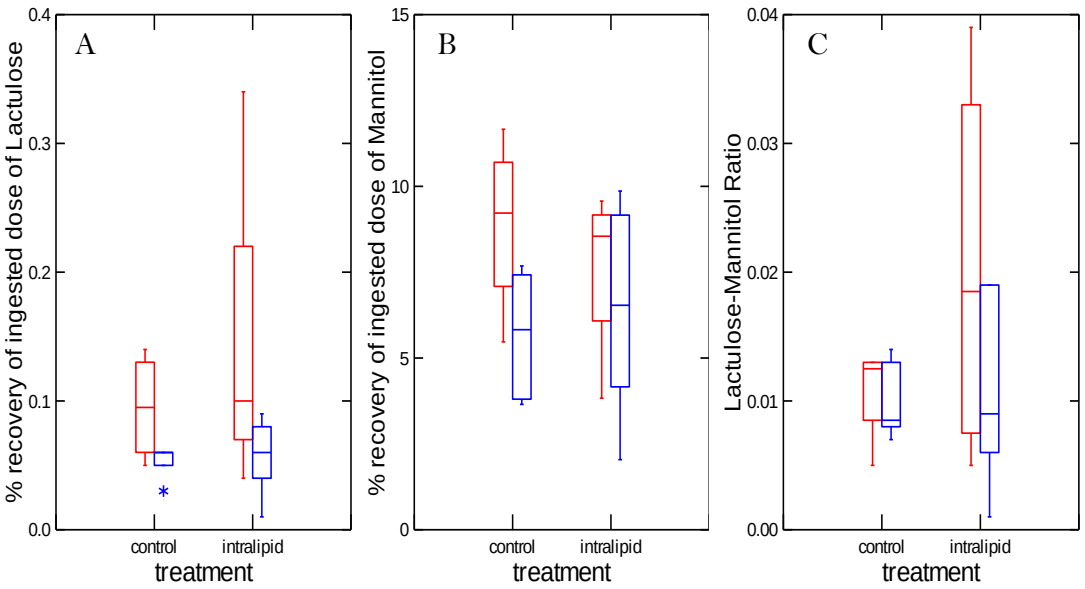

Figure 7-5. Box plots of percentage 3-h recovery of ingested dose of (A) lactulose and of (B) mannitol, and change in (C) LMR after consumption of water and of Intralipid for women diagnosed with CD (red box plots) and healthy women (blue box plots).

A) The excretion of lactulose was significant greater in women with CD than in healthy women after the consumption of either water or Intralipid (P = 0.033) (median CD $_{water}$ 0.095 % of dose, IQR 0.060-0.126 % of dose vs. median H $_{water}$ 0.058 % of dose, IQR 0.048-0.064 % of dose and median CD $_{lipid}$ 0.102 % of dose, IQR 0.71-0.224 % of dose vs. median H $_{lipid}$ 0.056 % of dose, IQR 0.039-0.078 % of dose). The excretion of lactulose after the consumption of water did not differ significantly from that after the consumption of Intralipid either in healthy women or in those with CD. B) The excretion of mannitol tended to be greater in women with CD than in healthy women but did not reach significance (median CD $_{water}$ 9.219 % of dose, IQR 7.085-10.703 % of dose vs. median H $_{water}$ 5.826 % of dose, IQR 3.802-7.415 % of dose and median CD $_{lipid}$ 8.547 % of dose, IQR 6.080-9.165 % of dose vs. median H $_{lipid}$ 6.532 % of dose, IQR 4.163-9.164 % of dose). There was no significant difference in the excretion of mannitol between the consumption of water and the consumption of Intralipid in both women with CD and healthy women. C) The LMR did neither significantly differ between women with CD and healthy women nor between the consumption of water and Intralipid (median CD $_{water}$ 0.013, IQR 0.009-0.013 vs. median H $_{water}$ 0.009, IQR 0.008-0.0013 and median CD $_{lipid}$ 0.018, IQR 0.007-0.033 vs. median H $_{lipid}$ 0.009, IQR 0.006-0.019).
* - value above 1 SD

7.3.6 Correlations between gut permeability and serum LPS and LBP levels

No significant correlation was observed between the 3-h percentage recoveries of the ingested dose of lactulose after the consumption of water and the levels of LPS in sera taken prior to consumption of water (Figure 7-6). Neither was there any significant correlation between the 3-h excretions of mannitol after the consumption of water and the levels of systemic LPS taken prior to consumption of water. Similarly, there was no correlation between the LMR and levels of systemic LPS taken prior to consumption of water (data not shown). Again, neither the 3-h excretions of lactulose,

nor those of mannitol, nor the LMRs after the consumption of water, correlated with the levels of systemic LBP taken prior to consumption of water (data not shown).

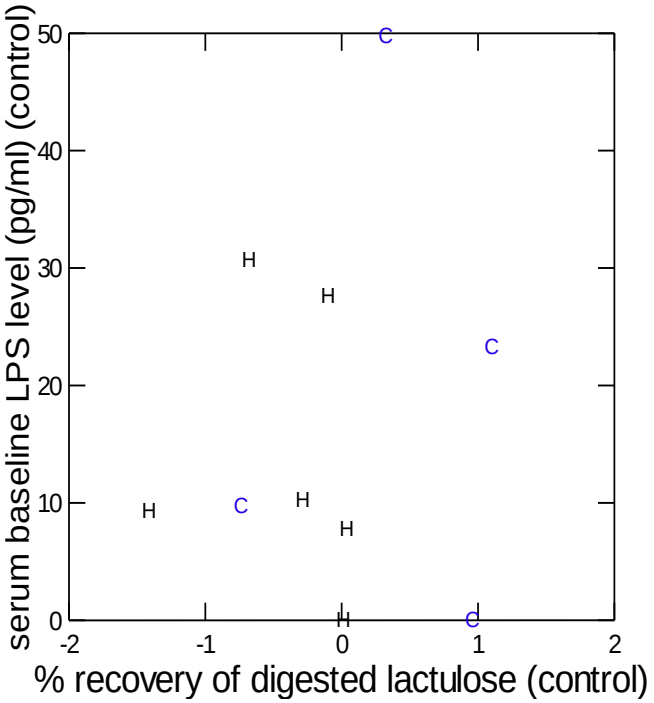

Figure 7-6. Correlation between the excretion of lactulose and serum baseline LPS level in women with CD (C) and healthy women (H).

There was no correlation between the excretion of lactulose and the serum baseline LPS level.

7.4 Discussion

As far as I am aware this is the first study to explore the relationship between small intestinal permeability and the levels of LPS and LBP in the systemic circulation of healthy subjects and in those with inactive CD, and the effect of consumption of water (control) or a HF drink on these relationships.

The fact that the paracellular permeability of the gut, as determined by the urinary excretion of lactulose, was increased in subjects with inactive CD is in line with previous reports [1-4]. It is noteworthy however, that one subject in the CD group had a level of paracellular permeability that was similar to those of healthy control subjects. This subject had been diagnosed with CD for over 20 years and had undergone two small bowel resections and had no relapse in the 12 years prior to current study. Hence, it is conceivable that normal permeability may eventually be restored in subjects with CD following suitable medical and surgical treatment.

Data indicated that there were no significant differences between healthy women and those with inactive CD in the baseline levels of systemic LPS. This observation is in agreement with previous reports showing that the levels of endotoxin in subjects with inactive and with active CD did not significantly differ to those of healthy control subjects [19]. There was however a greater degree of variation in the serum LPS levels of subjects with inactive CD than was found in healthy controls. The highest concentration of LPS in serum was determined in a subject with CD whose disease had last relapsed 8 months prior to study. Whereas those of other subjects with CD who had their last relapse at least 4 years ago, did not differ from those of healthy subjects. Hence it seems likely that the variation in levels of systemic LPS in subjects with CD reflects varying levels of recovery over time. The combined baseline levels of serum LPS from healthy subjects and from those with inactive CD showed no correlation

either with body mass or with BMI as has been previously reported [24, 29, 30] suggesting that variations in body mass and BMI between healthy subjects and those with inactive CD had no impact on level of systemic LPS. Altogether the finding suggested that whilst residual CD-related inflammation of the intestinal mucosa increased paracellular permeability, this did not influence the ability of LPS to transit from the lumen into the systemic circulation. However, this finding did not rule out the possibility that the increase in paracellular permeability allowed greater quantities of LPS to enter the portal circulation but that this was removed by the liver before it could enter the systemic circulation. This hypothesis is supported by the fact that the levels of endotoxin in the portal venous blood are reported to be significantly higher than in the systemic blood of human subjects at laparotomy [31] and that human subjects with hepatic cirrhosis have significant higher levels of endotoxin in the systemic circulation [32] compared to those in subjects with a healthy liver [31]. Again, the levels of endotoxin in the systemic circulation were found to vary with the level of liver damage in human subjects with non-alcoholic liver disease [33].

Results of this study showing there were no differences between the baseline levels of systemic LBP in healthy subjects and those with inactive CD further supported the finding that systemic levels of LPS were not elevated in subjects with inactive CD. However, our data again conflict with previous studies that reported that the levels of systemic LBP were elevated in subjects with both active and inactive CD [6, 19, 27]. Again, the baseline serum levels of LBP correlated neither with body mass nor with BMI. The strong correlation between the systemic levels of LPS and LBP may indicate that LBP is a useful indirect biomarker for systemic LPS. However, the correlation also raises questions as to whether the test responded to the same epitopes of free LPS and LPS that was bound in LBP.

The fact that the excretion of lactulose did not increase following ingestion of the HF drink whilst the serum triglyceride levels did, in either healthy subjects or those with inactive CD, does not fit in with a hypothesis that the consumption of HF meals induces increases in intestinal permeability [20, 34]. However, the urinary excretion of lactulose was markedly increased in one of the four subjects with inactive CD after the consumption of the HF drink. Hence, it is possible that the consumption of a fatty drink only influences the paracellular permeability of subjects with incomplete recovery from CD.

Published data indicate that LPS is absorbed from the gut lumen transcellularly within fat micelles and subsequently incorporated into chylomicrons [23, 35]. In contrast to previous studies [20-23, 36], we observed no significant difference between the levels of LPS in whole serum and those in either the chylomicron fraction or in the aqueous phase of the sera after the consumption of HF drink. Further, there was no correlation between the levels of systemic LPS with any of the parameters associated with the transport of lipids i.e. triglyceride, total cholesterol, HDL and LDL cholesterol. This contradictory finding may arise from the differences in the nature of circulating bacterial products that were determined. This study quantified levels of systemic LPS using a human-specific LPS - ELISA whereas previous studies have quantified the levels of systemic endotoxin using the LAL assay. The dose of LPS that is equivalent to that of endotoxin varies with the structural characteristics of the lipid A unit of the LPS molecule [37] which can be modified by several mechanisms in the human body [10]. Therefore it is conceivable that whilst levels of systemic LPS did not change after the consumption of a HF meal, the ratio of endotoxin to LPS did change. Hence, it appears to be more likely that the LAL assay did not accurately quantify the levels of systemic endotoxin as was reported previously by others [26]. Further work is necessary to investigate the relationship between levels of systemic LPS as determined

by LPS-specific ELISA, and levels of systemic endotoxin as determined by LAL assays.

It is also possible that the contradictory findings may be due to difference in the type of fat that was used. Hence, in previous studies fatty foods were consumed [20-23, 36] whereas in this study Intralipid® 20% was ingested. The latter comprises a lecithin stabilised emulsion of soya oil containing 20 % triglycerides.

Interestingly, subjects with inactive CD showed different temporal profiles of total cholesterol, LDL cholesterol and ratios of total to HDL cholesterol after the ingestion of a HF drink compared with those in healthy subjects. This could indicate that subjects with inactive CD absorbed and/or metabolised dietary fat at a different rate to that of healthy subjects. However, the sample size in the current study is too small to allow such conclusions.

7.5 References

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7.6 Work not idented for publication

The quantification of endotoxin in serum samples was conducted in all samples. The kinetic-QCL™ LAL assay manufactured by Lonza (Walkersville, MD, USA) was used for the endotoxin analysis in the Intralipid study. Data indicated that the pre-treatment of serum samples was not appropriate to assess the total quantities of (sequestered and free) endotoxin in serum samples. Data obtained with the LAL assay were therefore not included in the paper manuscript as they are not indented to be published in relation to the Intralipid study. Hence, the findings are presented in a separate section at the end of this chapter.

7.6.1 Determination of levels of endotoxin in serum samples

The sample of venous blood that was collected into glass 'serum' vacutainers (10.0 ml Ref. 366430; BD, Franklin Lakes, NJ, USA) was allowed to clot at room temperature over a period of 2 h before being centrifuged for 15 min at 3,500 g at 4°C. The serum was then decanted into endotoxin-free micro-centrifuge tubes. A 2 ml aliquot of each serum sample was further centrifuged at 13,201 g at 10°C for 30 min. Where applicable, sample of the chylomicron fraction (CM) (white supernatant layer) and of the aqueous phase (Aq) of the twice centrifuged serum sample were each carefully removed and transferred into fresh endotoxin-centrifuge tubes. In addition, 19 randomly chosen fresh whole serum samples were spiked with endotoxin at a concentration of 10 EU/ml at a ratio of 1:10 (v/v) and four randomly chosen fresh whole serum samples were spiked with endotoxin at a concentration of 50 EU/ml at a ratio of 1:10 (v/v) prior to storage. Aliquots of each of the (spiked and unspiked) whole serum sample and the CM and the Aq fraction were stored at -80°C overnight.

On the next day, aliquots of the (spiked and unspiked) whole serum sample and of the CM and Aq fraction were thawed and each sample then vortexed for 1 min. Four randomly chosen whole serum samples were also spiked with endotoxin at a concentration of 10 EU/ml. Each sample was then diluted with DA by factor 10 in a micro-centrifuge tube. The diluted sample was vortexed for 20 s before being transferred into endotoxin-free glass tubes and then heated in a water bath at 75°C for 10 min. Heated serum samples were allowed to cool down and were then vortexed for 20 s. The pre-treated sample was then transferred into endotoxin-free micro-centrifuge tubes. Positive Product Controls (PPC) were prepared by spiking pre-treated serum samples that have not been spiked with endotoxin prior to sample preparation, with endotoxin at a concentration of 1 EU/ml at a ratio of 1:10 (v/v) immediately prior to assay.

The four kinetic-QCL™ LAL kits from batch 0000386716 were used and the assay performed as previously described (3.2.4.2). The optical density (OD) of each sample was read at 405 nm every 15 seconds for a period of 120 min. Data were subsequently uploaded into Gene5™ software (BioTek Instruments, Inc., USA) and then transferred into Microsoft EXCEL 2010 for further data analysis. The concentration of endotoxin were calculated on the basis of the time difference between 0.5 units above the initial OD and 1.0 units above the initial OD (delta time). The standard curve was generated by plotting delta time against the logarithm function of the known concentration of endotoxin. The standard curve which behaved linearly up to 0.4 EU/ml, was then used to determine the endotoxin concentration in each sample using Microsoft EXCEL 2010.

7.6.2 Results

7.6.2.1 Levels of endotoxin in fasted serum samples in healthy women and in those with Crohn's disease

No endotoxin was detectable (< 0.06 EU/ml) in samples of fasted (whole) serum in either healthy women or women with inactive CD. The PPC in whole serum samples ranged broadly between 24.4 and 120.9 % (mean 70.9 %) indicating that the LAL reaction was variably inhibited by pre-treated serum samples.

7.6.2.2 The effect of Intralipid on levels of systemic endotoxin

In total 97 samples of whole serum were analysed for endotoxin. Only two out of these samples had detectable levels of endotoxin (> 0.06 EU/ml). An endotoxin concentration of 0.547 EU/ml was obtained in a woman diagnosed with CD 3 h after the ingestion of HF drink. An endotoxin concentration of 0.157 EU/ml was found in a healthy woman 2 h after the ingestion of water.

It was possible to visible separate the chylomicron fraction and an aqueous phase in 32 out of 97 serum samples. Endotoxin was detectable in the aqueous phase of one woman diagnosed with CD (0.513 EU/ml) whereas the remaining 31 samples were endotoxin-negative (< 0.06 EU/ml). No endotoxin was detectable in any of the samples of the chylomicron fraction. This data indicate that the ingestion of Intralipid had no effect on the levels of circulating endotoxin both in healthy women and in those diagnosed with CD. Again, the PPC varied broadly between 43.0 and 94.1 % (mean 72.2 %) in pre-treated samples of the aqueous phase, and between 27.2 and 159.9 % (mean 76.7 %) in pre-treated samples of the chylomicron fraction.

7.6.2.3 The recovery of endotoxin spiked into raw serum samples

Levels of systemic endotoxin of 0.185 EU/ml and 0.255 EU/ml were determined in two out of the 19 serum samples that were spiked with endotoxin at a concentration of 10 EU/ml at a ratio of 1:10 (v/v) prior to storage. These levels are equal to a recovery of endotoxin of 19 and 25 %. No endotoxin was recovered in the four thawed raw serum samples that were spiked with endotoxin at a concentration of 10 EU/ml at a ratio of 1:10 (v/v) prior to the treatment of serum. Endotoxin was detectable in two out of 4 samples that were spiked with endotoxin at a concentration of 50 EU/ml at a ratio of 1:10 (v/v) prior to storage. The recovery of endotoxin in those samples was 2 and 4 % of the expected amount of endotoxin.

7.6.3 Conclusion

Data indicated that detectable levels of endotoxin (> 0.06 EU/ml) are seldom in the systemic circulation both in healthy women and in those with inactive CD. Moreover, the fact that endotoxin spiked into raw serum samples, was not or hardly recovered suggested that the constituents of serum had the capacity to sequester spiked (free) endotoxin and made it therefore not available for detection by the LAL assay. However, the overall inhibition of the LAL reaction by constituents of pre-treated serum, as determined by the PPC, raised questions as to whether the lack of detectable amounts of endotoxin was due to the absent of any endotoxin in the systemic circulation or due to the inhibition of the LAL reaction. In order to properly describe any relationship between the quantities of endotoxin that enter the circulation and the quantity of fat in the diet it will be necessary to develop methods that can free any sequestered endotoxins into an environment that is favourable for quantification by LAL or other tests. Hence, the common used combination of sample dilution and

heating at 75°C prior to LAL assay is not an appropriate method by which to assess the total quantities of (sequestered and free) endotoxin in systemic blood.

CHAPTER 8 NOTE - INTRALIPID STUDY

SHORT COMMUNICATION: ENDOTOXIN DOES NOT PARTITION INTO LIPID DROPLETS IN AQUEOUS EMULSIONS

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This chapter is based on a paper manuscript which has not been submitted for publication yet.

Abstract

We investigated whether bacterial endotoxins partition into oil droplets in emulsions by incubating a series of dilutions of *Escherichia coli* endotoxin at concentrations between 0.1 and 2.5 EU/ml with Intralipid® emulsion. Between 95 to 106 % of the endotoxin in the aqueous phase of the suspension was recovered. Hence, it appears that significant qualities of endotoxin do not readily partition into fat emulsions and thus are unlikely to transit the endothelium in solution with dietary fats.

8.1 Introduction

Recent data indicate that the levels of endotoxin, also referred to as lipopolysaccharide (LPS), are elevated in the systemic circulation after the consumption of a high fat meal (post-prandial endotoxemia) [1-7]. It is thought that a diet-mediated increase in systemic endotoxin levels could provoke a low grade pro-inflammatory immune response that may lead in turn to the onset of metabolic disorders such as obesity, insulin resistance, type 2 diabetes and non-alcoholic fatty liver disease [10-17].

A possible molecular mechanism of association of endotoxin into the systemic circulation is that hydrophobic lipid A moiety of the LPS molecular will partition into an apolar environment whilst the associated hydrophilic carbohydrate element will partition into a polar environment. Hence, endotoxin will associate with the oil-water interfaces of lipid droplets, to be co-absorbed with emulsified fat by enterocytes and to be subsequently incorporated into chylomicrons [1, 8, 9]. Endotoxin containing chylomicrons are then transported via the lymphatic system into the systemic circulation [1, 9].

We tested the rate at which endotoxin partitions into the fat micelles via the oil water interface by determining the quantities of endotoxin that partition into the fatty phase of a proprietary lipid emulsion, Intralipid®. This emulsion, normally used for intravenous feeding, comprises of fat particles of 0.5 microns in diameter consisting of a core of soybean oil with an interfacial membrane composed of phospholipids derived from egg yolk [19]. Hence Intralipid® provides a source of lipid droplets with a high interfacial surface area in an aqueous phase that contains no proteins.

8.2 Materials and methods

8.2.1 Materials

LPS from *Escherichia coli* O55:B5 was reconstituted in LAL reagent water (LRW) (both sourced from Lonza, Walkersville, MD, USA) according to the supplier's recommendation. The stock solution contained 50 endotoxin units (EU)/ml of LPS.

Intralipid® 20 % (Fresenius Kabi Australia Pty Ltd, Pymble, NSW, Australia) consists of 20 % soybean oil, 1.2 % egg yolk phospholipids, 2.25 % glycerine suspended in sterile water. The soybean oil contains linoleic (44 - 62 %), oleic (19 – 30 %), palmitic (7 – 14 %), linolenic (4 – 11 %) and stearic (1.4 – 5.5 %) acids. The pH of the emulsion ranges between 6 and 8.9.

8.2.2 Experimental design

8.2.2.1 Assay of the effect of incubation period on the recovery of endotoxin from the aqueous phase

The stock solution of endotoxin was diluted with LRW to a final concentration of 1 EU/ml. Two ml aliquots of the sample were each mixed either with 20 % Intralipid® or with equivalent volume of LRW (negative control) in a ratio of 1:10 (v/v). The mixtures were then either vortexed for 10 s and subsequently incubated at room temperature for 30 s, 1 min, 5 min, 10 min, 15 min or 30 min, or were vortexed continuously for the entire period of incubation (30 s, 1 min, 5 min, 10 min, 15 min or 30 min). All mixtures were subsequently centrifuged at 20,106 g at 14°C for 15 min (Beckman Coulter, Inc., Palo Alto, CA, USA) and the clear aqueous phase used for endotoxin assay.

8.2.2.2 *Assays of recoveries of various concentrations of endotoxin from the aqueous phase*

Aliquots of the stock solution of endotoxin were each diluted with the required volume of LRW to produce overall concentrations of endotoxin of 2.5 EU/ml, 1 EU/ml, 0.4 EU/ml and 0.1 EU/ml. Aliquots of each dilution were subsequently mixed either with 0.2 ml of Intralipid® 20 % or of LRW (negative control) to a total volume of 2 ml. These samples were then vortexed for 10 s and incubated in a water bath at 37°C for 5 min before being centrifuged at 20,106 g at 14 °C for 15 min and the clear aqueous phase used for endotoxin assay.

8.2.3 Quantification of endotoxin in the aqueous phase

The concentrations of endotoxin that remained in the aqueous phase of the various suspensions were determined using the kinetic-QCL™ LAL kit (Lonza, Walkersville, MD, USA) in a 96-well micro-plate reader (ELx808, BioTek Instruments, Inc., USA) according to the manufacturer's instructions, with the exception that the optical densities (ODs) of each sample were recorded every 30 s for a period of 2 h. Data were subsequently uploaded into KC4 software (BioTek Instruments, Inc., USA) and transferred into Microsoft EXCEL 2010 for further analysis. The concentrations of endotoxin were calculated on a basis of the time it took for the OD to rise from 0.5 unit above the initial OD value to 1.0 unit above the initial OD value (delta time) after addition of the LAL reagents (see [18] for more details). The concentrations of endotoxin in all samples that had been mixed with Intralipid® 20 %, were each determined with respect to values in the corresponding standard curve.

The standard curve for the first experiment was based on delta time values obtained for known concentrations of endotoxin in LRW (between 0.1 and 1.0 EU/ml) plotted against the logarithms of the concentrations.

The standard curve for the second experiment was generated from the delta time values of the negative controls (0.1 – 2.5 EU/ml). The recoveries of endotoxin from the aqueous phase following admixture with Intralipid® 20 % were each compared with the negative control as a percentage.

8.3 Results and Discussion

8.3.1 The effects of the period of incubation with Intralipid® on the recoveries of endotoxin from the aqueous phase

The recoveries of the standard dose of endotoxin (1 EU/ml) from the aqueous phase of the Intralipid® suspension were identical 86.1 % regardless of the period of incubation, both in samples that were continuously vortexed during the period of incubation and those that were not. Hence, the recoveries of endotoxin from aqueous phase of the Intralipid® suspension were neither dependent on the incubation time nor on the intensity of mixing.

8.3.2 The effect of the dose of endotoxin on its recovery from the aqueous phase

Between 95.6 and 106.7 % of the endotoxin dose was recovered from the aqueous phase with dosages ranging from 0.1 to 2.5 EU/ml (Table 8-1). There was no consistent pattern of variation in % recovery with endotoxin dose.

Table 8-1. Levels of endotoxin observed in aqueous phase after admixture with Intralipid® 20 % and the corresponding values for the recovery of endotoxin.

Endotoxin concentration in EU/ml		Recovery of endotoxin in %
expected	observed	
0.1	0.102	101.6
0.4	0.389	97.1
1.0	1.067	106.7
2.5	2.389	95.6

Hence, little if any LPS was absorbed by the oil droplets within the Intralipid® emulsion regardless of the duration of incubation or the dose of endotoxin. The difference in the recoveries obtained from the first (86.1 %) and the second (95.6 – 106.7 %) experiment presumably results from the differences in the preparation of endotoxin standards for the standard curves i.e. freshly prepared standards prior to

LAL assay in the first experiment and standards that were prepared the day prior to assay and which were heated at the same time as the Intralipid samples..

Our finding suggests that significant quantities of LPS do not readily partition into fat micelles in an aqueous phase that contains no protein and has a neutral pH. However, apolipoproteins are suggested to bind endotoxin [20-22] and therefore mediate neutralisation of endotoxin by circulating lipoproteins such as chylomicrons [23], low density lipoproteins (LDL) [20, 24] and high density lipoproteins (HDL) [20, 22, 24]. Further, the recovery of endotoxin from Intralipid® solution is reported to decrease when apolipoproteins such as apoB and apoA-I are added [25]. Bile or its salts may also modify the oil water interface in a manner that promote uptake of endotoxin by fat micelles. Thus it is conceivable that the action of certain agents such as apolipoproteins and bile or its salts within the chyme could facilitate the partition of gut-derived endotoxin into fat micelles. Further work is needed to determine the manner in which gut-derived endotoxin can be absorbed by enterocytes and how it is subsequently enters the systemic circulation.

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CHAPTER 9 TECHNICAL NOTE: LPS - ELISA

TECHNICAL NOTE – THE EVALUATION OF A NEW ELISA BASED KIT FOR THE DETECTION OF LPS MANUFACTURED BY CUSABIO

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This chapter is based on a paper manuscript which has not been submitted for publication yet.

Abstract

We evaluated whether the LPS - ELISA manufactured by Cusabio is a viable method for quantifying LPS compared with the Limulus Amebocyte Lysate (LAL) assay for the detection of endotoxin. We used the kit provided LPS standard, two commercially available types of purified LPS from *Escherichia coli* O55:B5, a commercially available control standard endotoxin (CSE) that was derived from the same strain of *E. coli*, and a sample of human faecal water to determine the sensitivity of the LPS - ELISA and the LAL assay to those different sources of LPS and endotoxin, respectively. We also investigated the LPS - ELISA for cross-reactivity with LPS binding protein (LBP).

Our results indicated that the LPS - ELISA from Cusabio failed to detect LPS from any of the sources of LPS other than the LPS standard provided with the kit. Further that the LPS - ELISA detected LBP standards and LBP that was spiked into samples of human serum. In addition, there was a significant correlation between detected levels of LPS and LBP in fasted serum samples of 10 female subjects ($r^2 = 0.683$; $P < 0.001$). Hence, it was likely that the tested LPS - ELISA kit detected LPS that was bound to LBP rather than 'free' LPS.

9.1 Introduction

Endotoxin, also referred to as lipopolysaccharide (LPS), is a component of the outer cell membrane of Gram-negative bacteria that is released up on their lysis in the intestinal lumen [1, 2]. A number of publications suggest that elevated levels of circulating endotoxin may be responsible for the pro-inflammatory status associated with obesity and insulin resistance in mice [3-5] and in humans [6, 7]. The Limulus Amebocyte Lysate (LAL) assay has been used to detect endotoxin in plasma and serum but previous work by our work group has shown that it was confounded by serum [8] i.e. serum proteins may have sequestered the endotoxin. Hence, we wished to assess whether detection of LPS by a commercial LPS - ELISA is a viable alternative. We first examined the ability of the ELISA kit to quantify various crude and refined forms of LPS and endotoxins in aqueous solution. We then investigated the cross-reactivity of the LPS - ELISA with LPS binding protein (LBP) in aqueous solution. Finally we examined cross-reactivity with LBP in samples of human serum.

9.2 Methods

Endotoxin-free certified tubes (Biopur® Safe/Lock micro test tubes, Eppendorf AG, Germany) and pipette tips (Endosafe® tips, Charles River, France) were used throughout the analyses.

Taking blood samples for the analysis of levels of LPS and LBP in sera was approved by Massey University Human Ethic Committee Southern A (application number 14/11) (Appendix III - i) and informed written consent was given by all volunteers (Appendix III - vii).

9.2.1 Bacterial LPS and endotoxin

Two commercially available sources of Lipopolysaccharide from *Escherichia coli* O55:B5 were each reconstituted with endotoxin-free water (Sigma® Life Science, Sigma-Aldrich, Switzerland) either to a concentration of 1 mg/ml (L6529-1MG, purified by gel-filtration chromatography) or to a concentration of 5 mg/ml (L4524-5MG, purified by ion-exchange chromatography) (both from Sigma® Life Science, Sigma-Aldrich, Israel). Both LPS stock solutions were stored in 50 µl aliquots at -20°C and were subsequently thawed and diluted with the sample diluent provided with the LPS - ELISA kit to produce a range of concentrations between 6.25 pg/ml and 0.5 mg/ml prior to LPS - ELISA assay. Further, stock solution of both types of LPS were diluted to 1:100 and 1:1000 with LAL reagent water (LRW) (Lonza, Walkersville, MD, USA) prior to LAL assay.

Similarly, the control standard endotoxin (CSE) from *E. coli* O55:B5 that was provided with a commercial available LAL kit (Lonza, Walkersville, MD, USA) was reconstituted with LRW water to a concentration of 50 EU/ml. The stock solution was further diluted with LRW immediately prior to use in a range of concentrations

between 0.0125 EU/ml and 25 EU/ml for use with the LPS - ELISA assay and in a range between 0.00625 and 0.4 EU/ml for use in the LAL assay.

9.2.2 LPS-binding protein

The LPS binding protein (LBP) standard that was provided with the LBP - ELISA kit, manufactured by Cusabio® Biotech Co., Ltd., (Wuhan, Hubei Province, P.R. China), was diluted with the sample diluent provided with the LPS - ELISA kit to a range of concentrations between 0.625 and 40 µg/ml. The LBP standards where then assayed with the LPS - ELISA kit to determine whether there were any cross-reactivity of the LPS - ELISA kit with the LBP standard.

9.2.3 Faecal water

Aliquots of a single stool sample were transferred into 15 ml centrifuge tubes (BD Falcon™) and centrifuged at 10,000 rpm for 2 h. The supernatant (faecal water) was stored at -80°C pending further analysis. One aliquot of the faecal water was diluted with the sample diluent provided with the LPS - ELISA kit to 1:10, 1:100 and 1:1000 and a similar range of dilutions was prepared with LRW as the diluent. A further aliquot of faecal water was diluted 1:3 with the sample diluent provided with the LPS - ELISA kit and sonicated. The sonicated sample was subsequently centrifuged at 13,000 rpm for 2 min and the supernatant analysed.

9.2.4 Serum samples

Fasted venous blood samples of six healthy females and of four females who had been diagnosed with Crohn's disease by a medical practitioner, were collected into BD plastic serum vacutainers (6.0 ml, Becton Dickinson and Company, Oxford, UK) twice with a one week interval between samples. Each sample was allowed to clot for 2 h before centrifugation for 15 min at 3,500 g at 4°C. The serum was then decanted into endotoxin-free micro-centrifuge tubes (Biopur® Safe-Lock micro test tubes; Eppendorf, Hamburg, Germany) and aliquots of each sample were stored at -80°C pending analysis. Four randomly chosen serum samples were thawed and spiked with 40 µg LBP/ml with a volume ratio of 1:10 and assayed by LPS - ELISA.

9.2.5 Quantification of LPS/LBP by LPS - ELISA

Assay were performed using the Human Lipopolysaccharides (LPS) ELISA kit, manufactured by Cusabio® Biotech Co., Ltd. (Wuhan, Hubei Province, P.R. China), following the manufacturer's instructions. The optical density (OD) of each sample was determined by a micro-plate reader (ELx808, Bio-Tek Instruments, Inc., USA) at 450 nm.

The assay was calibrated with a range of LPS concentrations prepared from the standard provided with the kit. The LPS standard was reconstituted with sample diluent provided with the kit and subsequently six dilutions using the sample diluent as diluent were prepared in the range over 6.25 to 200 pg/ml which was within the range specified by the manufacturer (6.25 to 400 pg/ml). The OD values of each LPS standard were plotted against the known concentration of LPS in CurveExpert Professional version 2.02 to generate a standard curve. The concentration of LPS detected in various samples was determined by comparison with the standard curve.

9.2.6 Quantification of endotoxin by Limulus Amebocyte Lysate Assay

A kinetic chromogenic LAL assay (kinetic-QCL™ LAL kit, Lonza, Walkersville, MD, USA) was used to determine endotoxin levels in various samples. The detection range of the LAL assay was stated by the manufacturer to lie between 0.005 to 50 EU/ml. The endotoxin standard provided with the kit was diluted with LRW to concentrations between 0.00625 and 0.4 EU/ml. The assay was performed as described earlier [9] (see also 3.2.4.2) and the standard curve and subsequently the quantification of endotoxin in samples were determined by a modified calculation algorithm that was described in detail previously [9]. Briefly, the time in seconds it took for OD to increase from 0.5 OD above the baseline OD to 1.0 OD above the baseline OD were determined for each standard and for each sample. The determined values of each endotoxin standards were then plotted against the logarithm function of the endotoxin concentration in CurveExpert Professional version 2.02 to generate endotoxin standard curves. The standard curve was then used to determine the concentration of endotoxin in various samples.

9.2.7 LBP - ELISA

LBP was quantified by the Human LPS-binding protein (LBP) ELISA kit, manufactured by Cusabio® Biotech Co., Ltd. (Wuhan, Hubei Province, P.R. China), according to the manufacturer's instructions. The optical density (OD) of each sample was determined by a micro-plate reader (ELx808, Bio-Tek Instruments, Inc., USA) at 450 nm.

The test was calibrated using the LBP standard that was provided with the kit, in a range of dilutions in sample diluent between 0.625 and 20 µg/ml which was within the range specified by the manufacturer (0.625 to 40 µg/ml). The OD values of LBP

standards were plotted against LBP concentration in CurveExpert Professional version 2.02 to produce a standard curve. The concentration of LBP in each of the serum samples was determined by comparison with the standard curve.

9.2.8 Statistical analysis

Statistical analysis was only performed with serum levels of LPS and LBP in serum using the SYSTAT software package version 13 (Systat Software Inc., Chicago, IL, USA). The correlation between LPS and LBP serum levels was assessed by linear regression, Pearson correlation coefficient and the Bartlett Chi-Square Statistic.

9.3 Results

9.3.1 Detection of LPS and endotoxin by LPS - ELISA

The standard curve (Figure 9-1A) of OD values for absorption at 450 nm vs. known concentration of LPS standard, provided with the kit, up to 200 pg/ml was a second order polynomial curve (r^2 = 0.9938). No quantifiable colour changes occurred with either of the two types of LPS from *E. coli* O55:B5 at doses between 6.25 pg/ml and 0.5 mg/ml or with CSE from *E. coli* O55:B55 at doses between 0.0125 EU/ml and 25 EU/ml (Figure 9-1B + C). Similarly, no quantifiable colour change occurred with diluted faecal water or with diluted and sonicated faecal water (Figure 9-1D).

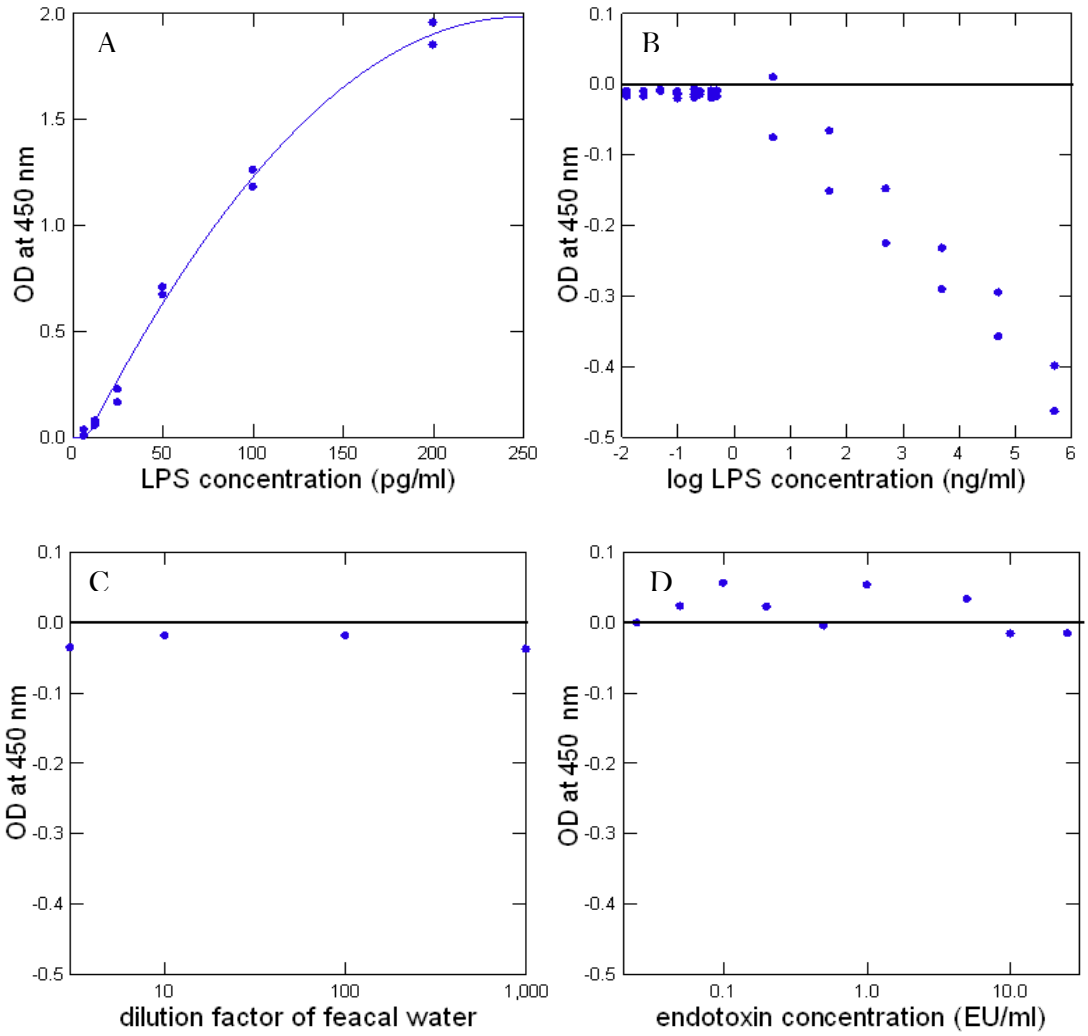

Figure 9-1. OD at 450 nm for A) standard LPS provided with the kit from Cusabio, B) LPS from *E. coli* O55:B5, C) CSE from *E. coli* O55:B5 and D) faecal water.

9.3.2 Detection of LPS by LAL assay

The levels of endotoxin detected by the LAL assay in the dilutions of both of the two types of *E. coli* LPS purchased from Sigma, and in all of the dilutions of faecal water were all above the highest standard of 0.4 EU/ml (Figure 9-2A).

When various LPS standards of the LPS - ELISA kit and the kit-provided sample diluent which was used for the dilution of the LPS standard, were assayed with the LAL assay the reaction curve of all of them were overlapping and were above the highest standard of 0.4 EU/ml (Figure 9-2B). Given the fact that the sample diluent provided with the LPS - ELISA kit alone showed similar reaction with the LAL assay then various LPS standards from the LPS - ELISA kit it seemed evident that the sample diluent in the LPS - ELISA kit interfered with the LAL assay. Hence the high level of 'background noise' caused by the sample diluent may have obscured the assay of endotoxin in the LPS standard provided with the LPS - ELISA kit at all concentrations between 6.25 pg/ml and 400 pg/ml.

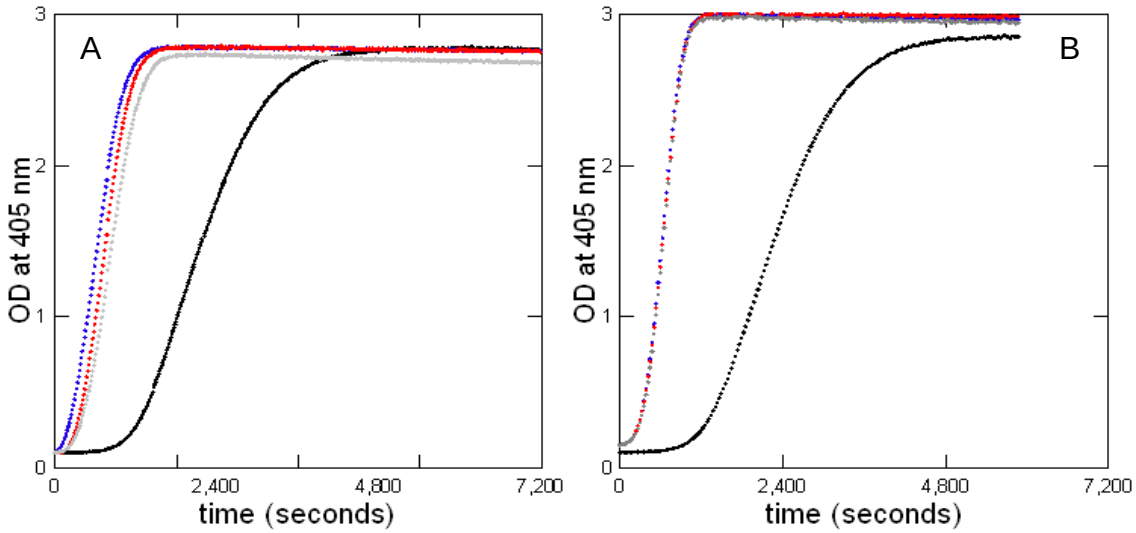

Figure 9-2. Reaction curves of the LAL assay A) of LPS from *E. coli* O55:5 and from faecal water; B) sample diluent and LPS standard from the LPS - ELISA kit.

A) black line - CSE 0.4 EU/ml, blue dots - 0.5 ng/ml LPS from E. coli O55:B5 (Sigma, 5mg/ml), red dots - 0.1 ng/ml LPS from E. coli O55:B5 (Sigma, 1 mg/ml) and grey dots - faecal water diluted 1:1000 with LRW. B) black line - CSE 0.4 EU/ml , blue dots - sample diluent from LPS - ELISA kit, red dots - 6.25 pg/ml standard LPS from LPS - ELISA kit and grey dots - 400 pg/ml standard LPS from LPS - ELISA kit.

9.3.3 Quantification of LBP standards by LPS - ELISA

The LBP standard curve of OD values at 450 nm obtained with dilutions of LBP standard up to 20 µg LBP/ml, on quantification with the LPS - ELISA, was a second order polynomial curve (r^2 =0.9863). The standard curve obtained from LBP was similar to the standard curve of OD values at 450 nm that was obtained with dilutions of LPS standard up to 200 pg LPS/ml. This was evident when the OD values of the dilution of LBP standard were plotted on the same x scale as OD values of the dilution of LPS standard (Figure 9-3).

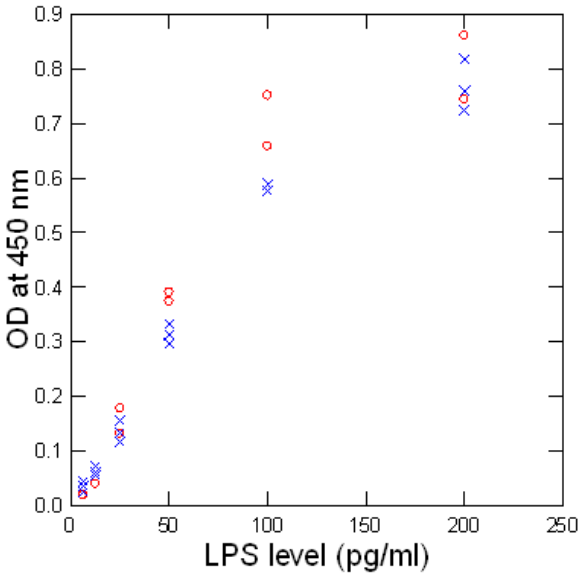

Figure 9-3. Comparison of LPS standard curve with the LBP standard curve obtained using LPS - ELISA.

Blue cross – LPS standard curve. Red circle – LBP standard curve.

9.3.4 Quantification of LBP spiked into serum samples by LPS - ELISA

No LPS was detected in two out of four unspiked serum samples whereas around 23 pg/ml of LPS was detected in the sera that was spiked with 40 µg LBP/ml with a volume ratio of 1:10 prior to LPS - ELISA assay. The remaining two unspiked serum samples had LPS level of 7.68 and 25.41 pg/ml. Similar to the other two samples, the

levels of LPS were higher in those serum samples that had been spiked with LBP than in unspiked serum (Table 9-1).

Table 9-1. Detected LPS levels in unspiked serum samples and their aliquots that have been spiked with 40 µg LBP/ml at a ratio of 1:10 prior to LPS - ELISA assay.

sample	LPS level in pg/ml (unspiked)	LPS level in pg/ml (spiked with 40 µg LBP/ml)
1	<6.25	22.43
2	<6.25	23.57
3	25.42	29.34
4	7.681	28.46

9.3.5 Correlation between ambient LPS and ambient LBP level in serum samples

The ambient concentrations of LPS and LBP in serum were determined using the same aliquot of each sample. There was a linear relationship (df = 1; F = 38.712; P < 0.001) between ambient levels of LPS and ambient levels of LBP in the sera within subjects (Figure 9-4). The correlation coefficient between the two was significant (df = 1, Bartlett Chi-Square = 20.083; P < 0.001; r^2 = 0.683).

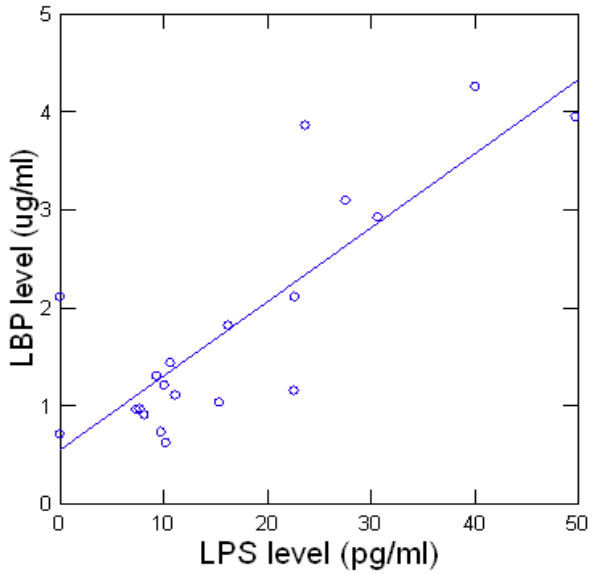

Figure 9-4. Correlation between serum LPS and LBP levels from repeated measurements from 10 subjects.

There was a strong correlation between serum LPS and LBP level within subjects (r^2 = 0.683; P < 0.001).

9.4 Discussion

Our data indicates that the proprietary LPS - ELISA kit was only able to detect the 'LPS' that was provided with the kit and failed to detect either of the two types of purified LPS from *E. coli* at any dilution, and failed to detect the LPS in faecal water at any dilution. Conversely using the LAL assay confirmed that endotoxin, and hence presumably LPS, was present in all of these materials. This finding raises the question as to the nature of the calibration material provided with the kit that one would presume to be pure LPS i.e. whether the test was sensitive only to a specific LPS provided by the manufacturer.

Further, the LPS - ELISA appears to detect the presumable pure dilutions of LBP standards that were provided with the LBP - ELISA kit which was supplied by the same manufacturer. Similarly, the kit was able to quantitatively detect the same presumable pure LBP when it had been spiked into serum samples. Moreover, there was a strong correlation between the ambient levels of LPS and the ambient levels of LBP in sera from 10 subjects (6 healthy women and 4 with Crohn's disease). Thus it is evident that the tested LPS - ELISA kit in its current form is unable to detect unbound LPS of a form that is normally found in microbial homogenates or in faecal water. Further, it seems likely that the LBP provided in the kit may have been combined with LPS and that the latter was the moiety detected by the LPS assay. At all events, this raises the question as to whether LPS or LBP were being detected by the LBP - ELISA.

9.5 Conclusion

These observations raised questions about the usefulness and reliability of the LPS - ELISA kit manufactured by Cusabio in research settings.

9.6 References

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CHAPTER 10 METHOD EVALUATION – CROHN'S DISEASE STUDY

THE EVALUATION OF OTHER METHODS FOR THE PRE-TREATMENT OF SERUM SAMPLES PRIOR TO LAL ASSAY AND THE EVALUATION OF ANOTHER ELISA FOR THE QUANTIFICATION OF LBP

10.1 Introduction

As previously discussed the LAL assay is interfered by constituents of the sera. Hence, the pre-treatment of serum samples prior to LAL assay and the adaptation of the calculation algorithm are required. Data of the method evaluation prior to the Intralipid study indicated that the combination of dilution with DA and heating at 75°C for 10 min appeared to be the best method for the preparation of serum samples prior to assay, to minimise inhibition or enhancement of the assay by constituents of the sera (Chapter 6). However, this conclusion was drawn based on Positive Product Controls (PPCs) of a series of diluted and heated serum samples. It is noteworthy that PPCs, spiking of pre-treated sample with endotoxin prior to assay, can only indicate whether the assay is interfered by the pre-treated sample but does not give any information whether the 'endogenous' concentration of endotoxin in the sample is accurately recovered. In order to determine the latter a series of raw serum samples of the Intralipid study were spiked with known concentrations of endotoxin prior to pre-treatment of those samples. Results of this set of experiments indicated that endotoxin spiked into raw serum samples was not or barely recovered after the pre-treatment of the samples. It seems conceivable that the spiked endotoxin was sequestered by constituents of the raw sera or that it was sequestered by serum proteins during the process of denaturation which reduces the amount of endotoxin to be detected by the assay. If the latter is the case, the method for the pre-treatment of serum has to be modified in order to minimise sequestration of endotoxin by serum proteins during the process of denaturation.

I therefore firstly investigated whether the digestion of serum proteins by Endotoxin Sample Preparation (ESP™) kit (BioDtech, Inc., Birmingham, AL, USA) or by proteinase K improves the recovery of endotoxin that has been spiked to raw serum samples prior to pre-treatment of the sample. The ESP™ kit is designed to improve

the recovery of endotoxin in plasma samples [1]. However, the test has not yet been used for the pre-treatment of serum samples according to information provided by the manufacturer. Proteinase K is routinely used to digest proteins in plasma or serum samples prior to isolation of RNA and DNA in those samples. Furthermore, I investigated whether a heating step at a lower temperature such as at 60°C, allows more endotoxin to be recovered than after a heating step at 75°C.

In addition, data of the Crohn's disease study indicated that the ELISA kit manufactured by Cusabio is not a reliable method for the quantification of LBP in human serum. I therefore evaluated the reliability of the human LBP – ELISA manufactured by Hycult.

10.2 Methods

10.2.1 Subjects and sampling of blood samples

For the work related with the modification of the method for the pre-treatment of serum prior to LAL assay, blood samples from four healthy volunteers were each drawn into glass 'serum' vacutainers (10ml, Ref. 366430; Becton, Dickinson and Company (BD), USA) after fasting. An extension of the number of blood samples taken from volunteers was approved by MUHEC: Southern A application number 12/61 (letter from 26 May 2014) (Appendix I - i) and informed written consent was given by volunteers (Appendix I - iii).

For the evaluation of the modified method for the pre-treatment of serum samples prior to LAL assay and for the experiment using the LBP – ELISA manufactured by Hycult, blood samples from 13 healthy women and from 12 women with Crohn's disease were each drawn into glass 'serum' vacutainer (10ml, Ref. 366430; Becton, Dickinson and Company (BD), USA) after fasting. Those samples were taken as part of the Crohn's disease study. This project was approved by MUHEC: Southern A application number 15/18 (Appendix IV - i) and informed written consent was given by volunteers (Appendix IV - vii).

10.2.2 Modification of the method for the pre-treatment of serum samples prior to LAL assay

Kinetic-QCL™ LAL kits (Lonza, Walkersville, MD, USA) from two different batches (lot 0000410350 and 0000438466) were used for the experiments described in this section. The LAL assay was conducted as previously described (Chapter 7) with the exception that the OD values were recorded every 30 s for a period of 2 h and that data were uploaded into KC4 software (Bio-Tek Instruments, Inc., USA).

10.2.2.1 The evaluation of the pre-treatment of serum samples by the Endotoxin Sample Preparation kit prior to the LAL assay

The Endotoxin Sample Preparation (ESP™) kit was used according to the manufacturer's instruction. Briefly, unspiked raw serum and serum that has been spiked with 50 EU/ml at a ratio of 1:10 (v/v) immediately prior to pre-treatment of samples, were each added into endotoxin-free certificated glass tubes (depyrogenated 13 x 100 mm borosilicate glass dilution tubes, Charles River Laboratories, Charleston, SC, USA) and samples then heated at 60°C for 30 min. Samples were allowed to cool down before an aliquot of 30 µl heat-inactivated serum was added to 270 µl buffer #1 in a fresh endotoxin-free glass tube. The sample had an acid pH value after dilution with buffer #1. The mixture was vortexed for 10 s and 30 µl of protease solution was then added to the mixture followed by further vortexing for 10 s. Negative controls were prepared by replacing the protease solution with an equal volume of buffer #1. Samples were then digested by incubating at 37° for 30 min. Again samples were allowed to cool down before an aliquot of 50 µl of digested serum sample were each added to 450 µl buffer #2 in endotoxin-free micro-centrifuge tubes. The usage of buffer #2 brought the pH value of the mixture back to a neutral pH level. An aliquot of each digested and pH neutral sample was also spiked with endotoxin at a concentration of 1 EU/ml at a ratio of 1:10 (v/v) prior to LAL assay to determine the PPC of each sample. Levels of endotoxin in all samples were then analysed using the LAL assay as described above.

10.2.2.2 Preparation of serum samples using proteinase K

Thawed raw serum samples were spiked with endotoxin at a concentration of 50 EU/ml at a ratio of 1:10. Proteinase K (Roche Diagnostic GmbH, Mannheim, Germany) was reconstituted with LAL reagent water (LRW) (Lonza, Walkersville, MD, USA) according to the manufacturer's instruction to give a proteinase K stock solution of 2 mg/ml. Aliquots of the stock solution were further diluted with LRW to total concentrations of 20 µg/ml and 2 mg/ml. Aliquot of 50 µl proteinase K was mixed with 500 µl endotoxin-spiked raw serum and with 450 µl Endosafe® Dispersing Agent (DA) (Charles River Laboratories, Charleston, SC, USA) in endotoxin-free certified glass tubes (depyrogenated 13 x 100 mm borosilicate glass dilution tubes, Charles River Laboratories, Charleston, SC, USA). For the negative controls proteinase K was each replaced by an equal volume of DA. The mixtures were then pulse-vortexed for 30 s and digested at 60°C for 30 min. Digested serum samples were then further diluted with DA to a total dilution factor of 10, 20 and 40. An aliquot of each dilution series was then transferred into a fresh glass tube and heated at 75°C for either 5 or 10 min to determine whether the denaturation of proteinase K by a second heating step was required prior to LAL assay. An aliquot of each sample (with and without a second heating step) was also spiked with endotoxin at a concentration of 1 EU/ml at a ratio of 1:10 (v/v) to determine the PPC of each mixture. Endotoxin levels in all mixtures were then analysed using the LAL assay as described above.

10.2.2.3 Preparation of serum samples with a heating step at 60°C

The sample preparation of the negative controls in the previous series of experiments with proteinase K included two dilution steps (one prior to and one after the initial heating step). I then investigated whether the recoveries of spiked-endotoxin in these samples differed from those in endotoxin-spiked serum samples that were diluted only once at the beginning of the sample preparation. Again, raw serum was spiked with 50 EU/ml at a ratio of 1:10 immediately prior to sample preparation. Samples with one dilution step were diluted with DA by factor 20, 40, 60 and 100 in endotoxin-free certified glass tubes (depyrogenated 13 x 100 mm borosilicate glass dilution tubes, Charles River Laboratories, Charleston, SC, USA). Samples were pulse-vortexed for 30 s and then heated at 60°C for 30 min. Samples were allowed to cool down and an aliquot was decanted into a micro-centrifuge tube. The remaining sample in the glass tube was subsequently heated for a second time at 75°C for 5 min. The latter samples were also decanted into a micro-centrifuge tube after samples were cooled down. In the case of samples with two dilution steps, the raw serum sample was initial diluted by an equal volume of DA and pulse-vortexed for 30 s. Samples were then heated at 60°C for 30 min. Samples were allowed to cool down and further diluted with DA to a total dilution factor of 20, 40, 60 and 100 in a micro-centrifuge tube. An aliquot of each sample was transferred into a fresh glass tube and then heated at 75°C for 5 min. Levels of endotoxin in samples were analysed using the LAL assay as described above.

I then investigated whether the recovery of endotoxin that was spiked into raw serum prior to pre-treatment of serum was a function of the spiked dose. Hence, raw serum samples of three volunteers (two healthy female subjects and one woman with inactive CD) were each spiked with endotoxin at concentrations of 5, 10, 25, 50 and 100 EU/ml at a ratio of 1:10 (v/v) immediately prior to pre-treatment of serum. Spiked and unspiked raw serum samples were then diluted 20-fold with DA in endotoxin-free

glass tubes and pulse-vortexed for 30 s. Diluted samples were then heated at 60°C for 30 min. Heated samples were allowed to cool down prior to transferring into endotoxin-free centrifuge tubes and were then analysed.

10.2.3 Evaluation of the modified method for the pre-treatment of serum samples prior to LAL assay

Kinetic-QCL™ LAL kits (Lonza, Walkersville, MD, USA) from batch # 0000438466 were used for the experiments described in this section.

An aliquot of each thawed serum sample was spiked with either 2.5 or 10 EU/ml, and with 25 and with 100 EU/ml (endotoxin standard provided with LAL assay) at a volume ratio of 1:10 immediately prior to preparation of serum samples. Endotoxin-spiked and unspiked sera were each diluted 20-fold with Endosafe® Dispersing Agent (DA) (Charles River Laboratories, Charleston, SC, USA) in a micro-centrifuge tube and pulse-vortexed for 20 s. The diluted samples were then transferred into endotoxin-free certified glass tubes (depyrogenated 13 x 100 mm borosilicate glass dilution tubes, Charles River Laboratories, Charleston, SC, USA) and heated in a water bath (SUB6, Grant Instruments (Cambridge) Ltd, England) at 60°C for 30 min. Heated samples were allowed to cool prior to transferring into fresh endotoxin-free micro-centrifuge tubes. The LAL assay was conducted as described above. The observed delta time (s) of a series of endotoxin standards were compared with those of pre-treated serum samples that were spiked with endotoxin, to allow the effect of the pre-treatment to be quantified.

10.2.4 The evaluation of the human LBP – ELISA manufactured by Hycult for the quantification of LBP in serum

10.2.4.1 Calibration of the LBP – ELISA manufactured by Hycult

The human-specific LBP - ELISA, manufactured by Hycult® Biotech (Uden, Netherlands), was used according to the manufacturer's instruction (3.4.1). The reported detection range of the ELISA was from 4.4 to 50 ng LBP/ml. However, the colour development, as assessed by optical density (OD) at 450 nm, was barely visible in the two lowest standards (4.4 and 6.6 ng/ml) (Figure 10-1A). Hence, the OD values of the latter two standards were not included for the generation of the standard curve. The standard curve for LBP in the range between 9.9 to 50 ng/ml was non-linear but was fitted by second order polynomial function over this range ($r^2 > 0.99$) (Figure 10-2B).

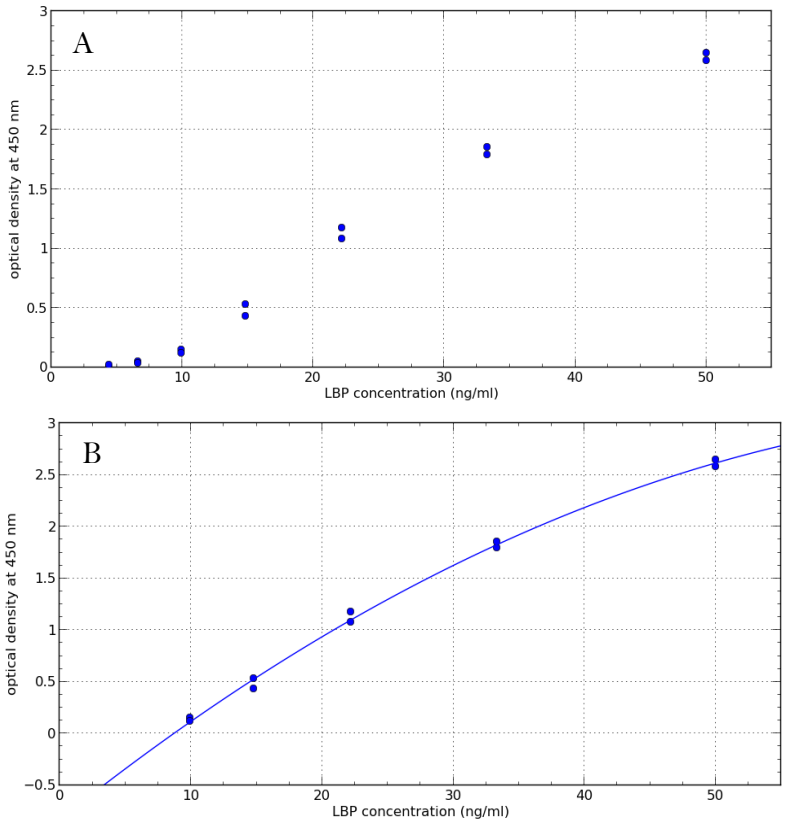

Figure 10-1. OD values of LBP at concentrations between 4.4 and 50 ng/ml (A) and the standard curve generated in the range from 9.9 to 50 ng/ml (B)

10.2.4.2 The determination of an appropriate dilution factor

The manufacturer of the LBP – ELISA kit recommends dilution of serum samples by at least factor 1,000 prior to assay. Four randomly chosen serum samples that were prepared from blood that was allowed to clot for 2 h, was used in this set of experiments. This clotting period was not recommended by the manufacturer of this LBP – ELISA kit. However, this clotting period was used for any other tests determining LPS, endotoxin and LBP in serum samples.

Aliquots of serum sample were each 10-fold diluted with dilution/wash (d/w) buffer that was provided with the kit, and subsequently further diluted with d/w buffer by factor 100 to give a total dilution factor of 1,000. Similarly, aliquots of serum samples were each diluted 20-fold followed by a further 100-fold dilution to give a total dilution factor of 2,000. The endogenous levels of LBP in those diluted serum samples were then determined using the LBP – ELISA according to the manufacturer's instruction (3.4.1).

10.2.4.3 Testing for interferences between constituents of serum samples and the ELISA

Thirteen randomly chosen serum samples prepared from blood that was again allowed to clot for 2 h, were used for this series of experiment. Human LBP (SRP6033-10UG, Sigma Aldrich®, SIGMA, Saint Louis, MO, USA) was reconstituted with MilliQ water according to the manufacturer's instructions to yield a concentration of 1 mg/ml. The stock solution was then diluted with d/w buffer to a concentration of 100 µg/ml. Aliquots of thawed raw serum samples were each spiked with LBP at a concentration of 100 µg/ml at a ratio of 1:10 (v/v). Aliquots of spiked and unspiked serum samples were 10-fold diluted with d/w buffer and immediately further diluted with d/w buffer by factor 100 to give a total dilution factor of 1,000. Levels of LBP in diluted (spiked

and unspiked) serum samples were determined using the ELISA according to the manufacturer's instructions (3.4.1) and the recovery of spiked LBP calculated in comparison to the endogenous concentration of LBP in the corresponding unspiked sample.

10.2.4.4 Testing the reliability of the ELISA to detect LBP from sources other than those provided with the ELISA

Aliquots of the solution of human LBP (SRP6033-10UG, Sigma Aldrich®, SIGMA, Saint Louis, MO, USA) with a concentration of 100 µg/ml were again diluted with d/w buffer, that was provided with the ELISA kit, to yield concentration of 9.9, 14.8, 22.2, 33.3, 50 ng/ml and 5,000 ng/ml. Aliquots of those dilution series and of the LBP solution with a concentration of 100 µg/ml were concurrently analysed with the LBP standards provided with the ELISA kit.

10.3 Results

10.3.1 Evaluation of other methods for the pre-treatment of serum samples prior to LAL assay

10.3.1.1 The effect of the ESP™ kit on the recovery of endotoxin spiked into raw serum

The observed levels of 'endogenous' endotoxin were higher in the negative controls (no protease solution added prior digestion step) than in digested serum samples (protease solution added). The recoveries of endotoxin spiked into raw serum samples in the negative control were similar to those in digested serum samples. However, the PPCs of digested serum samples indicated that the LAL reaction was enhanced by those samples whereas the LAL reaction was not significantly interfered by negative controls (Table 10-1).

Table 10-1. Observed endotoxin levels in serum and endotoxin-spiked serum after the sample preparation with the ESP™ kit.

sample	Observed endotoxin level (EU/ml)	Expected endotoxin level (EU/ml)	Recovery of spiked endotoxin (%)	Positive Product Control (%)
Negative control unspiked	1.2	NA	NA	119.0
Negative control spiked	1.3*	5.0	26.0	86.7
unspiked	0.0	NA	NA	220.3
Spiked	1.0*	5.0	20.6	253.2

* corrected by baseline value of unspiked sample

10.3.1.2 The effect of proteinase K digestion on the recovery of endotoxin spiked into raw serum

No endotoxin was recovered in serum samples that were pre-treated with proteinase K at a concentration of 2 mg/ml. It is noteworthy that PPC was 0.7 % when the serum samples were digested with 2 mg/ml of proteinase K which was not denatured by a second heating step whereas the PPC reached 91.3 % when the serum samples were digested by 2 mg/ml of proteinase K and the proteinase K subsequently denatured by a heating step at 75°C for 5 min (Table 10-2). In contrast, 1.6 – 46.1 % of the spiked dose of endotoxin was recovered when proteinase K at a concentration of 20 µg/ml was used. It is noteworthy that a significantly higher amount of spiked endotoxin was recovered when the serum samples were diluted 40-fold than when the serum samples were diluted by factor 10 (Table 10-2). However, the recoveries of spiked endotoxin were significant higher in the negative controls (serum samples pre-treated without proteinase K) than in serum samples digested with 20 µg/ml proteinase K (Table 10-2).

Table 10-2. Observed endotoxin levels in endotoxin-spiked serum after the sample preparation with and without proteinase K.

Concentration of Proteinase K	Dilution factor	2nd heating step	Observed endotoxin level (EU/ml)	Expected endotoxin level (EU/ml)	Recovery of spiked endotoxin (%)	Positive Product control (%)
Negative control	20	N	1.5	5.0	30.4	152.0
Negative control	20	5 min	3.8	5.0	75.9	138.9
Negative control	40	N	2.1	5.0	42.1	NA
Negative control	40	5 min	2.5	5.0	50.6	NA
20 µg/ml	10	N	0.1	5.0	1.6	131.3
20 µg/ml	10	5 min	0.3	5.0	5.9	143.8
20 µg/ml	10	10 min	0.2	5.0	3.8	154.4
20 µg/ml	20	N	0.7	5.0	14.8	136.5
20 µg/ml	20	5 min	0.7	5.0	14.8	179.3
20 µg/ml	20	10 min	0.7	5.0	14.8	179.3
20 µg/ml	40	N	1.2	5.0	23.8	143.8
20 µg/ml	40	5 min	2.3	5.0	46.1	115.9
20 µg/ml	40	10 min	1.2	5.0	23.8	186.7
2 mg/ml	20	N	0	5.0	0	0.7
2 mg/ml	20	5 min	0	5.0	0	91.3
2 mg/ml	40	N	0	5.0	0	NA
2 mg/ml	40	5 min	0	5.0	0	NA

10.3.1.3 The effect of a heating temperature at 60°C on the recovery of endotoxin spiked into raw serum

The recoveries of spiked endotoxin were higher in samples that were diluted by factor 20 and 40 in a single step compared to those where two dilution steps were applied to achieve the total dilution factor of 20 and 40. However, the recoveries of endotoxin spiked into raw serum declined to 21.1 % and 4.7 % when samples were diluted with DA by factor 60 and 100, respectively (Table 10-3). The PPCs values suggest that the sample matrix slightly enhances the assay (Table 10-3) but those values were within the manufacturer's recommended range between 50 and 200 % [2].

Table 10-3. Observed endotoxin levels in endotoxin-spiked serum samples that have been diluted once prior to the first heating or that have been diluted twice (before and after the first heating step).

Dilution steps	Dilution factor	2nd heating step	Observed endotoxin level (EU/ml)	Expected endotoxin level (EU/ml)	Recovery of spiked endotoxin (%)	Positive Product control (%)
1	20	N	3.2	5.0	63.2	115.7
1	20	Y	3.2	5.0	63.2	170.7
1	40	N	3.0	5.0	60.8	NA
1	40	Y	3.7	5.0	73.0	NA
1	60	N	1.1	5.0	21.1	NA
1	60	Y	1.1	5.0	21.1	NA
1	100	N	0.2	5.0	4.7	NA
1	100	Y	0.5	5.0	9.8	NA
2	20	N	1.5	5.0	30.4	152.0
2	20	Y	3.8	5.0	75.9	138.9
2	40	N	2.1	5.0	42.1	NA
2	40	Y	2.5	5.0	50.6	NA
2	60	N	2.2	5.0	43.8	NA
2	60	Y	1.8	5.0	36.5	NA
2	100	N	0.8	5.0	16.9	NA
2	100	Y	0.6	5.0	11.7	NA

10.3.1.4 The relationship between the dose of endotoxin spiked into raw serum and its recovery

The recoveries of endotoxin spiked into raw serum at various concentrations varied broadly from around 25 % to around 100 % between individuals. However, the percentages of recoveries within subjects were similar (Table 10-4).

Further, there was a significant linear regression (df = 1; subject 1: F = 687.795; subject 2: F = 360.674; subject 3: F = 537.162; P < 0.001 for all 3 subjects) between the concentration of added endotoxin (not corrected by dilution factor) and the reaction time of endotoxin-spiked serum samples within subjects and the correlation coefficient between the two was significant (df = 6, Bartlett Chi-Square Statistic = 31.932; P < 0.001; subject 1: r^2 = -0.998; subject 2: r^2 = -0.996 and subject 3: r^2 = -0.997). The linear trend line of each subject generated from the added amount of endotoxin (not corrected by dilution factor) and the corresponding reaction time were parallel to those of the endotoxin standard curve (Figure 10-2).

Table 10-4. Recoveries of endotoxin spiked into raw serum at a series of known concentration prior to sample preparation.

Subject #	Spiked with endotoxin (EU/ml)	Observed endotoxin level (EU/ml)	Expected endotoxin level (EU/ml)	Recovery of spiked endotoxin (%)
1	0	0.1	NA	NA
1	5	0.6	0.6	101.3
1	10	1.3	1.1	121.1
1	25	2.7	2.6	103.4
1	50	6.1	5.1	120.6
1	100	10.7	10.1	105.9
2	0	0.0	NA	NA
2	5	0.1	0.5	25.9
2	10	0.2	1.0	23.3
2	25	0.5	2.5	20.4
2	50	1.4	5.0	27.2
2	100	2.5	10.0	24.5
3	0	0	NA	NA
3	5	0.2	0.5	38.3
3	10	0.4	1.0	42.0
3	25	0.9	2.5	36.8
3	50	2.5	5.0	49.0
3	100	5.4	10.0	53.7

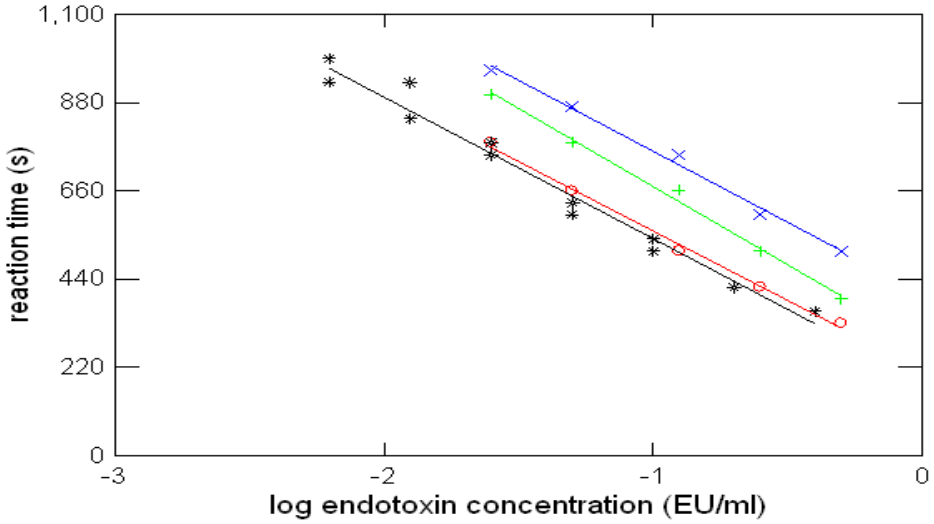

Figure 10-2. Reaction time of serum samples that have been spiked with a series of known concentrations of endotoxin prior to sample preparation.

Reaction time of spiked into serum samples at various concentrations compared to endotoxin standards. There was strong correlation between added levels of endotoxin and the corresponding reaction time on LAL assay. Linear trend lines of detectable spiked endotoxin within subjects were parallel to the endotoxin standard curve. Black * – endotoxin standards, red o – subject 1 (r^2 = -0.998; P < 0.001), blue x - subject 2 (r^2 = -0.996; P < 0.001) and green + - subject 3 (r^2 = -0.997; P < 0.001).

The reaction times of the endotoxin standards and those of serum spiked with endotoxin were then 'normalised' by subtracting the corresponding reaction time of the endotoxin at a concentration of 0.025 EU/ml (lowest common concentration of endotoxin between endotoxin-spiked serum and endotoxin standards). The normalised reaction times were plotted against the logarithm of endotoxin concentration for each subject and the endotoxin standards. The linear trend lines of endotoxin spiked into serum of three subjects were similar to those of the endotoxin standards (Figure 10-3A) and the 95 % confidence interval of the linear trend line generated from values of all three subjects was overlapping with the 95 % confidence interval of the linear trend line generated from endotoxin standards (Figure 10-3B).

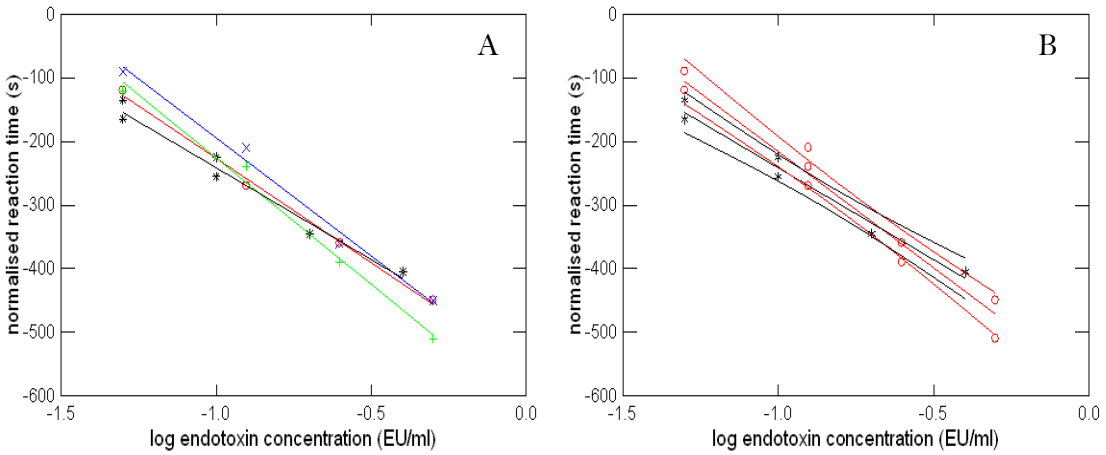

Figure 10-3. Normalised reaction times of serum samples that have been spiked with series of known concentrations of endotoxin prior to sample preparation compared to those values of the endotoxin standards.

Reaction times of endotoxin-spiked serum and endotoxin standards were normalised by the corresponding reaction time at a concentration of 0.025 EU/ml. A) Normalised reaction times of endotoxin-spiked serum of each subjects compared with those of the endotoxin standards. Black * - endotoxin standards, red o – subject 1, blue x – subject 2 and green + - subject 3. B) Trend line and 95 % confidence interval of pooled normalised reaction time of endotoxin-spiked serum (red) compared to those of endotoxin standards (black). The 95 % confidence interval of endotoxin-spiked serum overlaps with those of the endotoxin standards.

10.3.2 Comparison of delta time observed from a series of endotoxin standards and from pre-treated serum samples that were spiked with endotoxin

A linear curve for each subject was generated by plotting delta time (s) of spiked serum sample against the logarithm of the concentration of the spiked dose of endotoxin. There were no significant differences between the slopes of individual linear curves generated from spiked serum in healthy women from those in women with CD on ANOVA. Similarly, there were no significant differences between y-intercepts of individual linear curves from healthy women and those from subjects with CD on ANCOVA. However, the slopes of individual linear curve generated from serum samples spiked with endotoxin were significantly different ($df = 2,27$; $F = 4.25$; $p = 0.025$) from those obtained with a series of endotoxin standards in LRW on ANOVA (Figure 10-4).

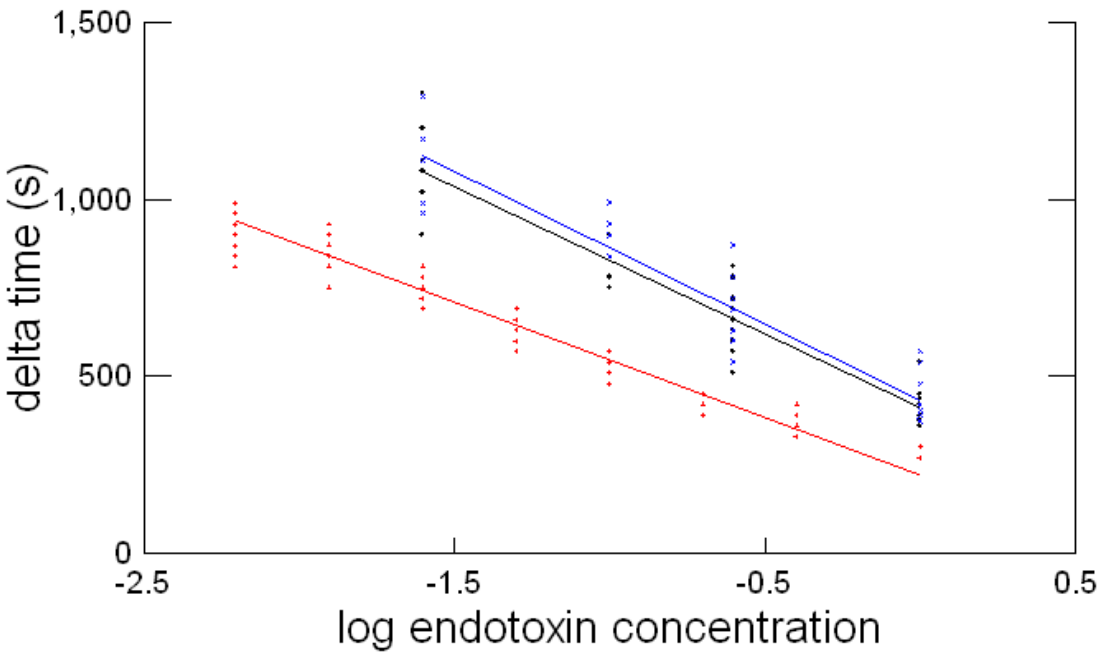

Figure 10-4. Comparison of the pooled linear curve obtained with a series of endotoxin standards in LRW (red line) with the pooled linear curve obtained with serum samples either from healthy women (blue line) or women with CD (black line) that were spiked with various concentrations of endotoxin.

10.3.3 The evaluation of the LBP – ELISA manufactured by Hycult

10.3.3.1 The determination of an adequate dilution factor

The levels of endogenous LBP were higher when serum was diluted by factor 2,000 compared to when it was diluted by factor 1,000, presumably as the levels of LBP were around the detection limit when serum was 2,000-fold diluted (Table 10-5).

Table 10-5. Levels of LBP in serum determined with different dilution factors.

Sample #	Dilution factor	LBP concentration (µg/ml)
1	1000	17.1
1	2000	20.8
2	1000	11.9
2	2000	18.8
3	1000	16.3
3	2000	20.7
4	1000	16.1
4	2000	20.8

10.3.3.2 The recovery of LBP spiked into raw serum samples

The observed levels of LBP were lower in all thirteen serum samples that were spiked with LBP solution from Sigma prior to dilution of serum, than in the corresponding unspiked serum sample (data not shown).

10.3.3.3 The quantification of dilution series of LBP sourced from a different company

No LBP was detected by the LBP – ELISA manufactured by Hycult, when dilution series of LBP sourced from Sigma were analysed at concentrations between 9.9 and 50 ng/ml. LBP levels of 16.1 ng/ml and of 55.9 ng/ml were determined in LBP solutions from Sigma containing LBP at concentrations of 5 µl/ml and of 100 µl/ml, respectively.

10.4 Discussion

The data regarding the evaluation of various methods for the pre-treatment of serum prior to LAL assay indicated that the digestion of serum proteins either by the ESP™ kit or by proteinase K did not improve the recoveries of endotoxin spiked into raw serum. Further that the LAL assay was interfered by proteinase K at concentration of 2 mg/ml and therefore denaturation of proteinase K i.e.by a second heating step at 75°C, was required prior to LAL assay. In contrast, proteinase K at a concentration of 2 µg/ml did not interfere with the LAL assay and therefore no additional heating step was required. However, the simple denaturation of constituents of serum by a combination of a 20-fold dilution with DA followed by heating at 60°C for 30 min was the most effective method for both inactivation of serum constituents that interfered with the LAL assay and recovery of endotoxin spiked into raw serum. It is noteworthy that plain DA inhibits the LAL assay, presumably as its pH is below the optimal pH range of the LAL assay. Hence, it seems likely that the high dilutions with DA were responsible for the low recovery of spiked endotoxin in serum samples that were diluted with DA by factor 60 and 100. Preliminary data indicate that there are individual variations in the recovery of spiked endotoxin as assessed by delta time (s) of each sample. This finding was later confirmed with endotoxin-spiked serum samples of the CD study.

With respect to the LBP – ELISA manufactured by Hycult, it was not possible to determine the recovery of LBP spiked into raw serum samples using the LBP standard provided with the ELISA kit, due to the high dilution of serum samples i.e. by factor 1,000. Hence, LBP from another source was used for the assessment of LBP recovery. In addition, a dilution series of LBP sourced from Sigma, in d/w buffer was concurrently analysed. Results of both experiments suggest that the ELISA kit from Hycult failed to detect LBP from Sigma at concentrations within the stated detection

range. However, it is possible that the chemical and structural characteristics of the human LBP standard provided with the ELISA differ from those of the human LBP sourced from Sigma i.e. human LBP sourced from Sigma is expressed by CHO (Chinese hamster ovary) cells [3] whereas the origin of LBP provided with the kit is unknown. There are a couple of known polymorphisms in the gene of the human LBP [4-6] and the structure of LBP also varies between species [7, 8]. Hence, it is conceivable that differences in the origin of LBP and thus in its structural characteristic resulted in reduced sensitivity of the assay to LBP sourced from Sigma.

10.5 Conclusion

The combination of dilution and heating at 60°C for 30 min might be a reliable method for the pre-treatment of sera prior to determination of endotoxin levels. With respect to the LBP – ELISA manufactured by Hycult, further work is needed to confirm its sensitivity.

10.6 Reference

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CHAPTER 11 CROHN'S DISEASE STUDY

AN EXAMINATION OF INDICES OF MUCOSAL INFLAMMATION, GUT PERMEABILITY AND ENDOTOXIN TRANSLOCATION AS INDICATORS OF CROHN'S DISEASE – RESULTS OF A PILOT STUDY

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This chapter is based on a manuscript of a paper that has not been submitted for publication yet.

ABSTRACT

The ability of various parameters based on immune function (faecal calprotectin and C-reactive protein (CRP)), gut permeability (lactulose and mannitol) and translocation of bacterial products (serum endotoxin and lipopolysaccharide binding protein (LBP)) to distinguish subjects with Crohn's disease (CD) in remission or with mild symptomatology (Harvey-Bradshaw Index) from healthy subjects was determined in 13 healthy female subjects and 13 female subjects with a history of Crohn's disease (CD). The ability of serum to sequester a standard spiked dose of endotoxin was also determined in a subset of these subjects. We compared the results by multivariate analysis and assessed whether combination of these parameters were better able to distinguish subjects with CD.

Faecal calprotectin levels ($p < 0.001$), lactulose excretion ($p = 0.009$), the lactulose-mannitol ratio ($p = 0.015$) were all significantly greater in subjects with a history of CD than in healthy subjects. Detectable levels of systemic endotoxin were found in 3 subjects with CD but none was detected in any of the healthy subjects. The serum levels of LBP and CRP of subjects with CD did not differ significantly from those in healthy subjects. The predictive capacity of combination of faecal calprotectin levels with lactulose and mannitol uptake was marginally better than faecal calprotectin alone in distinguishing low levels of activity of CD. The relative proportion of endotoxin spiked at a concentration of 10 EU/ml recovered from random sera of five subjects with mild to moderate CD was significantly higher than that recovered inform random the sera of eight healthy subjects ($p = 0.04$). Hence, whilst serum endotoxin levels were not significantly elevated in subjects with CD, on-going endotoxin entry may have reduced serum binding capacity.

11.1 Introduction

Crohn's disease (CD) influences the local (mucosal) immune response e.g. the release of calprotectin by neutrophils [1-4]; the mucosal integrity e.g. increased paracellular permeability to lactulose [5-9]; the translocation of enteral bacterial products into the systemic circulation [10-12] and the secondary systemic inflammatory response [12, 13]. It is noteworthy that there is a weak correlation between clinical symptoms e.g. Crohn's disease activity index (CDAI) or Harvey-Bradshaw Index (HBI), and various parameters of gut health [1-4, 14]. Hence, the quantification of the effects of the disease on any of the previous mentioned processes may provide a useful tool for monitoring the activity of the disease.

It would seem logical that the most sensitive pathological parameter would be that which is first affected in the advancing pathogenesis. However, analytic circumstances may conspire to defeat such logic. Hence for example, the assay of the levels of circulating endotoxin would seem to be an ideal indicator as it would provide quantitative insight into barrier compromise and immune challenge. However, the results regarding elevation of levels of endotoxin in subjects with CD are equivocal. One group has reported that endotoxin levels were elevated in subjects with either active or inactive CD [10] whilst others reported no significant differences between healthy subjects and those with CD [15]. Possible causes for the lack of reliability of this test include the rapid sequestration and clearance of endotoxin, also referred to as lipopolysaccharide (LPS); from the systemic circulation [16-18] and interference of serum components with the test during the assay [18, 19].

Other indirect indicators of immune challenge with endotoxin such as LPS-binding protein (LBP), an acute phase protein secreted by the liver appear to be more reliable as they are reported to be elevated in adults [10, 15, 20] and in children with CD

[21]. Again, the systemic inflammatory response that accompanies CD has been shown to be a useful marker. For example, the serum levels of C-reactive protein (CRP), an acute phase protein produced by the liver [22], are elevated in subjects with CD [1-3, 23].

Similarly, the mucosal inflammatory response can be quantified using faecal calprotectin [1-4, 24], a protein that is predominantly derived from neutrophils [24]. This biomarker has been successfully used as diagnostic [4, 25, 26] and prognostic indicator [14, 27], as well as to monitor progress following treatment [14, 27-29]. Of all of the available tests, faecal calprotectin has been found to be the best non-invasive indicators of intestinal inflammation [1-3, 23].

The aim of the current study was to quantify and compare the capacity of various immune and other parameters that are influenced by the pathogenesis of CD by assessing their ability to distinguish female subjects with CD in remission or who had mild symptomatology from healthy female subjects. Hence, we simultaneously determined faecal calprotectin levels, 3-h lactulose and mannitol absorption/excretion rates, and systemic levels of CRP, LBP and endotoxin and related these parameters to a clinical index of severity of CD, the Harvey-Bradshaw Index (HBI). We also devised a novel quantitative assay of the ability of the serum to sequester a standard dose of endotoxin based on a proprietary Limulus Amebocyte Lysate (LAL) endotoxin assay.

11.2 Materials and methods

11.2.1 Subjects

A total of 13 female subjects with confirmed Crohn's disease (CD) at the age between 21 and 60 years (mean age 38 years), of body heights between 1.59 and 1.76 m, and body masses between 52 and 111 kg, were recruited from the area of Palmerston North and Wanganui for the CD group. Eight of those subjects had CD in remission (Harvey-Bradshaw Index (HBI) < 5) and five of those subjects had mild to moderate disease activity (HBI 5 – 16). All subjects with CD, had been diagnosed at least two years prior to the study, had experienced no flare ups over the last 6 months prior to the study and their CD remained under control during the period of study. A total of 13 healthy female subjects, aged between 20 and 56 years (mean age 37 years), of body heights between 1.58 and 1.80 m, and body masses between 50 and 106 kg, were recruited for the control group from the same catchment area.

Each subject completed a health screening questionnaire (Appendix IV - iv and Appendix IV - v). Subjects with CD also underwent a clinical interview and were scored according to the Harvey-Bradshaw Index (HBI) (Appendix IV - vi). The clinical details of participants with CD are given in Table 11-1. Healthy females were questioned to exclude those with a family or personal history of gastrointestinal disorder. Similarly, any subjects in either groups with a history of endocrine or metabolic disorder including diabetes; hepatic disorder or recent abdominal pain, nausea, vomiting, diarrhoea, the passage of blood or mucus or urinary or vaginal discharge, or who consumed more than one alcoholic drink per day were excluded.

Table 11-1. Clinical details of participants with Crohn's disease.

Subject	HBI	affected Area	Diagnosis	last Relapse	intestinal Surgery	Medication
1	0	Terminal ileum	2010	2010	N	Pentasa
2	1	Ileum	1976	2013	Y	None
3	1	Ileum + colon	2011	2013	N	None
4	2	Colon	1987		N	Pentasa Azathioprine Vitamin B12 injections
5	2	Unknown	2000	2014	N	Pentasa
6	3	Terminal ileum + colon	2010	2014	Y	Azathioprine Budesonide
7	3	Small intestine	2013	2014	N	Pentasa
8	4	Small intestine + colon	1999	2012	N	None
9	5	Unknown	2006	2013	N	Pentasa Infliximab
10	6	Terminal ileum	1993	2003	Y	Pentasa Azathioprine Colestipol
11	6	Ileum	2000	2012	Y	Infliximab Azathioprine Vitamin B 12 injections
12	7	Colon	2002	2015	N	Pentasa Azathioprine Prednisone
13	9	Ileum	2008	2011	N	Pentasa Prednisone

11.2.2 Study protocol and design

The participants attended the Human Nutrition Lab at Massey University, Palmerston North Campus, New Zealand, for the experimental session. All participants were asked to refrain from taking any antibiotics for at least 2 weeks prior to experimental session, not to consume any alcohol for at least 3 days prior to experimental session, to avoid exercise on the day prior to attending the experimental session and to fast overnight prior to experimental session.

Participants were asked to collect a faecal sample prior to experimental session. On arrival at the laboratory, participants were instructed to empty their bladder into a container, after which a 15 ml control sample of venous blood was drawn. Each

participant then ingested a solution containing 10 g lactulose (Laevolac; Douglas Pharmaceuticals Ltd, Auckland, New Zealand) and 5 g mannitol (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 100 ml of water. This was followed immediately by a further 300 ml of water. At 1.5 h and at 3 h after the administration of the lactulose-mannitol solution, each participant was instructed to empty their bladder into a container. The study protocol was approved by the Massey University Human Ethics Committee (Southern A 15/18) (Appendix IV - i) and written consent was given by all volunteers (Appendix IV - ii).

11.2.3 Determination of endotoxin

Venous blood was collected into glass 'serum' vacutainers (10.0 ml, Ref. 366430; BD, Franklin Lakes, NJ, USA) and allowed to clot at room temperature over a period of 2 h before centrifuging for 15 min at 3,500 g at 4°C (Megafuge 1.0R, Heraus Instruments GmbH, Hanau Germany). Serum was decanted into micro-centrifuge tubes and aliquots of each sample were stored at -80°C pending endotoxin analysis.

Three types of subsamples were prepared for each serum sample prior to endotoxin assay. Firstly, for the determination of endogenous concentration of endotoxin in serum each sample was diluted by factor 20 with Endosafe® Dispersing Agent (DA) (Charles River Laboratories, Charleston, SC, USA) in a micro-centrifuge tube and pulse-vortexed for 20 s. The diluted samples were subsequently transferred into endotoxin-free certified glass tubes (depyrogenated 13 x 100 mm borosilicate glass dilution tubes, Charles River Laboratories, Charleston, SC, USA), heated in a water bath (SUB6, Grant Instruments (Cambridge) Ltd, England) at 60°C for 30 min and allowed to cool prior to being transferred into fresh micro-centrifuge tubes.

For the second type that was used to determine the capacities of the sera to sequester a known concentration of endotoxin and the dose at which the endotoxin binding capacity is 'saturated', a series of subsamples of each thawed raw serum were spiked with different doses of endotoxin i.e. with either 2.5, 10, 25 or 100 EU/ml at a volume ratio of 1:10. Spiked samples were similarly diluted and heated as the unspiked samples and the percentages of recovery of the spiked dose were calculated.

The third type of test, the Positive Product Control (PPC), was prepared by spiking an aliquot of the unspiked diluted and heated sample with 1 EU/ml of endotoxin at a volume ratio of 1:10 immediately prior to assay. Again, the percentages of recovery of the spiked dose were calculated. The PPC was used to quantify any inhibitory or enhancing effect of constituents of individual (diluted and heated) serum samples on the assay. The acceptable range for PPC is said to lie between 50 and 200 % [30]. The endotoxin used for spiking was the standard provided in the LAL assay kit produced by Lonza (Walkersville, MD, USA).

A kinetic-QCL™ LAL kit from Lonza (Walkersville, MD, USA) (batch 0000438466) was used to quantify endotoxin in serum. Briefly, 100 µl of each standard, spiked and unspiked diluted and heated serum sample or PPC was pipetted into a 96-well micro-plate (flat bottomed, sterile non-pyrogenic polystyrene; Coasta® 3596, Corning Incorporated, USA) and incubated in a micro-plate reader (EL_X808_{IU}, Bio-Tek Instruments, Inc., USA) at 37°C for 10 min. 100 µl of freshly prepared LAL reagent was then added to each well. The plate was shaken for 30 s and the optical density (OD) of each sample was read at 405 nm immediately after shaking and every 30 s thereafter for a period of 2 h. Data was subsequently uploaded into KC4 software (Bio-Tek Instruments, Inc., USA). Data sets were then transferred into Microsoft EXCEL 2010 to determine the reaction time of each standard, each (spiked and unspiked) sample and each PPC. The reaction time was defined as the time it takes for OD to

increase from 0.5 OD above the baseline OD to 1.0 OD above the baseline OD as previously described in detail [18]. OD values of standards with known endotoxin concentrations between 0.00625 EU/ml to 0.4 EU/ml in endotoxin-free water were then plotted against logarithms of endotoxin concentrations in CurveExpert Professional version 2.0.2 to generate the standard curve. The standard curve was linear up to 0.4 EU/ml, and was subsequently used to determine the endotoxin concentration in each sample using Microsoft EXCEL 2010. There were no significant differences between plates either in the slopes and y-intercepts of the standard curve on ANCOVA. The intra- and inter-assay precision of the LAL assay were < 15 %. The PPC ranged from 117.5 to 202.4 % (mean 158.2 %).

11.2.4 Determination of LBP

An aliquot of each serum sample was also diluted by 1,000-fold with dilution/wash (d/w) buffer (provided by Hycult for use with their LBP – ELISA kit) and the concentration of circulating LBP determined using a human specific LBP - ELISA kit (Hycult Biotech, Uden, The Netherlands), according to the manufacturer's instructions (3.4.1). The range of detection of the LBP assay was reported to lie between 4.4 and 50 ng/ml. Standards with known concentrations of LBP between 9.9 and 50 ng/ml in the d/w buffer provided were run concurrently with the serum samples to provide standard curves. Standard curves of the optical densities (OD) of standards determined at 450 nm plotted against the known concentrations of LBP (CurveExpert Professional version 2.02) were prepared and the levels of LBP in serum samples then determined by comparison. The LBP standard curve up to 50 ng/ml was a second-order polynomial. The intra- and inter-assay precision of the LBP – ELISA were < 5 %.

11.2.5 Determination of inflammatory markers in samples of blood and faeces

Samples of venous blood that had been drawn into plastic SST™ II Advance vacutainers (5.0 ml Ref. 367954; BD, UK) were sent to an accredited medical laboratory (Medlab Central, Palmerston North, New Zealand) for the determination of circulating levels of C-reactive protein (CRP) using the immunoturbidimetric assay CRPL3 (Roche Diagnostics). All faecal samples were sent to Canterbury Health Laboratories, Christchurch, New Zealand for the quantification of faecal calprotectin using an ELISA with an upper limit of 500 µg/g [31].

11.2.6 Determination of urinary excretion rates lactulose and mannitol

The volumes of the initial urine samples and those of urine samples taken 1.5 and 3 h after the ingestion of the lactulose-mannitol solution were each determined in a measuring cylinder. A 15 ml subsample was centrifuged at 3,500 g at 4°C for 10 min (Megafuge 1.0R, Heraus Instruments GmbH, Hanau Germany), and the supernatant transferred into micro-centrifuge tubes. Aliquots of each supernatant were stored at -80°C pending HPLC analysis.

A 1 ml aliquot of each supernatant of urine sample was diluted with one ml of MilliQ filtered water, desalted with 1 g Amberlite resin (Amberlite IRA-410 chloride form and Amberlite IR120 hydrogen form; Fluka Analytical, Sigma Aldrich, St. Louis, MO, USA) and the supernatant filtered into a 2 ml HPLC vial (Thermo Scientific, Thermo Fisher Scientific, Hemel Hempstead, UK) as previously described [18, 32]. HPLC analysis was performed on a 20-µl aliquot of filtrate of each sample in a HPLC system from Agilent Technologies (1200 Series) equipped with Degasser Model G1322A, a Quant Pump Model G1311A, an Autosampler Model ALS-G1329A and a Column Oven Model TCC-G1316A. The component sugars in each standard and each filtrate

were detected with a Refractive Index Detector (RID) Model RID-G1362A (Agilent Technologies). Filtered MilliQ water was used as the mobile phase at a flow rate of 0.3 ml/min at 55 bar and at 60°C over 20 min for each standard of lactulose and mannitol and over 40 min for each sample filtrate, respectively.

The quantities of lactulose and mannitol excreted were calculated from the areas under the curve relative to those of a series of concurrently run lactulose and mannitol standards as described previously [33]. The total quantity of each sugar (mg/ml) excreted in 3 h by each subject was determined by adding the quantity of each sugar determined 1.5 and 3 h after the ingestion of the sugar solution. The percentages of ingested dose (10 g and 5 g, respectively) excreted by each subject were then calculated and the ratios of lactulose to mannitol (LMR) determined.

The calibration curves for sugars were linear up to 2000 µg mannitol/ml and 200 µg lactulose/ml, respectively. The accuracy of the quantification of these sugars in our laboratory by HPLC analysis has been previously described [34]. The intra-assay coefficient of variation was in the range of 0.1 to 7.7 % for lactulose and 0.1 to 1.6 % for mannitol.

11.2.7 Statistical analysis

Test for normality of the distribution on all data were examined in the Minitab software version 17 (Minitab Inc., PA, USA) prior to statistical analysis. Statistical analyses were performed in SYSTAT version 13 (Systat Software Inc., Chicago, IL, USA). The levels of endotoxin in sera of healthy subjects were compared with those in subjects with CD by non-parametric Kruskal-Wallis test. The recoveries of various doses of endotoxin spiked from serum of healthy subjects were compared with those of subjects with inactive and mild to moderate active CD by repeated measure ANOVA.

The levels of CRP in the sera of healthy subjects were compared with those of subjects with CD by non-parametric Kruskal-Wallis test. The levels of LBP in serum and the levels of faecal calprotectin in healthy subjects were each compared by ANOVA with those with CD. Similarly, the rates of excretion of lactulose and of mannitol, and the ratio of lactulose to mannitol of healthy subjects were each compared by ANOVA with those of subjects with CD. Differences were considered significant at the $P < 0.05$ level.

The correlation between HBI and any determined parameter were each assessed by Pearson correlation coefficient and the Bartlett Chi-Square statistic obtained on linear regression. Correlation coefficients were considered significant when P was < 0.05.

Normalised data for all parameters, expect serum endotoxin levels and serum CRP levels, were subject to multivariate analysis by classical discriminant analysis to determine the relative ability of each parameter to distinguish between healthy subjects and those with a history of CD. This was judged on a basis of the F-to-remove values and canonical discriminant function standardised within variances the latter providing an indication also of the direction of the effect. Further, we calculated sensitivity and specificity on a basis of the Jackknifed classification matrix from these analyses.

11.3 Results

11.3.1 Body composition

The heights of the 13 healthy female subjects ranged from 1.58 – 1.80 m (mean 1.66 m), their body masses ranged from 50 – 106 kg (mean 71 kg) and their Body Mass Index (BMI) varied between 18.5 – 41.9 (mean 25.9). The heights of female subjects with CD ranged similar from 1.59 – 1.76 m (mean 1.65 m), their body masses varied between range 52 – 111 kg (mean 76.3 kg) and their BMIs between 20.1 – 41.7 (mean 27.9).

11.3.2 Serum endotoxin levels and recovery of endotoxin spiked into raw serum

The levels of systemic endotoxin were not normally distributed and not amenable to transformation using the Johnson algorithm (Minitab version 17). Hence, comparisons were performed on non-parametric methods.

None of the thirteen healthy subjects had detectable levels of endotoxin (< 0.125 EU/ml) in their sera. Endotoxin was detected in the sera of three of the thirteen subjects with CD (one with HBI < 5 had 0.24 EU/ml; and two with HBI > 5 had 0.13 and 0.71 EU/ml). Comparison of results from all subjects by Kruskal-Wallis test indicated that endotoxin levels were not significantly greater in subjects with CD than in healthy subjects.

The overall percentages recoveries of spiked endotoxin increased significantly on repeated measures ANOVA with increases in the spiked dose of endotoxin ($df = 2,14$; $F = 13.58$; $p = 0.001$). When raw serum was spiked with endotoxin at a concentration of 2.5 EU/ml at a volume ratio of 1:10 endotoxin was recovered in one of five healthy subjects (52.4 %) and in one of seven subjects with CD (one with HBI > 5 had 18.4 %).

In contrast, endotoxin was recovered from all samples from all subjects when the raw sera were spiked with endotoxin at concentrations of either 10 EU/ml, 25 EU/ml or of 100 EU/ml at a volume ratio of 1:10 (Table 11-2). Hence, these results indicated that the doses of endotoxin spanned the range over which the capability for sequestration became saturated.

Table 11-2. Overview of percentage recoveries of endotoxin spiked into raw serum at various concentrations

Group	Spiked endotoxin concentration (EU/ml)	Recovery of spiked endotoxin (%) Mean	SD
Control	10	15.9	10.3
	25	36.7	19.4
	100	51.3	17.2
Inactive CD	10	29.5	10.7
	25	46.9	22.1
	100	58.0	11.1
Mild to moderate active CD	10	43.0	4.7
	25	34.8	25.5
	100	48.1	19.4

There were no significant differences on repeated measures ANOVA when the percentage recoveries of endotoxin of all spiked doses in sera from all healthy subjects were compared with those from sera from all subjects with CD. However, the percentage recoveries of endotoxin from sera from healthy subjects spiked with 10 EU/ml endotoxin at a volume ratio of 1:10 were significantly lower ($p= 0.04$) than those of subjects with mild to moderate CD on post hoc Bonferroni testing of a similar ANOVA ($df = 2.7$; $F= 5.78$; $p= 0.033$) restricted to these two groups.

11.3.3 Serum LBP levels

The levels of serum LBP were not normally distributed and required Asinh conversion by Johnson Transformation in Minitab version 17 to render them amenable to parametric statistical analysis.

The levels of systemic LBP in healthy subjects (median raw data13.6 µg/ml; 25 -75 % interquartile range (IQR) 12.7 – 17.7 µg/ml) were not significantly different on ANOVA of Asinh transformed values to those of subjects with CD (median raw data 16.9 µg/ml; IQR 14.5 – 21.05 µg/ml) (Figure 11-1A).

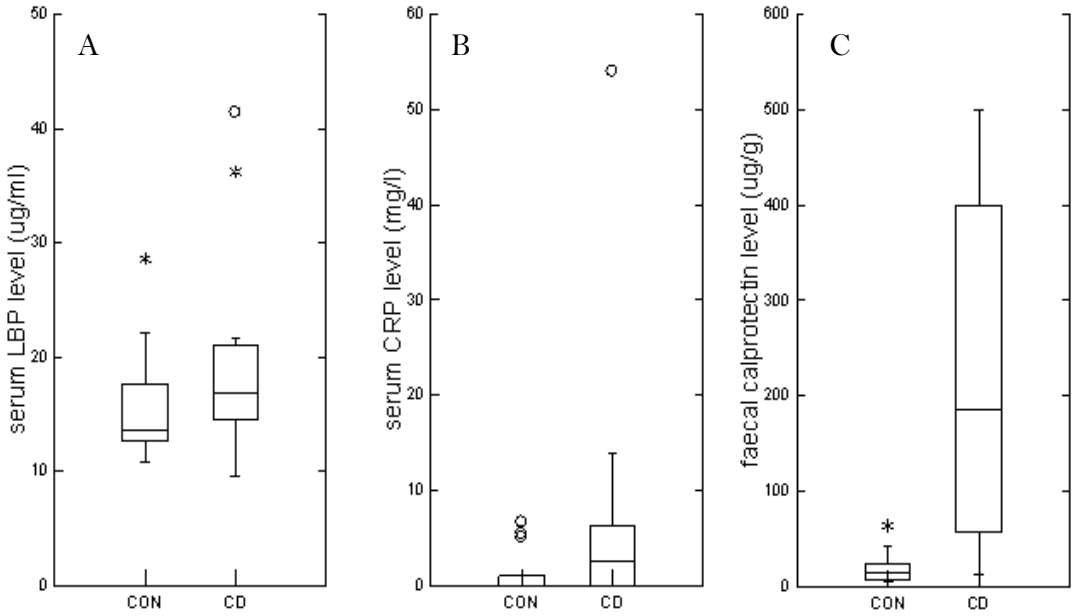

Figure 11-1. Levels of systemic LBP (A) and CRP (B) and faecal calprotectin (C) in healthy women (CON) and in women with CD (CD).

*, value above 1 SD; °, value above 2 SD.

11.3.4 Levels of systemic and intestinal inflammation markers

The levels of CRP in serum were not normally distributed and not amenable to transformation using the Johnson algorithm (Minitab version 17). Hence non-parametric methods were used for comparison.

The levels of serum CRP in healthy women ranged from < 1.0 mg/l to 6.7 mg/l (median raw data < 1.0 mg/l), and those in women with CD ranged from < 1.0 mg/l to 54 mg/l (median raw data 2.6 mg/l) (Figure 11-1B). The serum levels of CRP in healthy subjects did not differ from those of subjects with CD on Kruskal-Wallis test.

The faecal calprotectin levels required natural logarithmic conversion by Johnson Transformation in Minitab version 17 to render them amenable to parametric statistical analysis.

The faecal calprotectin levels in all subjects with CD (median raw data186 µg/g; IQR 58 – 400 µg/g) were significantly higher (df = 1,24; F = 23.946; p < 0.001) than those of healthy subjects (median raw data 15 µg/g; IQR 8 – 25 µg/g) on ANOVA of transformed values (Figure 11-1C).

11.3.5 The excretion of lactulose and mannitol

The percentages of recoveries of administered dose of lactulose were normally distributed whereas these for mannitol and lactulose-mannitol Ratio (LMR) required Asinh and logarithmic transformation, respectively, (Johnson Transformation in Minitab version 17) to render them amenable to parametric statistical analysis.

The 3-h excretions of lactulose were significantly greater in subjects with CD (mean 0.40 % ± 0.25 %) (df = 1,24; F = 8.067; p = 0.009) than in healthy subjects (mean 0.17 % ± 0.14 %) on ANOVA (Figure 11-2A: median $_{\text{control group}}$ 0.11 %; IQR 0.07 – 0.25 % vs. median $_{\text{CD group}}$ 0.40 %; IQR 0.28 -0.45 %). There were no significant differences in the 3-h excretions of mannitol between the control group (median raw data 9.35 %; IQR 7.67 – 11.13 %) and the CD group (median raw data 8.69 %; IQR 8.05 – 9.80 %) on ANOVA of transformed values (Figure 11-2B). The LMRs of subjects with CD (median raw data 0.05; IQR 0.03 – 0.05) were significantly higher on ANOVA of transformed values (df = 1,24; F = 6.827; p = 0.015) than those of healthy subjects (median raw data 0.01; IQR 0.01 – 0.03) (Figure 11-2C).

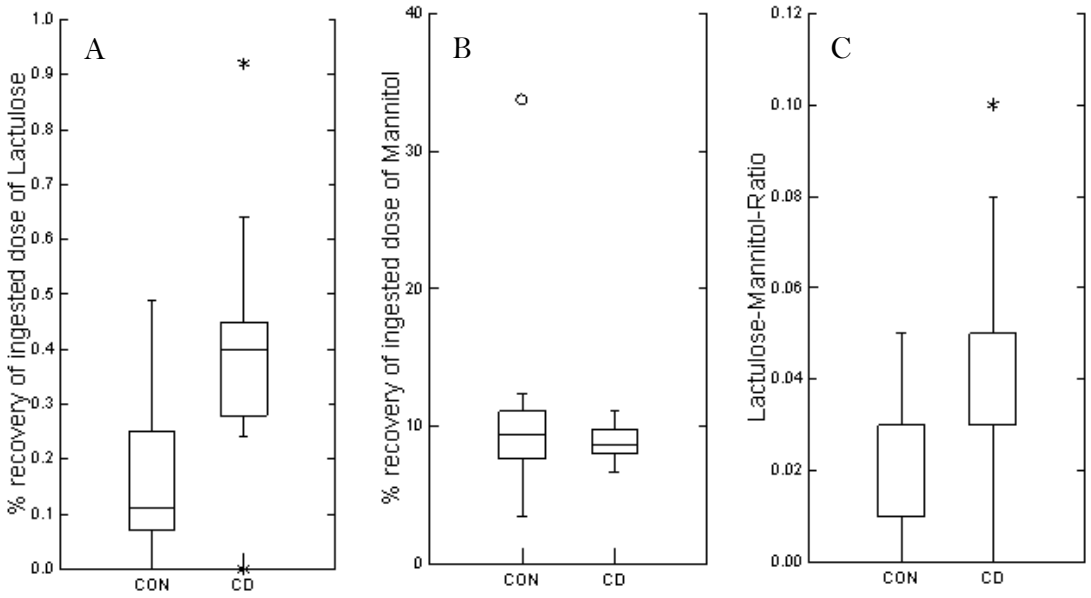

Figure 11-2. Percentage of 3-h recovery of total dose of A) lactulose and B) mannitol, and C) Lactulose-Mannitol-Ratio in healthy women (CON) and those with CD (CD).

11.3.6 Relationship between HBI and parameters of gut health

Neither the levels of faecal calprotectin nor the excretion rates of lactulose and the LMR correlated with the disease activity as assessed by HBI. Similarly, all other tested parameters did not correlate with HBI.

11.3.7 Assessment of tested parameters to distinguish between healthy subjects and those with a history of CD

Normalised data was used in the classical discriminant analysis with exception of the serum levels of endotoxin and CRP as those data were not amenable to transformation. The data for recovery of endotoxin with a spiked dose of 10 EU/ml were not included in classical discriminant analysis owing to the small number of samples for which these were available.

Classical discriminant analysis of all six parameters i.e. serum levels of endotoxin, LBP and CRP, levels of faecal calprotectin and the excretion of lactulose and mannitol, was

significant (df = 6, 1, 23: F-ratio = 4.587; p = 0.005) showing that this combination was able to distinguish between healthy subjects and those with a history of CD on Jackkniefed classification (CD = 67 % correct, health = 92 % correct) though with less specificity than when the analysis was conducted on a reduced data set (Table 11-3). In this analysis it is noteworthy that only parameters relating to permeability and faecal calprotectin had significant F-to remove vales and canonical discriminant functions that exceeded 0.3. In the first two multivariate analyses the vales for mannitol had greater vales for F-to remove values and canonical discriminant function that did those for either endotoxin, LBP or CRP. Hence, fluctuation in the serum levels of the latter three parameters did not significantly augment the capacity of the analysis to discriminate healthy subjects form those with a history of CD.

Reduced classical discriminant analysis considering only the three parameters faecal calprotectin and permeability to lactulose and mannitol gave better discrimination with 77 % specificity and 92 % sensitivity (Table 11-3) and higher levels of significance (df = 3, 1, 24; F-ratio = 10.708; = 0.0015). It is noteworthy that the value for specificity of the third discriminant analysis was less (77 %) but sensitivity was greater (92 %) than those obtained when the same analysis was conducted on faecal calprotectin alone (specificity 85 %; sensitivity 85 %) (Table 11-3). Furthermore, faecal calprotectin and 3-h excretion of lactulose run contrary to 3-h excretion of mannitol (Table 11-3).

Table 11-3. Results of classical discriminant analysis

Variable	F-to Remove	Canonical Discriminant Function	Variable	F-to Remove	Canonical Discriminant Function	Variable	F-to Remove	Canonical Discriminant Function	Variable	F-to Remove	Canonical Discriminant Function
Endotoxin	0.632	0.253	**LBP**	0.293	0.182	**Calpro-tectin**	16.219	0.851	**Calpro-tectin**	23.946	
LBP	0.165	-0.162	**Calpro-tectin**	11.366	-0.806	**Lactulose**	4.003	0.517			
CRP	0.006	-0.030	**Lactulose**	4.274	-0.605	**Mannitol**	1.471	-0.332			
Calpro-tectin	8.500	0.792	**Mannitol**	1.310	0.354						
Lactulose	3.039	0.562									
Mannitol	1.296	-0.371									
P = 0.005			P = 0.001			P = 0.00015			P = 0.00005		

	Jackknifed Classification			Jackknifed Classification			Jackknifed Classification			Jackknifed Classification		
	CD	Control	% correct	CD	Control	% correct	CD	Control	% correct	CD	Control	% correct
CD	8	4	67	9	3	75	10	3	77	10	3	85
Control	1	12	92	1	12	92	1	12	92	1	12	85
Total	9	16	80	10	15	84	11	15	85	11	15	85

11.4 Discussion

Of the various parameters tested only the direct and non-invasive indicators of gut health such as the levels of faecal calprotectin, the excretion rates of lactulose and the LMR differed significantly between healthy subjects and those with CD. These observations are in line with previous reports [4-9, 25, 26] and highlight the importance of those parameters as diagnostic tool of which the lactulose-mannitol test is the cheapest.

The finding that discriminant analyses showed the highest specificity for faecal calprotectin alone is in line with the findings of other studies that the test is most accurate in identifying CD [2, 4]. The levels of faecal calprotectin are reported to increase in proportion to the degree of local inflammation and the extent of lesions along the intestinal mucosa [1-4, 14] this is in line with our finding that faecal calprotectin levels are associated positively with the excretion of lactulose, an indicator of paracelluar permeability [36, 37], and are negatively associated with the excretion of mannitol, an indicator of mucosal surface area [38, 39]. In addition, our data suggest that the use of combination of parameters notably levels of faecal calprotectin and excretion of lactulose and mannitol, in discriminant analysis gives the best level of sensitivity.

The levels of faecal calprotectin and the excretion rates of lactulose were not correlated with disease activity as assessed by HBI suggesting that clinical symptoms do not reflect disease-associated on-going mucosal inflammation and barrier function. The same finding has been reported by others [4].

In contrast to other work showing that subjects with CD had elevated systemic levels of LBP [10, 15, 20] and CRP [1-3, 23] we did not observed such elevations. The lack of this finding may be due to the small sample size in the current study. Like others

[15] we found that the levels of systemic endotoxin in subjects with active or inactive CD did not differ from those of healthy subjects. Further, our data suggest in general that the capacity to sequester endotoxin is becoming saturated both in healthy subjects and those with CD when serum is spiked with more than 2.5 EU/ml of endotoxin at a volume ratio of 1:10. The finding that the percentage recoveries of a spiked 10 EU/ml dose of endotoxin at a volume ratio of 1:10 were greater in subjects with CD than in healthy subjects suggests that the capacity of serum to sequester endotoxin may be reduced in conditions where increased quantities of endotoxin are able to traverse the gut wall. Hence, elements that bind endotoxin such as LBP may be relatively depleted in subjects with CD. This hypothesis is in line with the scaling of canonical discriminant functions obtained on multivariate analysis showing that the serum levels of LBP are inversely correlated with indicators of mucosal inflammation and paracellular gut permeability. This finding is in contrast to published work showing that endotoxin spiked at a concentration of 1,000 EU/ml at the same volume ration (1:10) is recovered at lower levels in subjects with CD than in healthy subjects [35]. This inconsistency may be due to differences between the two studies in sources, doses of endotoxin and techniques. For example, whilst we spiked a 10 EU/ml dose of purified endotoxin from *E. coli* O55:B5, into raw serum and processed samples within 10 min, the other group spiked crude endotoxin extracted from *Salmonella enterica* serovar *typhimurium* LT2 [35] at a concentration of 1,000 EU/ml to diluted plasma and incubated the spiked sample for 2 h [35].

Further work with larger sample size, subjects with more pronounced disease activity and cohorts that include both sexes is needed to confirm the function of those biomarkers alone or in combination with others in subjects with CD.

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CHAPTER 12 SUMMARY - ENDOTOXIN MEASUREMENT

CHASING A GHOST? – ISSUES WITH THE DETERMINATION OF CIRCULATING LEVELS OF ENDOTOXIN IN HUMAN BLOOD

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MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Anne Gnauck

Name/Title of Principal Supervisor: Prof Roger G. Lentle

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In which Chapter is the Published Work: Chapter 12

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

  and / or

- Describe the contribution that the candidate has made to the Published Work:
  - Conducting the literature search
  - Statistical data analysis and presentation of data
  - Writing the draft of the paper

A. Gnauck
Candidate's Signature

10/12/2015
Date

Principal Supervisor's signature

10/12/15
Date

GRS Version 3– 16 September 2011

Abstract

Reliable quantification of bacterial products such as endotoxin is important for the diagnosis of Gram-negative infection and for the monitoring of its treatment. Further, to identify patients with persistent subclinical level of bacterial products in their systemic circulation as data from animal studies also suggest this may be correlated with the onset of metabolic syndrome.

In this chapter I review published data regarding endotoxin levels in healthy subjects and those with sepsis, inflammatory bowel disease, liver disorders and metabolic disorders such as obesity and diabetes. I also review the evidence regarding influence of macronutrients in augmenting the levels of systemic endotoxin. The results of this review show that reported mean levels of endotoxin in the systemic circulation of healthy humans and of those with various clinical disorders vary over a wide range. Further that a significant proportion of this variation can be related to the method that was used to prepare plasma and serum samples prior to assay and its ability to reduce the effect of various blood borne factors that interfere with the LAL assay.

12.1 Introduction

The human gut contains around 10^{14} commensal bacteria, a number that is greater than the total number of human somatic cells. Under normal physiological conditions these microbiota live in a symbiotic relationship with their human hosts [4]. The majority of gut-derived bacteria and their products that are able to penetrate the intestinal mucosa are promptly detoxified in the liver [5-9]. On occasion, for example after traumatic injury, significant quantities of these bacteria or their products may transit from the hepatic portal to the systemic circulation where they can induce sepsis and septic shock [10] (Figure 12-1). Further, work with mouse models indicates that other 'non-traumatic' phenomena such as a fat-rich diet may chronically increase such transit and allow on-going entry of low concentrations of bacterial products in the systemic circulation. In this latter case it remains unclear whether such elevation results from an increase in paracellular permeability of the gut mucosa [11, 12] or from transcellular passage concurrent with the absorption of fatty acids (Figure 12-1). However, the latter route is favoured as it is thought to culminate in incorporation of the bacterial products into chylomicrons [13, 14] with subsequent transport via the lymphatic system and thoracic duct into the systemic circulation effectively circumventing hepatic detoxification [15, 16] (Figure 12-1) [17, 18].

The continued presence of small numbers of bacteria or their products in the systemic circulation may lead to on-going (local) low grade inflammation i.e. the chronic production of subclinical levels of pro-inflammatory cytokines by circulating innate immune cells, epithelial cells or adipocytes, and may promote the development of disorders associated with the metabolic syndrome such as insulin resistance, type 2 diabetes, atherosclerosis and cardiovascular disease [19-23].

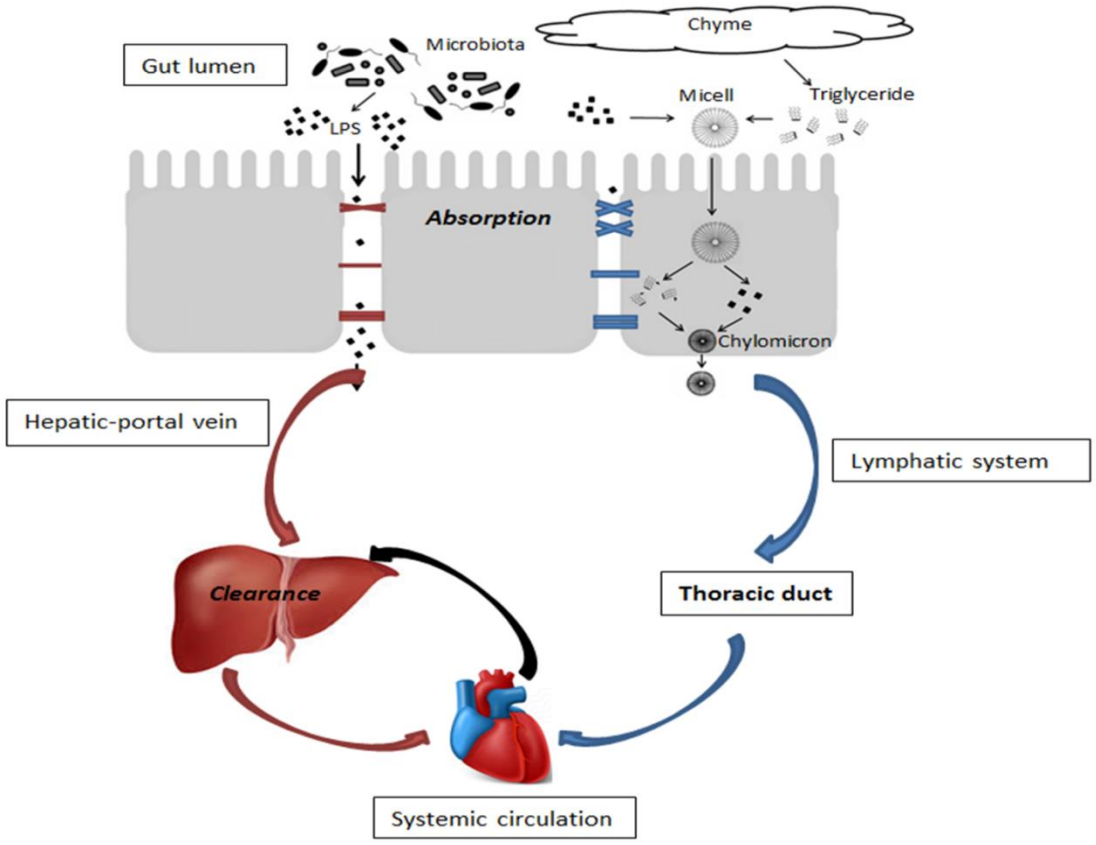

Figure 12-1. Schematic drawing of the transportation route of bacterial products such as lipopolysaccharide (LPS) from the gut lumen into the systemic circulation.

Showing paracellular (red: left) and transcellular (blue: right) routes of absorption of LPS. The former undergoing immediate detoxification and removal by the liver via the portal circulation, the latter transports LPS via fat directly into the systemic circulation (figure after Figure 1 from Kelly et al. [3] with modifications).

Lipopolysaccharide (LPS), the major component of the outer cell membrane of Gram-negative bacteria, is of particular interest in this matter as the contained lipid A fraction act as ligand for the immune receptor toll-like receptor 4 (TLR4) that promotes a pro-inflammatory response in immune and other somatic cells [2, 24]. The dose of endotoxin within a sample of LPS is quantified either by weight (ng) or in endotoxin units (EU), the use of the latter being currently predominant. The endotoxin unit (EU) reflects the endotoxic activity of the mixture of LPS molecules within a given sample relative to that of the control standard endotoxin. The WHO International Standard for endotoxin currently defines one EU as the biological activity induced in the endotoxin test by around 120 pg of LPS from Escherichia coli O113H10:K [33].

The concurrence of on-going low systemic levels of endotoxin with chronic inflammation that was reported in mice [19-23] has not been conclusively demonstrated in human subjects with metabolic disorders as the data varies with the particular medical condition. Hence, the levels of plasma endotoxin in subjects with the hepatic manifestation of the metabolic syndrome i.e. non-alcoholic fatty liver disease (NAFLD) are reported to be elevated compared to those of healthy controls [34, 35]. Conversely Leber and co-workers [36] were unable to detect any circulating endotoxin (< 0.05 EU/ml) either in obese subjects with type 2 diabetes or in healthy subjects [36], whilst five other studies reported that the levels of circulating endotoxin were elevated in subjects with type 2 diabetes [37-41]. However, the mean levels of circulating endotoxin reported in the latter five studies ranged widely from 5.19 to 70.73 EU/ml as did those of the healthy control subjects in the same five studies which ranged from 3.89 to 61.06 EU/ml [37-41]. It is important to note that the range of means in the healthy subjects from these five studies was greater and the mean was higher than the means of healthy subjects reported by other workers (Figure 12-3) [11, 12, 34, 35, 42-52] and higher than those reported in patients with NAFLD, sepsis or septic shock (which latter ranged between 1 to 4 EU/ml with maximum levels of 12.5 EU/ml) [42, 50, 52-55]. Further, that other studies report that between 89 and 11 % of patients with sepsis or septic shock [42, 50, 53-58] and more than 50 % in other ill and hospitalised subjects [59] have no detectable levels of endotoxin in their blood. These high degrees of variation may result, either from technical difficulties in adapting the currently available assays that are primarily designed for testing endotoxin in pharmaceuticals [60, 61] for use with plasma or serum, or may be biological. Hence, a reliable assay of endotoxin in blood is much needed for prompt diagnosis and management of acute sepsis, and for detecting

subclinical levels of circulating endotoxin in subjects with impaired gut barrier, liver dysfunction or metabolic syndrome.

In this review I will review, in the context of reliability of the results, the body of research that describes the levels of circulating endotoxin in healthy subjects and subjects with various conditions where these levels may be acutely and chronically elevated.

12.2 Selection of studies

A systematic literature search was conducted on Medline and Google Scholar for studies that reported circulating levels of endotoxin in healthy subjects and in subjects with various diseases or states that may promote uptake of endotoxin from enteral microbiota in the lumen of the gut. Hence, these included acute sepsis or septic shock, inflammatory bowel disease i.e. Crohn's disease and Ulcerative colitis, hepatic disorders including acute and chronic hepatitis, alcoholic and non-alcoholic (fatty) liver disease, hepatic cirrhosis (alcoholic and non-alcoholic) and metabolic disorders such as obesity and diabetes (type 1 and type 2). The search also included studies that reported the effects of consumption of various macronutrients that may augment absorption of enteral endotoxins. Further, the reference lists of all identified reports were searched for additional publications that were not directly identified on electronic searches. Studies published in English and German were included in this review.

Only studies that determined endotoxin using either the chromogenic or the turbidimetric method for LAL assay were included. Where the methodology of the LAL assay was not described but references regarding the methodology were provided, the study was classified by methodology only if it could be reliably sourced from the original publication. Studies with either quantitative or qualitative data (either in pg/ml or in EU/ml) were used.

Formal statistical evaluation by meta-analysis could not be undertaken for a number of reasons. Firstly, as data from some reports were not normally distributed. Secondly, as the bulking of results was confounded by differences in the methods used to prepare samples prior to LAL assay. Thirdly, as reported levels of endotoxin appeared to vary to a greater degree with sample size and with the method of sample preparation than between a healthy and a diseased state. The results from healthy subjects were

grouped on forest plots, with suitable classification and identification of methodologies and other relevant factors, in order to evaluate consistency of results in this respect.

12.3 The levels of systemic endotoxin in health and disease

In this section I review and discuss published findings regarding the levels of systemic endotoxin in healthy human subjects and in subjects with conditions where the enteral microbial barrier is compromised and there is consequent acute elevation in circulating endotoxin levels. These conditions include sepsis, inflammatory bowel disease and compromised hepatic function. I also discuss work relating chronic low grade increase in endotoxin levels to the genesis and maintenance of metabolic disorders such as obesity, type 1 and type 2 diabetes.

My literature search identified a total of 63 publications during the period between 1984 and 2014, in which systemic levels of endotoxin were determined by chromogenic or turbidimetric LAL assay, either in healthy subjects, or in subjects with the disorders mentioned above.

12.3.1 Systemic endotoxin levels in healthy humans

The literature search identified 47 studies in which the levels of systemic endotoxin were determined in healthy subjects. Forty five mean/median plasma or serum endotoxin levels for healthy subjects were found in a total of 38 publications. Of these, 19 studies reported levels in pg/ml (reported mean/median levels: n = 21) (Figure 12-2) and 19 studies in EU/ml (reported mean/median levels: n = 24) (Figure 12-3). The data of another study were not included in the forest plot as the data were from the same study population as reported in a separate paper [50]. Further, eight studies were identified in which data were only presented in figures [35, 110] or in which either quantitative data i.e. the percentage of samples with detectable level of endotoxin [34, 57], or qualitative data i.e. the range of detectable levels without any information about the mean or median [51, 101, 111, 112], were given. The values

covered a broad range that varied significantly according to method used to prepare the plasma or serum samples prior to assay (see below).

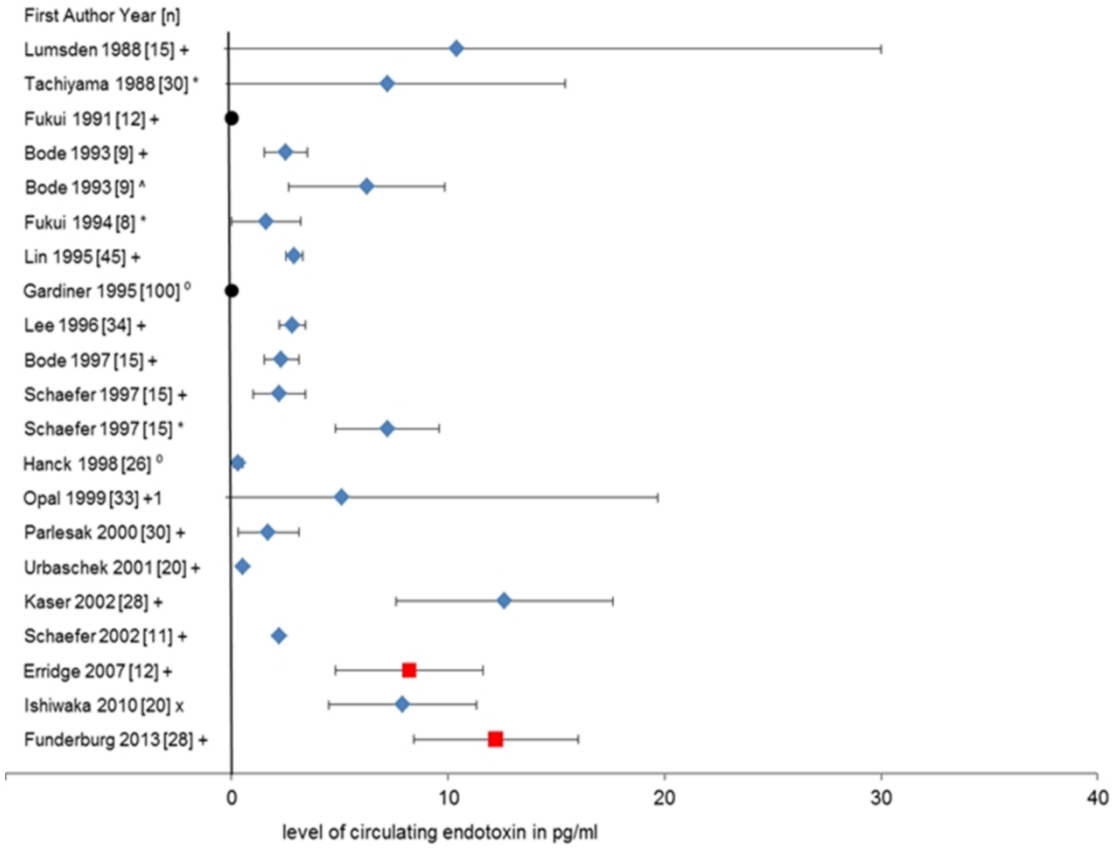

Figure 12-2. Mean levels of systemic endotoxin levels reported in pg/ml in healthy subjects.

black circles, no endotoxin detected; blue diamonds, display means and 95 % confidence intervals; red rectangles, display medians and 25 - 75 % interquartile ranges.

Bracket number next to authors indicates the sample size.

Symbols next to the sample size indicate methods of sample preparation: +, dilution and heating; *, perchlorid acid method; ^, Tween 80; x, Trition X-100; °, no details provided.

Number next to the methods of sample preparation indicates: 1, no endotoxin detectable in more than 50 % of the samples.

Note: The portions of the 95 % confidence intervals that lie below zero have been removed.

Three out of the 47 studies failed to detect any endotoxin (<0.005 EU/ml, < 0.015 EU/ml or < 10 pg/ml) in plasma or sera from healthy subjects [36, 49, 113]. In three other studies the proportion of healthy subjects with detectable levels of systemic endotoxin ranged from 16 % to less than 50 % [34, 50, 114].

The overall mean levels of systemic endotoxin in healthy subjects ranged widely from 0.15 to 61 EU/ml (median 0.32 EU/ml, 25 – 75% interquartile range (IQR) 0.23 – 3.89 EU/ml) (Figure 12-4A) or ranged from 0.5 to 65 pg/ml (median 5.1 pg/ml, IQR 2.2 – 7.9 pg/ml) (Figure 12-4B).

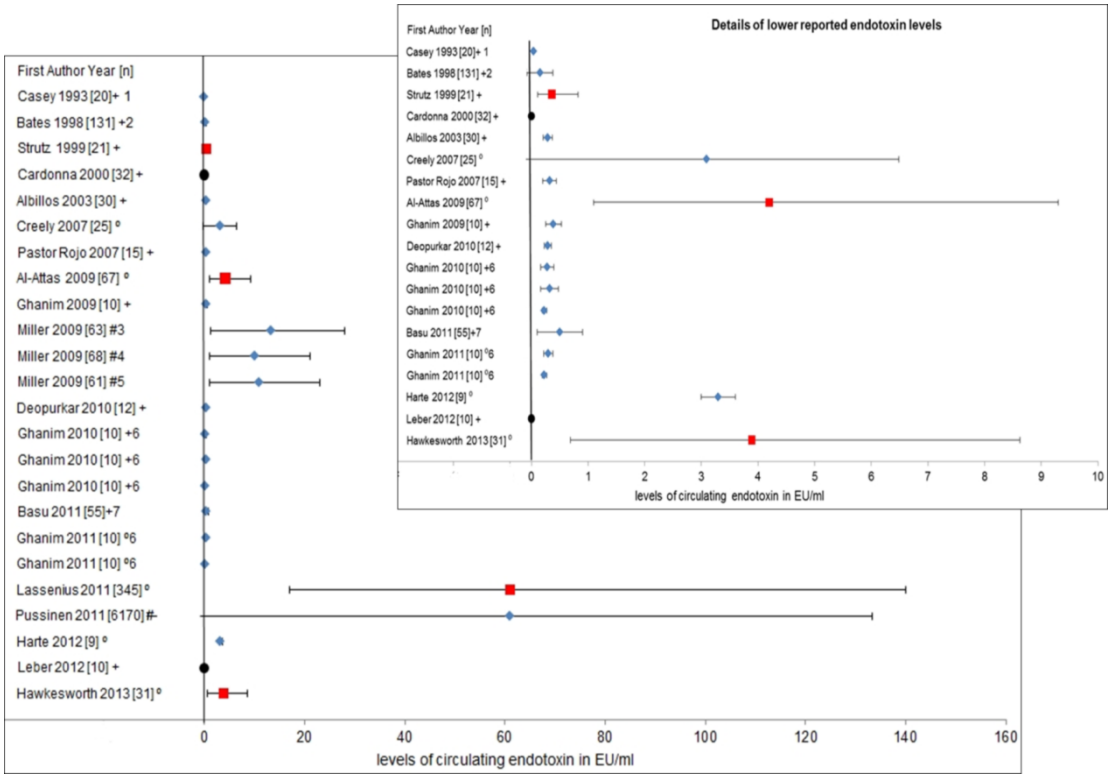

Figure 12-3. Mean levels of systemic endotoxin reported in EU/ml in healthy subjects. Inset shows reports with values below 10 EU/ml.

black circle, no endotoxin detected; blue diamonds, display mean and 95 % confidence interval; red rectangle, display median and 25 - 75 % interquartile range.

Bracket number next to authors indicates the sample size.

Symbols next to the sample size indicate methods for the preparation of samples: + dilution and heating, # simple dilution, ° no details provided

Numbers next to the method of sample preparation indicate as follows: 1, no SD given; 2, levels determined in whole blood sample; 3, levels of British with South Asian ethnicity; 4, levels of British with African ethnicity; 5, levels of British with Caucasian ethnicity; 6, fasted levels of different treatment groups; 7, levels of pregnant women.

Note: The portions of the 95 % confidence intervals that lie below zero have been removed.

In the studies that reported data in pg/ml, various methods were used to pre-treat the plasma or serum prior to LAL assay; 15 used a combination of dilution and heating, six a treatment with perchloric acid, one a treatment with Tween 80 and one a treatment

with Triton X-100. Two of the studies provided no information regarding the method that was used for the pre-treatment of the samples [113, 115] (Figure 12-2). The distributions of the mean levels of systemic endotoxin (in pg/ml) from healthy subjects varied according to the method that was used (Figure 12-4A). The median of the mean levels of plasma endotoxin from healthy subjects was slightly higher when samples were treated with the perchloric acid method (median 6.6 pg/ml, IQR 3.8 – 7.2 pg/ml) or with Tween 80 or Triton X-100 (mean 7.075 pg/ml) (Figure 12-4A) than when they were treated with dilution and heating (median 5.1 pg/ml, IQR 2.2 – 10.4 pg/ml) prior to LAL assay. Hence, with all treatments the mean levels of systemic endotoxin were generally below 10 pg/ml in healthy subjects (Figure 12-2).

The studies that reported results in EU/ml used either a combination of dilution and heating (n = 11) or dilution alone (n = 2) for sample preparation (Figure 12-3). Six of these studies provided no details regarding the method for preparation of the samples (Figure 12-3). The median of the mean levels of systemic endotoxin in healthy subjects was higher, and the distribution wider, when samples were simply diluted (mean 12.10 EU/ml) than when samples were diluted and then heated (median 0.29 EU/ml, IQR 0.177 – 0.34 EU/ml) or in the group where methods of treatment were not reported (median 3.195 EU/ml, IQR 0.265 – 4.045 EU/ml) (Figure 12-4B).

Taken together these results indicate that the method that was used for the plasma or serum sample preparation prior to LAL assay, had a marked effect on the levels of endotoxin detected and, in the case of dilution alone, markedly increased the range. It is therefore difficult to determine whether significant levels of endotoxin are normally present in the systemic circulation of healthy persons, and if so, what the levels are. Further, in studies of disorders that involve acutely and chronically elevated levels of systemic endotoxin, it is important to compare the levels obtained with those of

healthy individuals that were determined using the same method of sample preparation, preferably concurrently.

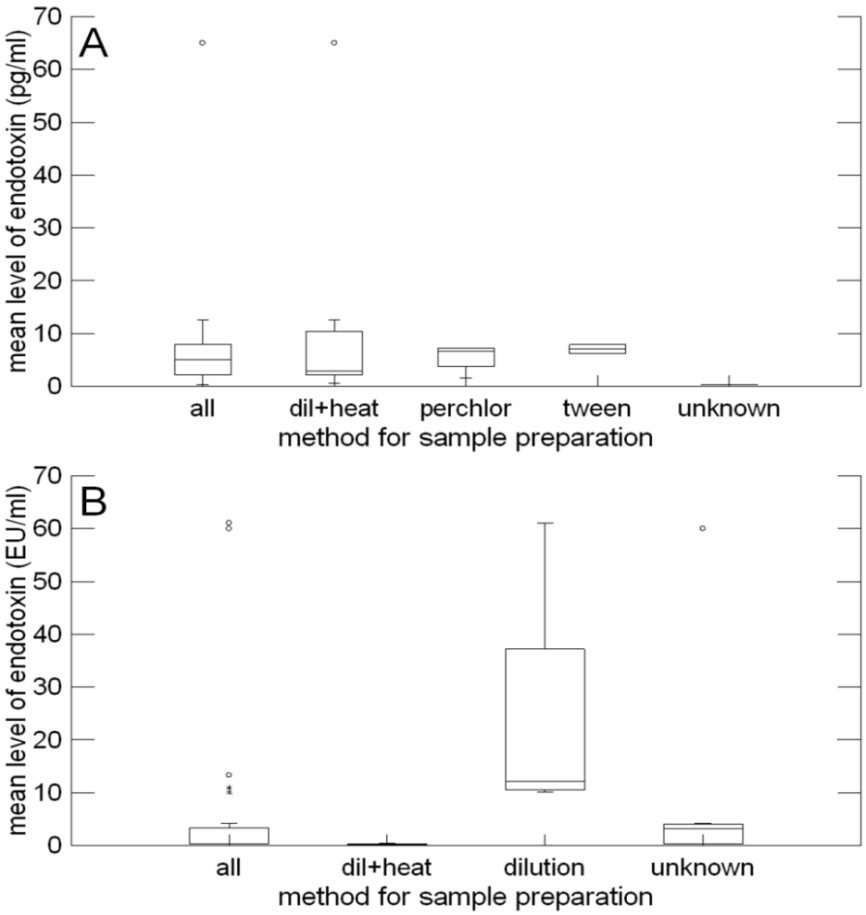

Figure 12-4. Reported mean levels of systemic endotoxin in healthy subjects classified by method of preparation of samples prior to assay.

A) All publications that reported endotoxin levels in pg/ml (n = 21). dil+heat, diluted and heated samples (n = 14); perchlor, samples pre-treated with perchloric acid (n = 4); tween, samples pre-treated with either Tween 80 or Trition X-100 (n = 2); unknown, data from work that provided no details regarding method used for preparation of samples) (n = 1). B) All publications that reported endotoxin levels in EU/ml (n = 24). dil+heat, diluted and heated samples (n = 12); dilution, samples diluted with endotoxin-free water (n = 4); unknown, no details provided method used for preparation of samples)(n = 8).

* - value above 1 SD. ° - value above 2 SD.

12.3.2 Acute elevation of systemic endotoxin

Sepsis or septic shock is a condition characterised by an overwhelming pro-inflammatory immune response to an acute elevation of systemic endotoxin which can be fatal [10]. I found a total of 12 studies that had reported some form of data regarding systemic endotoxin in patients admitted to hospital with sepsis or septic shock. Significant levels of systemic endotoxin were detected in the circulation of between 11 and 89 % of patients in these studies [42, 48, 50, 52-58, 114, 116]. Hence, circulating endotoxin was not detected in all patients. All these studies had endeavoured to eliminate any source of endotoxin contamination i.e. had used endotoxin-free blood collection tubes and a strict protocol that avoided endotoxin contamination of any sources.

Some of this variation may result from variation in the levels of the factors that sequester endotoxin such as circulating levels of LBP, BIP and the relative proportion of certain lipoproteins [90, 93, 95-98]. Further some patients may have developed temporary tolerance to LPS a condition that is accompanied by an increase in the rate of bacterial clearance [117-119]. However, it is hard to find a biological explanation for the complete absence of endotoxin in some sera.

Four out of the 12 studies did not provide the mean or median levels of systemic endotoxin [48, 56, 57, 116]. One other study reported a median value of 300 pg/ml, the choice of statistical parameter suggesting that the values were not normally distributed [114]. The remaining seven studies provided mean levels in EU/ml. The median of the mean levels of systemic endotoxin reported in these seven studies was 1.99 EU/ml with an IQR from 0.84 – 3.47 EU/ml (Figure 12-5B) [42, 50, 52-55, 58]. It is noteworthy that in one study the levels of endotoxin detected in plasma of subjects with sepsis or septic shock were tested with two LAL kits from different

manufacturers which gave mean values of 1.99 EU/ml and 3.50 EU/ml although the significance of this difference was not reported [55].

Five of the 12 studies compared the systemic levels of endotoxin detected in patients with sepsis or septic shock with those of healthy control subjects that were determined concurrently by the same method. The mean levels of systemic endotoxin in these healthy subjects ranged from 0.04 to 0.36 EU/ml in four studies [42, 48, 50, 52] and was 5.1 pg/ml in the other study [114] whilst the mean levels detected in subjects with sepsis or septic shock in these four studies ranged from 0.96 to 3.45 EU/ml [42, 48, 50, 52] and was 300 pg/ml in the other study [114].

Six of those seven studies that reported mean endotoxin values in EU/ml and the one study that reported median endotoxin level in pg/ml determined the levels of systemic endotoxin with reference to endotoxin standards prepared by spiking endotoxin into endotoxin-free pooled plasma from healthy donors [42, 52-55, 114]. Two of these six studies also repeated the analysis when the levels of recovery of product positive controls (PPCs) (endotoxin spiked into diluted and heated plasma/serum) were below 75 % or above 125 % [54, 55]. Hence, the reported levels of endotoxin in subjects with sepsis or septic shock seem reliable. All in all it seems that higher levels of systemic endotoxin are detected in plasma from patients with sepsis or septic shock than in healthy controls. However, it is noteworthy that different proprietary kits were used in these studies and thus there may be superadded variation from differences between kits.

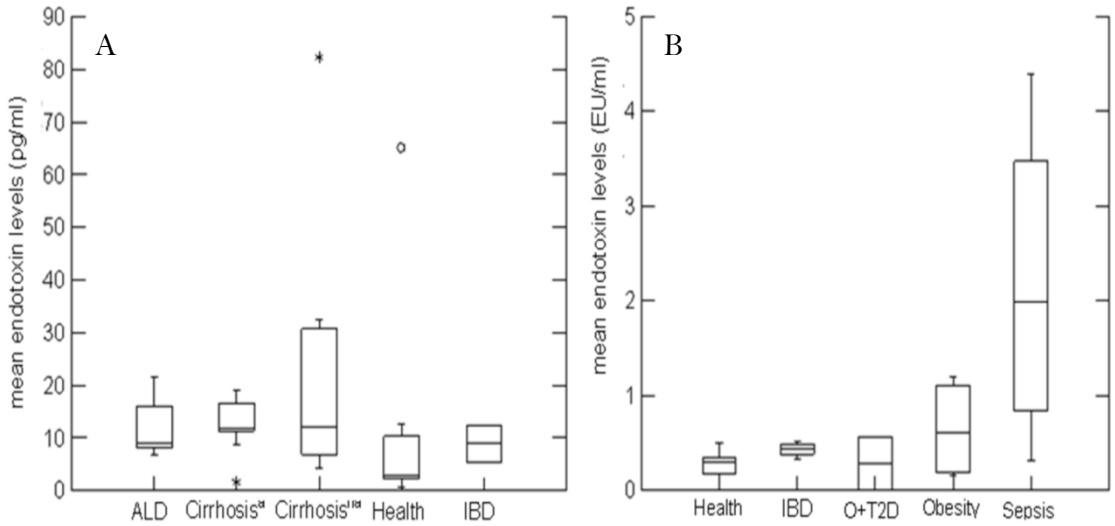

Figure 12-5. Comparison of reported mean levels of systemic endotoxin in healthy subjects and those in subjects with various diseases where samples were diluted and heated prior to LAL assay.

A) Mean levels of systemic endotoxin reported in pg/ml. Health- healthy non-obese (n = 14); IBD,- inflammatory bowel disease (n = 2); ALD- alcoholic liver disease (n = 10); cirrhosis [a]- alcoholic (n = 9); and cirrhosis [na] non-alcoholic (n = 9). B) Mean levels of systemic endotoxin reported in EU/ml. Health, healthy non-obese (n = 12); IBD, inflammatory bowel disease (n = 4); O+T2D, obese subjects with Type 2 diabetes (n = 2); obesity, obese subjects (n = 4); sepsis, hospitalised subjects with sepsis or septic shock (n = 7).

° - value above 2 SD

12.3.3 Chronic elevation of systemic endotoxin in intestinal and hepatic disorders

12.3.3.1 Inflammatory Bowel Disease

Inflammatory bowel diseases (IBD) such as Crohn's disease (CD) and ulcerative colitis (UC) are characterised by chronic and relapsing inflammation in sections of gastrointestinal mucosa in genetically susceptible subjects. This inflammation may be triggered or exacerbated by an unduly aggressive pro-inflammatory immune response to bacterial antigens that permeate from the gut lumen [120]. The ability of antigens to penetrate the gut wall is thought to be facilitated in patients with IBD [45, 49] by a general increase in the permeability of their gut mucosa, which has been identified on oral dosage with inert sugar probes [121-125].

I found five studies published between 1986 and 2013 that assayed the systemic levels of endotoxin using a chromogenic type of LAL assay in patients who had been diagnosed with IBD [45, 49, 113, 126, 127]. The results of these studies are inconsistent. One study reported that 17 out of 18 patients (94 %) with active CD had detectable levels of endotoxin in their plasma [127] whilst another reported only 31 % of patients with CD (18 % with active CD and 47 % with non-active CD) and 17 % of patients with UC (22 % with active UC and 10 % with non-active UC) had detectable amounts of endotoxin (> 0.015 EU/ml) in their plasma [87]. Two other studies failed to mention whether all of the patients they tested had detectable levels of systemic endotoxin [45, 126].

Four studies published values for the levels of systemic endotoxin and all four studies compared their results with those of healthy controls that were determined concurrently by the same method. However, only two of these studies provided details regarding the preparation of serum or plasma (dilution and heating) prior to endpoint chromogenic LAL assay that was used. One of these studies reported the results in EU/ml and the other in pg/ml [45, 126]. In the first of the latter two studies, the mean level of systemic endotoxin in subjects with CD or with UC was higher than that in healthy controls (mean CD = 0.46 EU/ml, mean UC 0.45 EU/ml and mean control = 0.32 EU/ml). Further, this study reported levels of systemic endotoxin were greater than two standard deviations from the mean of the concurrently determined levels of systemic endotoxin in healthy controls. Hence, these levels were > 0.44 EU/ml in 63 % of subjects with active CD, in 36 % of subjects with inactive CD, in 41 % of subjects with active UC and in 5 % of subjects with inactive UC [45]. However, in the second study the mean endotoxin level from healthy controls did not differ from those with CD and UC (mean control = 12.2 pg/ml, mean CD = 12.4 pg/ml and mean UC = 5.5 pg/ml) [126]. Since one of those studies reported endotoxin levels in EU/ml and the

other in pg/ml (without providing the requisite conversion factor to EU) the two cannot be directly compared. Pooling the means from the latter two studies of subjects with both active or inactive CD and UC gave a median level of systemic endotoxin in subjects with IBD of 0.44 EU/ml with an IRQ between 0.38 and 0.48 EU/ml and of 8.90 pg/ml with an IQR between 5.5 – 12.4 pg/ml (Figure 12-5A+B). Hence, the overall median level of systemic endotoxin in the IBD group (0.44 EU/ml or 8.90 pg/ml) is slightly greater than the overall median of healthy subjects in all other studies (0.29 EU/ml or 2.85 pg/ml) (Figure 12-5A+B).

When the data from the remaining two studies that provide no details on method used for sample preparation, were combined with those from the former two studies that provided this information, according to unit used, the mean levels of endotoxin detected in subjects with CD ranged from 0.1 to 0.52 EU/ml [45, 49] and from 12.4 to 29.1 pg/ml [113, 126]. Similarly, the levels of systemic endotoxin in subjects with UC ranged from 0.136 to 0.45 EU/ml [45, 87] and from 5.5 to 26.1 pg/ml [113, 126]. The mean levels of endotoxin in the systemic circulation in concurrently determined healthy controls ranged from non-detectable (< 0.015 EU/ml) to 0.32 EU/ml [45, 87] and from non-detectable (< 10 pg/ml) to 12.2 pg/ml [113, 126]. The interpretation of bulked data from all four studies is complicated both by the lack of information regarding the methods of sample preparation and by the form of the chromogenic LAL assay that was used in three of the studies. Given the shortcomings and difficulties in comparing data it is unclear from these studies whether patients with CD or UC had either, greater levels of systemic endotoxin or greater proportions of subjects with detectable levels of endotoxin, than did healthy subjects.

12.3.3.2 Liver disease

Since the liver has an important role in the clearance of endotoxin from the portal blood and systemic circulation (Figure 12-1)[5, 9, 15] it is likely that subjects with impaired liver function will be exposed to greater levels of systemic gut-derived LPS than are healthy subjects [8, 9, 84, 128, 129]. I found 25 studies published between 1984 and 2014 that determined the levels of systemic endotoxin in the plasma of patients with acute or chronic hepatitis, with early and advanced stage of chronic degenerative disease of the liver i.e. alcoholic liver disease (ALD), non-alcoholic fatty liver disease (NAFLD), and with alcoholic and non-alcoholic cirrhosis, using either the chromogenic (n = 22) or the turbidimetric (n = 2) LAL assay. Five out of 25 studies reported their results in EU/ml [5, 34, 35, 130, 131]. The remaining studies reported their results in pg/ml. The latter group used mainly LPS from either *E. coli* O111:B4 [9, 80-82, 85, 101, 112, 129, 132, 133] or LPS from *E. coli* O55:B5 [83, 84] or NP3 as reference endotoxins [134].

The mean levels of endotoxin and ranges of endotoxin concentrations obtained in plasma with the various methods of preparation and various conditions are summarised in Table 12-1. The data from three studies in the survey were not included in Table 12-1 either because they provided no information regarding the method of sample preparation [115, 130] or because the reported levels of systemic endotoxin were significantly higher than those reported in the remaining 21 studies [5].

In sum, the findings suggest that the levels of endotoxin in the systemic circulation increase with severity of disease in both acute disorders of the liver e.g. in acute hepatitis [84, 101, 112] and in chronic hepatic pathologies i.e. from mild to moderate degeneration of the liver such as ALD (median 9.015 pg/ml, IQR 8.2 – 16.0 pg/ml) to

more advanced chronic disorders such as hepatic alcoholic cirrhosis (median 11.7 pg/ml, IQR 11.1 – 16.75 pg/ml) and hepatic non-alcoholic cirrhosis (median 12.0 pg/ml, IQR 6.9 – 30.7 pg/ml) (all three sets of values being determined from diluted and heated plasma), and with the extent of complications such as bleeding from the gastro-intestinal tract [80, 133]. Hence, the medians of the mean levels of systemic endotoxin were increased according to the level of hepatic compromise in comparison to that from healthy subjects reported in 14 other studies (median 2.85 pg/ml, IQR 2.2 – 10.4 pg/ml) that also used diluted and heated plasma (Figure 12-5A). However, it is noteworthy that those studies that used a kinetic chromogenic type of the LAL assay but did not employ individual endotoxin standards (i.e. addition of known concentrations of endotoxin to plasma from each subject), nor a modified method of calculation, reported higher levels of endotoxin in plasma from subjects with chronic hepatitis and liver cirrhosis [8] than did the studies that did use refinements [81, 82, 85, 86, 93].

It is less clear whether chronic active hepatitis causes the systemic levels of endotoxin to increase. A number of reports in which plasma samples were diluted and heated prior to assay, indicate that the levels of endotoxin are higher than those concurrently assayed in healthy subjects [8, 84]. Other work in which plasma was treated with perchloric acid prior to assay found no such differences [101, 112] (Table 12-1).

Table 12-1. Range of reported mean levels of systemic endotoxin in subjects with various hepatic diseases with concurrently determined mean levels in healthy control subjects.

disease group/ sample preparation	healthy	hepatitis		fatty liver		liver cirrhosis	
		chronic	acute	non-alcoholic	alcoholic	non-alcoholic	alcoholic
dilution and heating							
pg/ml	0.5 - 12.6 [9, 86, 93, 129, 134-138]	4.6 - 57.1 [135, 136]	10.1 [136]		6.9 - 52.3* [86, 129, 134, 135, 139]	5.7 - 82.3 [9, 86, 134-136, 138]	8.8 - 19 [86, 93, 104, 134, 137, 139]
EU/ml	ND – 0.29 [8, 35, 66, 137, 140]			0.28 - 1.52^ [8, 35, 128]	0.1 – 0.2* [134, 137]	0.14 – 0.36 [135, 140]	0.13- 0.23 [134, 137]
perchloric acid method							
pg/ml	ND – 10 [72, 80, 86, 93, 101, 141]	ND – 10 [101, 141]	<5 – 86 [101, 141]	ND – 10^ [101]	12.4* [86]	9.2 – 75 [72, 80, 86, 101]	18.1 - 40.9 [86, 93, 104]
Tween 80 method							
pg/ml	6* [93]						25.4 - 40.7* [93, 104]

ND = non-detectable

* data obtained with kinetic chromogenic or turbidimetric LAL assay

^ data obtained with endpoint chromogenic LAL assay

The consumption of alcohol may have a direct effect on hepatocytes as well as engendering chronic hepatic cell dysfunction. Both of these effects are associated with elevation of endotoxin [82, 134]. The bulk of available data indicate that subjects with alcohol induced fatty degeneration of liver have elevated levels of endotoxin in their systemic circulation compared to those concurrently determined in healthy controls (mean 8.7 – 18.4 pg/ml vs. mean < 5 pg/ml) [82, 85-87]. However one study reported similar levels of plasma endotoxin in subjects with this condition (9.3 pg/ml) to those concurrently determined in healthy subjects (12.6 pg/ml) [8]. It is noteworthy that, in all these studies, the assays were carried out on diluted and heated plasma samples but that those which reported a relative increase in the systemic levels of endotoxin used individual endotoxin standard curves for each sample. This discrepancy highlights the influence of the diluent that is used for endotoxin standards on the quantification of endotoxin.

The plasma endotoxin levels, determined with dilution and heating of plasma samples prior to LAL assay, were higher in subjects with alcohol induced hepatic cirrhosis (mean 11.7 - 19 pg/ml) than in those with non-alcoholic of cirrhosis (mean 5.7 - 12 pg/ml) [82, 86].

It is noteworthy that the range of mean levels of plasma endotoxin in healthy subjects in all studies overlapped with those reported in subjects with liver disorders. This makes it difficult to determine, either a cut-off point for pathological elevation of plasma endotoxin or (as was discussed previously) a valid 'normal' range of plasma endotoxin levels that can be used in clinical practise. Bilirubin has been shown to interfere with the LAL assay [142]. Hence, the detection of endotoxin in the plasma of patients with biliary obstruction or acute hepatocellular disease may be complicated by elevated levels of bilirubin. Further, since the majority of studies on the effects of liver disease on endotoxin levels expressed their results in pg/ml and did not provide any

information regarding a suitable for conversion into endotoxin units, it is difficult to compare these values with those for other health conditions in which the results are reported in EU/ml.

12.3.4 Elevation of systemic endotoxin in chronic metabolic disorders

12.3.4.1 Obesity

I found six studies that determined systemic levels of endotoxin in obese adults (defined as BMI > 30) [41, 47, 110, 135, 143, 144] and two studies in obese adolescents (defined as subjects with a BMI z-score ≥ 95th percentile for age and gender) [131, 145]. Comparison of data showed that the mean levels of systemic endotoxin in obese subjects ranged widely between 0.16 and 5.16 EU/ml [41, 47, 135, 143-145] and were around 80 pg/ml in another study [110]. It is noteworthy that none of the eight studies reported the percentage of obese subjects in which endotoxin were not detectable. This implies that all obese subjects had detectable levels of endotoxin in their systemic circulation. When only the results from studies that used a combination of dilution and heating for the preparation of plasma or serum (n = 4) were included, the median of pooled mean levels of systemic endotoxin was 0.60 EU/ml with an IQR 0.181 – 1.10 EU/ml (Figure 12-5B). The studies that reported the highest levels of systemic endotoxin in obese subjects were those that did not describe the method by which the serum/plasma samples were prepared [41, 144, 145]. Hence, it is possible that the higher levels and greater variation of endotoxin in these studies resulted from undisclosed differences in the methods of preparation of serum/plasma.

Four of the eight studies that concurrently determined the levels of systemic endotoxin in obese and in lean healthy (defined as BMI ≤ 25) subjects, using the same

methodology reported higher levels of systemic endotoxin in obese than in the lean healthy subjects [41, 47, 110, 144]. However, with one exception, the method by which plasma or serum samples were prepared was not described. Moreover, the systemic levels of endotoxin in healthy subjects that were determined concurrently in these three studies, were higher than those reported by other workers that diluted and heated their plasma or serum samples prior to assay (Figure 12-4B). The mean levels of plasma/serum endotoxin in obese subjects from the four studies that diluted and heated the plasma or serum samples before assay and reported their results in EU/ml, had a broad range which overlapped with that of the variously reported range in healthy subjects (Figure 12-5B).

Hence, it is unclear from this comparison whether obese subjects had significantly higher levels of systemic endotoxin than did healthy subjects.

12.3.4.2 Diabetes

A review of the eight studies that were found showed that the mean levels of systemic endotoxin in subjects with type 1 diabetes ranged between 56 and 67 EU/ml [146] whilst those in subjects with type 2 diabetes ranged from non-detectable (< 0.005 EU/ml) to 77 EU/ml [36, 37, 39-41, 51, 144]. However, five of these studies did not provide any information regarding the method by which plasma or serum samples were prepared prior to LAL assay whilst one simply diluted them prior to testing [39]. Again, the study that simply diluted the samples, and those that did not provide information regarding sample preparation, were those that reported the highest levels of endotoxin. Hence, the mean was 77.03 EU/ml for sample preparation by simple dilution [146] and varied between 4.6 to 9.2 EU/ml [37, 40, 41, 144] with the five studies that did not indicate the method of sample preparation. In contrast, in the

remaining two studies in which plasma or serum samples were diluted and heated prior to LAL assay, one failed to detect any endotoxin in the systemic circulation of subjects with type 2 diabetes [36] and the other reported a much lower mean level of 0.567 EU/ml [51]. It is noteworthy that the study that failed to detect any endotoxin in the serum of diabetic subjects also found no endotoxin in the systemic circulation of healthy controls [36, 147] whereas the other study found elevated levels of systemic endotoxin in the plasma of diabetic subjects (mean 0.567 EU/ml) compared with those in concurrently tested healthy subjects (mean 0.15 EU/ml) [51]. Given that diluted serum can accelerate the LAL reaction and that dilution and heating of serum can delay the commencement of the LAL reaction [73], and assuming that the recommended method for calculation (based on the time it takes to initiate the LAL reaction), is likely to have been used in these studies, it seems likely that the overall results are influenced by the differences in method and are correspondingly suspect.

12.4 The effect of macronutrients on the levels of systemic endotoxin

I found seven studies published between 2007 and 2012 that investigated the effects of consuming foods that contained differing proportions of macronutrients on the levels of endotoxin in plasma/serum of either healthy subjects (n = 6), obese subjects (n = 2), subjects with type 2 diabetes (n =1) or subjects with impaired glucose tolerance (n = 1). None of the seven studies reported any subjects in whom there were no detectable levels of systemic endotoxin either before or after the meal. Five of the seven studies diluted and heated their plasma or serum samples prior to LAL assay [11, 12, 43, 143, 148] whilst the two remaining studies provided no information regarding the method by which samples were pre-treated [44, 144]. In these five studies, the ingestion of either, a 900 kcal meal containing around 50 g of fat (which comprised around 50 % of the calories), or of a drink of plain cream (33 g), lead to a post-prandial rise in median plasma levels of endotoxin from 0.305 EU/ml to 0.455 EU/ml [11, 12, 43, 44, 148] (Figure 12-6). Conversely, neither the ingestion of 300 ml of water, a 300 calorie solution containing 75 g of glucose (Glucola) or an equicaloric quantity of pure orange juice, caused the levels of systemic endotoxin to rise [11]. Another study showed that the levels of serum endotoxin rose from 3.3 EU/ml to 6.3 EU/ml when healthy non-obese subjects consumed a single meal containing 75 g of fat, 5 g of carbohydrate and 6 g of protein, the fat comprising 94 % of the calories [144]. Similarly, consumption of the same meal by obese subjects (defined as BMI > 30) caused serum endotoxin levels to rise from 5.1 to 7.7 EU/ml, whilst it rose from 5.7 to 7.5 EU/ml in subjects with impaired glucose tolerance, and from 5.3 to 14.2 EU/ml in subjects with type 2 diabetes [144]. However, this study provided no details regarding the method used for preparation of the samples. Moreover, the study used an endpoint chromogenic LAL assay, so that any variation in reaction kinetics from those of the standards would not have been detected.

Two studies examined the effect of co-consumption of other nutrients on these changes. Hence, after a co-consumption of a 900 kcal meal containing around 50 g fat (equal to around 50 % of calories from fat) with either a drink of orange juice (300 kcal), or a muscadine grape extract containing a mixture of resveratrol (100 mg) and other polyphenols to a total of 75 mg, systemic endotoxin levels were assayed by endpoint chromogenic LAL kit following dilution and heating of the plasma samples. Neither of these two treatments caused post-prandial systemic endotoxin levels to rise [12, 44]. Similarly, after the consumption of a 900 kcal meal in which the fat content had been reduced to around 27 g (equal to around 27 % of calories from fat) there was no significant rise in levels of plasma endotoxin [43]. These findings suggest that either co-consumption of other dietary items or a reduction in the relative proportions of fat in the meal may ameliorate any post prandial rise in systemic endotoxin although the problems outlined previously must be borne in mind.

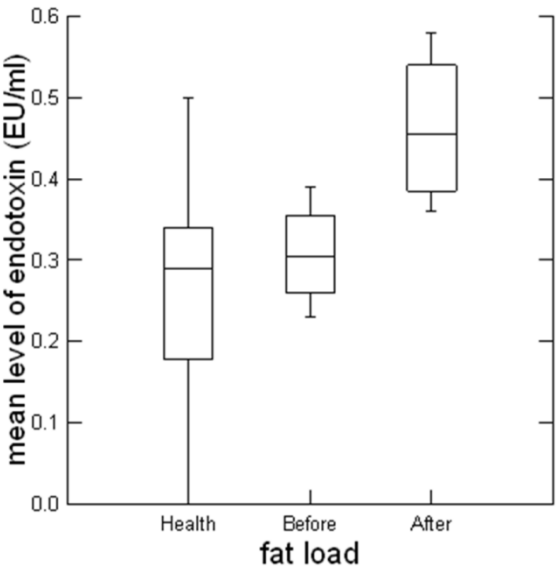

Figure 12-6. Comparison of reported mean levels of systemic endotoxin in healthy subjects before and after consuming a fatty meal where samples were diluted and heated prior to assay.

Reported mean levels of systemic endotoxin in fasted and non-fasted healthy subjects. Health, healthy non-obese subjects from other studies (n = 12); before, level in healthy subjects prior to consuming fatty meal (n = 4); after, level in healthy subjects after consuming a fatty meal (n = 4).

12.5 Conclusion

Adaptation of the LAL assay to accurately quantify endotoxin in samples of plasma or serum is challenging as is the interpretation of the data obtained using currently available methodologies. Pooling and comparison of the results from various studies indicate that the methods used for the pre-treatment of the sample prior to assay significantly influence the levels of endotoxin that are detected and in many cases confound meaningful comparison. Hence for example, meaningful comparison of systemic endotoxin levels in subjects with various medical disorders that compromise the barrier function of the intestine with those of healthy subjects is dependent upon selecting data that have been obtained using identical assay methodologies. Even then there may be difficulties. For example in studies where plasma or serum were simply diluted, or when the method of sample preparation was not described, the levels of systemic endotoxin in diabetic subjects (range of means 4.6 – 77.0 EU/ml) were elevated compared with those from simultaneously tested healthy subjects (range of means 3.1 – 61.1 EU/ml) [37, 39-41, 144, 146], but both these levels were higher than those reported in other studies in which plasma or serum were diluted and heated prior to endotoxin test (Figure 12-5A+B).

Whilst, a distinction between direct interference by constituents in serum or plasma with components of the LAL assay and interference by the sequestration of endotoxin by components of the blood would be useful in optimising methods for pre-treating samples, little work has been done in this regard. Some data suggest that a combination of dilution and heating is sufficient to reduce both direct interference with the assay by constituents of serum and direct interaction between (spiked) endotoxin and constituents of serum [73]. However, I can find no studies showing that such treatments are able to release sequestered endotoxins and render them detectable by current commercial LAL assay. In contrast, two publications indicate that the

treatment of plasma by either perchloric acid or the ESP kit is able to release sequestered endotoxin and to improve recoveries [100, 101].

A particular problem in the reporting and interpretation of data lies in the reported absence of endotoxin in samples of plasma or serum from a significant proportion of both healthy [36, 49, 50, 113, 114] and diseased subjects [36, 42, 49, 50, 53-58]. This may again result from the method of sample preparation prior to assay. However, it may equally represent a real finding in the population that is being studied. At all events studies should report and compare both the proportions of negative tests and the levels of endotoxin in the various study groups. It should also be borne in mind that significant proportions of negative tests may preclude the use of parametric statistical methods as well as rendering the mean a poor descriptor of that data.

It is possible that comparisons of healthy and ill populations using the same standard method of sample preparation prior to LAL assay will be sufficient to detect when higher levels of endotoxin are entering the systemic circulation in clinical conditions where the intestinal barrier or hepatic function are significantly compromised. Caution is nevertheless advised in interpreting the clinical relevance of elevated systemic endotoxin levels as the magnitude of LAL reaction may not directly correlate with the pro-inflammatory potential of LPS in human subjects. Further, it is important to keep in mind that the LAL test determines the endotoxicity of LPS molecules in a given sample but does not give any information about the quantity of LPS in a given sample. Hence, results obtained with the LAL assay should be reported in endotoxin units (EU) rather than in pg/ml. If results are reported in pg/ml the relevant conversion factor from pg/ml into EU/ml should be provided.

A considerable improvement on existing levels of accuracy of the LAL test on use with biological fluids would be required for adequate discrimination of more subtle effects

such as the detection of chronic low levels of endotoxin. A number of manufacturers state that a variation in the recovery of endotoxin spiked into (diluted and heated) plasma or serum (Positive Product Control, PPC) of between 50 and 200 % (i.e. between half and double the spiked dose) is acceptable [67, 78]. However, calculations based on the levels of endotoxin reported in healthy and in obese subjects following ingestion of a fatty meal [11, 12, 43, 44, 143, 144, 148] indicate that an assay with a CV of less than 15 % would be required to reliably distinguish a 50 % difference in levels of circulating endotoxin in these two groups ($p < 0.05$) [149].

Given the shortcomings of the currently available assays, methods that indirectly assess LPS by its effects are being increasingly used, even though they cannot directly establish causation. One example is the endotoxin activity assay (EAA) which is based on the quantification of free radicals released from neutrophils in response to endotoxin [150]. The assay is approved by the FDA for assessing the risk of septic shock [151] and has been used in this respect [147, 152-157], in healthy human subjects [158] and subjects with disorders such as obesity [159-161]. However, the assay must be completed within 3 h of collection [e-mail correspondence with the manufacturer] and may be awkward to use in surveys containing large numbers of subjects. Alternatively the quantification of LPS-binding protein (LBP) may be useful in inferring a chronic low level increase of systemic LPS as the production of LBP, an acute phase reactant produced by hepatocytes [162], is up-regulated following immune challenge with LPS [162]. Human specific LBP-ELISAs have been used to indirectly assess the systemic levels of LPS in various infections and in obesity [45, 163-175].

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CHAPTER 13 DISCUSSION

The original aim of my PhD project was to investigate the relationship between gut permeability and the quantities of bacterial products such as LPS/endotoxin in the systemic circulation in humans. However, reliable methods for the determination of both parameters are required before this can be done.

The work in my project showed that the commercial tests for the direct quantification of systemic LPS i.e. the LPS - ELISA and the LAL assay were not suitable for use with serum samples. Given the fact that the latter assay is based on the clotting cascade of the horseshoe crab it is likely that it would be subject to interference by constituents of the blood of other species such as in human serum. As was discussed in Chapter 12 it is necessary to evaluate the suitability of both the LAL assay and any method used to pre-treat the samples if results are to be reliable. It is noteworthy that heating serum at 70°C or above caused serum to form a gel whilst heating at 60°C had no such effect. Hence, it is conceivable that the very low recoveries of spiked endotoxin when the serum was heated at 75°C, resulted from physical sequestration of endotoxin within denatured proteins. Further work is needed to validate this hypothesis and to find a heat treatment that inactivates those constituents in serum that interfere with the LAL assay, whilst at the same time limiting sequestration of endotoxin. It is possible that such improvements will allow accurate assay of endotoxin levels in samples derived from blood and lead to a more accurate assessment of the 'sequestration' capacity of sera in healthy and in diseased individuals.

A number of the findings that I obtained using the commercial ELISA kits for LPS and LBP are worrying and highlight the importance of thoroughly testing the specificity of commercial kits before using them for clinical assays. For example, it was surprising that around 100 % of 'LPS' standard that was provided with the ELISA was

recovered when it was spiked into raw serum. Given the fact that sera has a high capacity to 'neutralise' LPS [1] this raises the question whether the supplied standard had the same chemical characteristics as LPS or whether it was another material that had the chemical moiety necessary for detection by the assay. Further, the LPS – ELISA kit manufactured by Cusabio, not only failed to detect any LPS or endotoxin from other sources but also cross-reacted with the LBP standards that were provided with a LBP - ELISA kit produced by the same manufacturer. Again, it seems likely that other commercially available LPS - ELISA kits are similarly unreliable in detecting LPS as 'antibodies-online', a distributer of such assays, reduced the number of ELISA kits that they recommended for the quantification of LPS in human blood from seven in September 2012 to one in November 2015.

Apart from the problems with the LPS – ELISA there were also problems with the specificity of the LBP – ELISA kit manufactured by Cusabio as this was found to vary significantly between batches, and again with the sensitivity (rather than the specificity) of the LBP – ELISA kit manufactured by Hycult. Hence, due precautions must be taken to quantify and factor out such series of variation. Such a process is both cumbersome and expensive. All in all the work in this thesis suggests even when tests produced by ISO classified manufacturers are used, the results are not always reliable. At all events researchers should ask the manufacturers to provide evidence validating the specificity and sensitivity of the assay under the conditions specified by the researcher. Ideally such evidence should be provided by an independent laboratory or agency and a reference antigen other than that provided by the manufacturer used for such validation. It does seem that this problem is becoming more widely known. For example, the companies 'antibodies-online' and 'Science Exchange' launched an 'Independent Validation Initiative' in July 2013 [2] that was specifically designed to address this problem. Ideally an independent central agency should administer this

and allow researchers to report unsatisfactory performances of ELISAs and other test kits allowing such problems to be efficiently communicated to the research community.

Clearly the current evidence based on assays of systemic LPS for its role in the pathogenesis of metabolic syndrome is unconvincing. My findings regarding the fidelity of the LAL assay and my review of results obtained from published studies regarding elevation in levels of endotoxin that used the LAL assay, indicate that a number of studies must be repeated for their conclusions to be validated. This may be done either with modified test or a different type of test i.e. the detection of increase in LBP in response to elevations in LPS. However, the preliminary testing conducted in this thesis shows there may also be similar problems regarding the reliability of LBP – ELISAs.

One useful approach to validate hypotheses regarding the effects of elevated systemic levels of LPS on the pathogenesis of metabolic syndrome, is to compare the various methods for assaying the levels of systemic LPS or LBP against other tests that quantify various aspects of the pathogenesis of the disease. Hence, for example quantifying the increase in mucosal permeability and comparing this with levels of LPS or with correlated variables such as the levels of LBP. Again as enterocytes and the constituents of the GALT have the ability to detoxify and neutralise LPS [1] thus it may be useful to examine covariance of mucosal permeability with the capacity for detoxification of LPS.

An incidental finding of my work is that the levels of faecal calprotectin and the rate of lactulose and mannitol excretions are useful diagnostic indicators in assessment of a patients gut health. However, further work with greater numbers of participants is needed to confirm this.

It is noteworthy that the intervals between urine collections varied between all of my three studies. Urine samples in my pilot study [38] (Chapter 5) were collected prior to ingestion of the treatment (water or aspirin) and 4 h after the ingestion of the treatment (3 h after the ingestion of the sugar solution). The quantification of lactulose in those samples by HPLC was difficult as the amount of lactulose in the pooled 4-h sample was at the limit of detection and the profile of the peaks observed by Sequeira (personal communication) were not evident, presumably owing to the pooled overall 4-h volume of urine i.e. urine produced between the ingestion of the treatment and the ingestion of the sugar solution was not discarded. Urine samples for the Intralipid study (Chapter 7) were therefore collected at three time points i.e. prior to ingestion of the treatment (water or Intralipid drink), prior to ingestion of the sugar solution (2 h after the ingestion of the treatment) and 3 h after the ingestion of the sugar solution (5 h after the ingestion of the treatment). The first sample was 'discarded' and the latter two were used for the quantification of lactulose and mannitol by HPLC. The peaks of mannitol were easy to identify but the lactulose peaks were still hard to identify and to interpret in the samples of healthy subjects. Nevertheless, the lactulose peaks in the pooled 3-h urine samples of the Intralipid study were more pronounced than in the pooled 4-h urine samples of the pilot study. Urine samples of the Crohn's disease study (Chapter 11) were collected 1.5 h and 3 h after the ingestion of the sugar solution in order to improve the identification of lactulose peaks. Both samples were separately analysed by HPLC and the amount of lactulose and mannitol excreted in both subsamples added to get the total amount of lactulose and mannitol excreted in 3 h. I found that 11 out of 13 healthy subjects and 5 out of 13 subjects with CD had detectable levels of lactulose in one of the two subsamples whereas mannitol was detectable in all subsamples. The amount of lactulose excreted in the urine was more accurately identifiable in subsamples than in the pooled 3 h urine samples and

subsequently the total 3-h excretion of lactulose higher in healthy subjects of the current study than in those of the pilot and Intralipid study (Chapter 5 and Chapter 7). It is further noteworthy that the excretion of lactulose in urine was around ten times lower than those of mannitol. Hence, the standard curves of the two sugars had to be adjusted compared to those used by Sequeira i.e. standards of lactulose in the range between 12.5 and 200 µg/ml and of mannitol in the range between 125 and 2,000 µl were used for the generation of standard curves in the Crohn's disease study (Chapter 11). Further work is needed to evaluate whether an interval between collection time points of 30 min, as conducted by Sequeira, allows a more precise quantification of the trace sugars than in those with an interval of 90 min between collections, as conducted in the Crohn's disease study (Chapter 11). This information will be useful with respect to the standardisation of a gut permeability test that can be used in clinical settings.

In sum, it is my opinion that researchers investigating hypotheses that endeavour to link the pathogeneses of various diseases with the penetration of bacterial products or other immunomodulatory substances through the gut mucosa may be better employed to quantify the fundamental characteristics on which such hypothesis hinge. Quantification of increases in mucosal permeability to LPS depends on the assumption that such substances can readily traverse the gut mucosa and that they remain immunologically active whilst in the systemic circulation in spite of the sequestration by substances such as LBP. My work suggests that the permeability of the gut to lactulose may provide such an index.

13.1 References

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APPENDIX I DOCUMENTS – EVALUATION OF METHODS

Appendix I - i MUHEC: Southern A - Approval Application 12/61

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA

6 December 2012

Anne Gnauck
IFNHH
PN452

Dear Anne

Re: HEC: Southern A Application – 12/61
Establishment of an assay to measure lipopolysaccharide (LPS) in human plasma

Thank you for your letter dated 3 December 2012.

On behalf of the Massey University Human Ethics Committee: Southern A I am pleased to advise you that the ethics of your application are now approved. Approval is for three years. If this project has not been completed within three years from the date of this letter, reapproval must be requested.

If the nature, content, location, procedures or personnel of your approved application change, please advise the Secretary of the Committee.

Yours sincerely

Dr Brian Finch, Chair
Massey University Human Ethics Committee: Southern A

cc Prof Roger Lentle
IFNHH
PN452

Prof Richard Archer, HoI
IFNHH
PN452

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA

19 April 2013

Anne Gnauck
IFNHH
PN452

Dear Anne

Re: HEC: Southern A Application – 12/61
Establishment of an assay to measure lipopolysaccharide (LPS) in human plasma

Thank you for your letter dated 15 April 2013 outlining the change you wish to make to the above application.

The change has been approved and noted, as follows:

- Amendment approved for taking 4ml blood samples from 5 additional volunteers to establish an LPS kit using serum samples.

If the nature, content, location, procedures or personnel of your approved application change, please advise the Secretary of the Committee. If over time, more than one request to change the application is received, the Chair may request a new application.

Yours sincerely

Dr Brian Finch, Chair
Massey University Human Ethics Committee: Southern A

cc Prof Roger Lentle
IFNHH
PN452

Prof Richard Archer, HoI
IFNHH
PN452

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA

26 May 2014

Anne Gnauck
IFNHH
PN452

Dear Anne

Re: HEC: Southern A Application – 12/61
Establishment of an assay to measure lipopolysaccharide (LPS) in human plasma

Thank you for your letter dated 23 May 2014 outlining the change you wish to make to the above application.

The change has been approved and noted, as follows:

- Amendment approved to take venous blood samples from a further 10 volunteers.

If the nature, content, location, procedures or personnel of your approved application change, please advise the Secretary of the Committee. If over time, more than one request to change the application is received, the Chair may request a new application.

Yours sincerely

Dr Brian Finch, Chair
Massey University Human Ethics Committee: Southern A

cc Prof Roger Lentle
IFNHH
PN452

Prof Richard Archer, HoI
IFNHH
PN452

Appendix I - ii Information Sheet

Version 1

Institute of Food, Nutrition and Human Health
Massey University
PO Box 11 222
Palmerston North 4442
New Zealand

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

Evaluation of an assay to measure lipopolysaccharides (LPS) in human plasma

I would like to invite you to participate in my project. Below is important information to tell you more about my research. Thank you for your interest.

INFORMATION SHEET

Who am I?
I am Anne Gnauck undertaking research in the Gut Physiology group for my PhD studying the impact of diet on gut permeability and plasma lipopolysaccharides (LPS) level in human. Our group is part of The Institute of Food, Nutrition and Human Health/ Massey University.

Why am I doing this project?
Our group is studying the effect that molecules/ nutrients have on absorption of nutrients by the small intestine. The current project is needed to evaluate an assay for plasma lipopolysaccharides (LPS) measurement. For that I need blood samples which will be taken by a qualified person.
Lipopolysaccharides form part of the surface of gut bacteria and are associated with a low grade chronic inflammation. I will use the LPS assay in further projects to describe the impact of diet on gut permeability and plasma LPS concentration.
If you are keen on participating you will be asked to attend the laboratory once in the morning and you have to fast overnight before blood taking.

Would you like to participate?
I would like to invite persons aged between 20 and 50 years to join my project. To be suitable to join in my project you should NOT:

- Be a smoker or tobacco chewer.
- Have a history of gastrointestinal diseases including a recent gut infection evidenced by a recent history of abdominal pain, nausea, vomiting, diarrhea, passage of blood and mucus in stool.
- Have a history of significant pain with venipuncture or difficult veins.
- Have an earlier diagnosis of diabetes, cancer, Crohn's diseases, ulcerative colitis, irritable bowel diseases.
- Have a BMI < 18.5 or > 30 kg/m².
- Be pregnant or lactating.

What you have to do to join the project?
You will be instructed to commence the study between 8:00am and 9:00am at the Human Nutrition Unit and you have to fast overnight (10- 12 hours) before having a 10 ml blood sample taken. A cup of tea or a glass of water and a biscuit will be provided.

Is taking blood sample harmful or painful?
This is a routinely procedure in clinical tests and should not cause any more than minimal discomforts. On rare occasions slight pain of venipuncture may occur. If you have a history of such pain you will be excluded from the project.

What am I going to measure?
I will measure the plasma lipopolysaccharides (LPS) using a specific ELISA kit.

Who will see the information about you?
When you join the trial you will be given a unique identifier and thereafter all information will be filed with the code number, and stored in a locked filing cabinet accessed by the research team only. Your anonymous blood sample is only used to evaluate the LPS assay. No data of the test will be used for presentation to academic societies and scientific publications.

Who is funding this research?
The project is funded by Massey University.

Compensation of Injury
If physical injury results from your participation in this project, you should visit a treatment provider to make a claim to ACC as soon as possible. ACC cover and entitlements are not automatic and your claim will be assessed by ACC in accordance with the Accident Compensation Act 2001. If your claim is accepted, ACC must inform you of your entitlements, and must help you access those entitlements. Entitlements may include, but not be limited to, treatment costs, travel costs for rehabilitation, loss of earning, and7 or lump sum for permanent impairment. Compensation for mental trauma may also be included, but only if this is incurred as a result of physical injury.

If your ACC claim is not accepted you should immediately contact the researcher. The researcher will initiate processes to ensure you receive compensation equivalent to that to which you would have been entitled had ACC accepted your claim from Massey University.

Will you get any financial compensation?
You will get no financial compensation.

What are your rights?
You are under no obligation to accept this invitation. If you decide to participate, you have the right to:

- Decline to answers any particular questions
- Withdraw from the study at any time
- Ask any questions about the study at any time during participation
- Provide information on the understanding that your name will not be used unless you give permission to the researcher.

If you are interested in participating please contact Anne Gnauck who will answer your questions.

Anne Gnauck
Institute of Food, Nutrition and Human Health
Massey University
Riddet Road
Palmerston North 4474
Tel/Text: 021 1507654
E mail: A.Gnauck@massey.ac.nz

This project has been reviewed and approved by the
Massey University Human Ethics Committee: Southern A, Application 12/61.
If you have any concerns about the conduct of this research, please contact
Dr Brian Finch, Chair, Massey University Human Ethics Committee: Southern A,
telephone 06 350 5799 x 8717, email humanethicsoutha@massey.ac.nz.

Version 2

Institute of Food, Nutrition and Human Health
Massey University
PO Box 11 222
Palmerston North 4442
New Zealand

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

Evaluation of an assay to measure lipopolysaccharides (LPS) in human serum

I would like to invite you to participate in my project. Below is important information to tell you more about my research. Thank you for your interest.

INFORMATION SHEET

Who am I?
I am Anne Gnauck undertaking research in the Gut Physiology group for my PhD studying the impact of diet on gut permeability and serum lipopolysaccharides (LPS) level in human. Our group is part of The Institute of Food, Nutrition and Human Health/ Massey University.

Why am I doing this project?
Our group is studying the effect that molecules/ nutrients have on absorption of nutrients by the small intestine. The current project is needed to evaluate an assay for serum lipopolysaccharides (LPS) measurement. For that I need blood samples which will be taken by a qualified person.
Lipopolysaccharides form part of the surface of gut bacteria and are associated with a low grade chronic inflammation. I will use the LPS assay in further projects to describe the impact of diet on gut permeability and serum LPS concentration.
If you are keen on participating you will be asked to attend the laboratory once in the morning and you have to fast overnight before blood taking.

Would you like to participate?
I would like to invite persons aged between 20 and 50 years to join my project. To be suitable to join in my project you should NOT:

- Be a smoker or tobacco chewer.
- Have a history of gastrointestinal diseases including a recent gut infection evidenced by a recent history of abdominal pain, nausea, vomiting, diarrhea, passage of blood and mucus in stool.
- Have a history of significant pain with venipuncture or difficult veins.
- Have an earlier diagnosis of diabetes.
- Have a BMI < 18.5 or > 30 kg/m^2.
- Be pregnant or lactating.

What you have to do to join the project?
You will be instructed to commence the study between 8:00am and 9:00am at the Human Nutrition Unit and you have to fast overnight (10- 12 hours) before having a 10 ml blood sample taken. A cup of tea or a glass of water and a biscuit will be provided.

Is taking blood sample harmful or painful?
This is a routinely procedure in clinical tests and should not cause any more than minimal discomforts. On rare occasions slight pain of venipuncture may occur. If you have a history of such pain you will be excluded from the project.

What am I going to measure?
I will measure the serum lipopolysaccharides (LPS) using a specific ELISA kit.

Who will see the information about you?
When you join the trial you will be given a unique identifier and thereafter all information will be filed with the code number, and stored in a locked filing cabinet accessed by the research team only. Your anonymous blood sample is only used to evaluate the LPS assay. No data of the test will be used for presentation to academic societies and scientific publications.

Who is funding this research?
The project is funded by Massey University.

Compensation of Injury
If physical injury results from your participation in this project, you should visit a treatment provider to make a claim to ACC as soon as possible. ACC cover and entitlements are not automatic and your claim will be assessed by ACC in accordance with the Accident Compensation Act 2001. If your claim is accepted, ACC must inform you of your entitlements, and must help you access those entitlements. Entitlements may include, but not be limited to, treatment costs, travel costs for rehabilitation, loss of earning, and7 or lump sum for permanent impairment. Compensation for mental trauma may also be included, but only if this is incurred as a result of physical injury.

If your ACC claim is not accepted you should immediately contact the researcher. The researcher will initiate processes to ensure you receive compensation equivalent to that to which you would have been entitled had ACC accepted your claim from Massey University.

Will you get any financial compensation?
You will get no financial compensation.

What are your rights?
You are under no obligation to accept this invitation. If you decide to participate, you have the right to:

- Decline to answers any particular questions
- Withdraw from the study at any time
- Ask any questions about the study at any time during participation
- Provide information on the understanding that your name will not be used unless you give permission to the researcher.

If you are interested in participating please contact Anne Gnauck who will answer your questions.

Anne Gnauck
Institute of Food, Nutrition and Human Health
Phone: 021 1507654
E mail: A.Gnauck@massey.ac.nz

This project has been reviewed and approved by the
Massey University Human Ethics Committee: Southern A, Application 12/61.
If you have any concerns about the conduct of this research, please contact
Dr Brian Finch, Chair, Massey University Human Ethics Committee: Southern A,
telephone 06 350 5799 x 8717, email humanethicsoutha@massey.ac.nz.

Appendix I - iii Participant Consent Form

Version 1

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

Evaluation of an assay to measure lipopolysaccharide (LPS) in human plasma

PARTICIPANT CONSENT FORM

I have read the Information Sheet and have had the details of the study explained to me. My questions have been answered to my satisfaction, and I understand that I may ask further questions at any time.

I agree to participate in this study under the conditions set out in the Information Sheet..

Signature: _____ **Date:** _____

Full Name - printed _____

Version 2

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

Evaluation of an assay to measure lipopolysaccharide (LPS) in human serum

PARTICIPANT CONSENT FORM

I have read the Information Sheet and have had the details of the study explained to me. My questions have been answered to my satisfaction, and I understand that I may ask further questions at any time.

I agree to participate in this study under the conditions set out in the Information Sheet.

Signature: _____ **Date:** _____

Full Name - printed _____

Appendix I - iv Confidentiality Agreement

Institute of Food, Nutrition and Human Health
Massey University
PO Box 11 222
Palmerston North 4442
New Zealand

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

Evaluation of an assay to measure lipopolysaccharide (LPS) in human plasma

CONFIDENTIALITY AGREEMENT

- to be completed by the person taking blood samples -

I ..... (Full Name - printed)

agree to keep confidential all information concerning the project 'Establishment of an assay to measure lipopolysaccharide (LPS) in human plasma'.

I will not retain or copy any information involving the project.

Signature: _____ **Date:** _____

APPENDIX II DOCUMENTS – PILOT STUDY

Appendix II - i MUHEC: Southern A – Approval Application 13/31

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA

5 June 2013

Anne Gnauck
IFNHH
PN452

Dear Anne

Re: HEC: Southern A Application – 13/31
The effect of aspirin on LPS/endotoxin level in blood

Thank you for your letter dated 4 June 2013.

On behalf of the Massey University Human Ethics Committee: Southern A I am pleased to advise you that the ethics of your application are now approved. Approval is for three years. If this project has not been completed within three years from the date of this letter, reapproval must be requested.

If the nature, content, location, procedures or personnel of your approved application change, please advise the Secretary of the Committee.

Yours sincerely

Dr Brian Finch, Chair
Massey University Human Ethics Committee: Southern A

cc Prof Roger Lentle
IFNHH
PN452

Prof Richard Archer, HoI
IFNHH
PN452

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Appendix II - ii Information Sheet

MASSEY UNIVERSITY
COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

The effect of aspirin on LPS level in blood

I would like to invite you to participate in my project. Below are important information to tell you more about my research. Thank you for your interest.

INFORMATION SHEET

Who am I?

I am Anne Gnauck, undertaking research in the Gut Physiology group for my PhD. I am studying the impact of diet on gut permeability and serum lipopolysaccharide (LPS) and endotoxin levels in humans. Our work group is part of the Institute of Food, Nutrition and Human Health at Massey University.

Why am I doing this project?

Our work group is studying the effect of gut bacteria and their products on absorption of nutrients by the small intestine. Gut colonizing bacteria constantly release bacterial components such as lipopolysaccharides (LPS)/ endotoxin into the gut lumen. LPS form part of the surface of gut bacteria and are associated with low grade inflammation in the body. It seems that diet may influence the absorption of this LPS from the gut.

The current work is focused on the effect of aspirin on gut permeability and the absorption of LPS/ endotoxin. We will measure changes in permeability of the gut using simple ingestible sugars that are excreted in the urine. We will determine changes in LPS and endotoxin concentration in the blood and the excretion of these sugars in the urine four hours after you consume either a drink of water or a single dose of aspirin.

Would you like to participate?

I would like to invite women aged between 20 and 40 years with a body height between 160 to 170 cm and a body mass between 60 and 80 kg.

To be suitable to join my project you should NOT:

- Have a history of allergic reaction to aspirin
- Be a smoker or tobacco chewer
- Be pregnant or lactating
- Take vitamin/ mineral supplements
- Take non-steroidal anti-inflammatory drugs
- Have a history of diabetes, renal (kidney) disorder, cancer, Crohn's diseases, ulcerative colitis and irritable bowel diseases
- Have a recent history of abdominal pain, nausea, vomiting, diarrhea, passage of blood and mucus in stool

- Have an aversion to venipuncture, difficult veins, a history of bleeding disorder or be on anticoagulant therapy

If you are interested in joining the study you will be asked to come for a health screening during office hours at the Human Nutrition Lab. The health assessment will take about 30 to 45 min to complete and includes questions about your medical history. If you pass the health check we will also measure your body height, body mass and body fat by using BOD POD. For the measurement of the body fat you will be asked to wear a skin tight lycra suit.

What you have to do to join the project?

If you chose to participate you will be asked to attend the Human Nutrition Lab on Palmerston North Campus at 8 am for two sessions one week apart. Each experimental session will take 4.5 to 5 hours during what you will be expected to stay in the laboratory (video will be available) You will be asked not to eat anything after 8 pm and not to drink anymore after 10 pm on the night before the lab session. You will be also asked to refrain from urinating after 6 am on the day of the lab session. You will be asked not to eat any food during the study period but a meal will be provided at the end of each lab session. You will receive a control (water) or aspirin solution plus some sugar solution (lactulose and mannitol) and water during the lab session.

The following is scheduled for both lab sessions:

- At first, one 4 ml blood sample will be taken.
- You will be asked to empty your bladder.
- You will then receive 100 ml water or a single dose of 600 mg aspirin dissolved in 100 ml water.
- One hour later, you will receive a 100 ml solution containing 10 g lactulose and 5 g mannitol followed immediately by 300 ml of water.
- 3 hours after the intake of the sugar solution, one 4 ml blood sample will be taken and you will be asked to empty your bladder. If you have to urinate before the urine collection point, an additional urine collection bottle will be provided.
- The study is over at this point and you will receive a meal.

Some question you may want to answer:

- *Is the measurement of body fat using BOD POD harmful or painful?*

Measuring body composition using BOD POD is a harmless procedure. You will be asked to breath through a small tube in the closed BOD POD wearing a skin tight lycra suit. If you have any fear of sitting in the closed BOD POD, i.e. claustrophobia or you would feel embarrassed wearing a skin tight lycra suit, you will not be able to participate in the project.

- *Is taking blood samples harmful or painful?*

Blood will be taken from the vein in your arm and could cause slight discomfort on rare occasions. If you have a history of difficult veins or aversion to venipuncture you will not be able to participate in the project.

- *What we are going to measure in your blood and urine?*

We will measure the lactulose and mannitol excretion in your urine sample using HPLC to assess the permeability of your gut. We will also measure the serum LPS concentration in your blood using a LPS-specific ELISA kit and the serum endotoxin concentration using LAL-kit.

- *Who will see the information about you?*

When you join the trial you will be given a unique identifier and thereafter all information will be filed with the code number. The list with the codes will be stored in a locked filing cabinet accessed by the research team only. You will receive a summary of anonymised result of the study. The outcome of the study will be published as peer reviewed article in a scientific journal and/ or be presented to academic societies.

- *Does the increased LPS level during the experiment affect my well being?*

The extent that LPS levels are correlated with chronic illness such as obesity is not known. Thus we will not be providing you with the LPS result as it currently has no value as a health predictor.

- *Who is funding this project?*

The project is funded by Massey University.

Compensation of Injury

If physical injury results from your participation in this project, you should visit a treatment provider to make a claim to ACC as soon as possible. ACC cover and entitlement are not automatic and your claim will be assessed by ACC in accordance with the Accident Compensation Act 2001. If your claim is accepted, ACC must inform you of your entitlement, and must help you access those entitlements. Entitlements may include, but not be limited to, treatment costs, travel costs for rehabilitation, loss or earning, and/ or lump sum for permanent impairment. Compensation for mental trauma may also be included, but only if this is incurred as a result of physical injury.

If your ACC claim is not accepted you should immediately contact the researcher. The researcher will initiate processes to ensure you receive compensation equivalent to that to which you would have been entitled had ACC accepted your claim from Massey University.

Will you get any financial compensation?

You will receive a $ 50 voucher at the end of each lab session.

What are your rights?

You are under no obligation to accept this invitation. If you decide to participate, you have the right to:

- Decline to answer any particular question;
- Withdraw from the study (specify timeframe);
- Ask any questions about the study at any time during participation;
- Provide information on the understanding that your name will not be used unless you give permission to the researcher;
- Be given access to a summary of the project findings when it is concluded.

If you are interested in participating please contact Anne Gnauck who will answer your questions.

Anne Gnauck
Institute of Food, Nutrition and Human Health
Massey University
Riddet Road
Palmerston North 4474

Phone 06 350 81386
Mobile 021 1507654
Email A.Gnauck@massey.ac.nz

or

Roger G. Lentle
Institute of Food Nutrition and Human Health
Massey University
Riddet Road
Palmerston North 4474

Phone 06 350 81402
Email R.G.Lentle@massey.ac.nz

This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application 13/31. If you have any concerns about the conduct of this research, please contact Dr Brian Finch, Chair, Massey University Human Ethics Committee: Southern A, telephone 06 350 5799 x 84459, email humanethicsoutha@massey.ac.nz.

Appendix II - iii Health Screening Questionnaire

MASSEY UNIVERSITY
COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

The effect of aspirin on LPS level in blood

HEALTH SCREENING QUESTIONNAIRE

for

First name: _____ Birthday: _____

Surname: _____ age: _____ years

Phone: _____ Email: _____

Address: _____

Body height: ____ m Body weight: ____ kg Body fat: ____ %

A) GENERAL HEALTH

Please answer the following questions with a tick or cross in the YES or NO box.

	YES	NO
Do you suffer from heartburn, indigestion or have a history of any other digestive disease other than appendicitis?		
Do you suffer from any endocrinological disease e.g. diabetes (NIDDM/IDDM), persistent sugar in the urine or thyroid disease like goitre or have hormonal trouble?		
Do you have any renal (kidney) disorders e.g. renal stones, proteinuria (protein in urine) or suffer from uraemia or recurrent UTI's?		
Do you have any hepatic (liver) disorders?		
Have you had any major abdominal surgery in the last 3 months?		
Do you suffer from impaired immunity or have had any immunization in the last 3 months?		
Have you had a gut infection in the last 3 months?		
Do you have any bleeding from the bowel or any blood in the urine?		
Have you had any chronic or recent constipation (in the last 3 months)?		
Have you had diarrhoea in the last 1 month?		
Do you have nausea, vomiting?		
Have you had mucus in stools?		
Have you had any UTI's in the last 3 months?		
Are your periods regular? Please provide details e.g. the number of days between cycles (we need this information so as to schedule your visits to the lab on the days you are not menstruating) _____ _____		
Are you pregnant or lactating?		

B) **<u>BOD POP</u> – during the health screening you will be asked for measuring your body composition using BOD POD**

Do you have any inconvenience in siting in the closed BOD POD, claustrophobia or embarrassment wearing a lycra suit? Y / N

_____

_____

C) **<u>TAKING BLOOD SAMPLE</u> – during each experimental session you will be asked for 3 blood samples**

Do you have any inconvenience with taking blood samples or any aversion of injections? Y / N

_____

_____

Do you have difficult veins? Y / N

_____

_____

Do you have any history of significant pain with venipuncture? Y / N

_____

_____

D) **<u>URINATION</u> – during each experimental session you will be asked to provide a specimen of urine every hour (you are assured privacy for the same)**

Do you have any difficulty in urinating that would prevent you from providing the required specimen? Y /N

_____

_____

E) **<u>VAGINAL DISCHARGE</u> – Vaginal discharge during the experiment will interfere with the test.**

Do you have any current condition that causes you to have an abnormal vaginal discharge? (asked as this will interfere with the test) Provision for paper towels for use by you prior to you providing the specimen will be made. Y / N

_____

_____

F) **ASPIRIN SENSITIVITY - as part of this study you will be asked to take a single dose of 600mg soluble aspirin.**

Are you allergic to aspirin, unable to take aspirin or have a tendency to develop any problems after having taken it (if so please give details)? Y / N

_____

_____

Are you currently taking any medications that interfere with aspirin, notably any Anti-coagulants? Y / N

_____

Do you take aspirin regularly or any other NSAIDs e.g. ibuprofen (Neurofen), indomethacin, naproxen, diclofenac (Voltaren), Ponstan? Y / N

_____

G) **LIFESTYLE HABITS**

Do you smoke (cigarettes, pipe tobacco, or any other herbs)? Y / N

How many drinks containing alcohol do you consume a day/ if a weekend drinker how many drinks do you take? Please state the kind of drink and amount e.g. 3 cans/pints of beer (330ml), one glass of wine etc.

_____

_____

Have you ever been told you have a high alcohol consumption rate? Y / N

_____

H) **SUPPLEMENTION**

Do you take any of the following supplements: Please answer the following questions with a tick or cross in the YES or NO box.

	YES	NO
Vitamin supplements		
Mineral supplements		
Iron supplements		
Other health foods		
Extra calcium		
Pills for anaemia		

I) MEDICATION

Do you take any of the following medications:

Pills for gastric ulcer or stomach disorder	
Blood thinning pills e.g. warfarin or aspirin	
Probiotic or prebiotic supplements e.g. lactulose, fibre supplements	
Immuno-suppressants or steroids (oral, spray or cream)	
Antibiotics (within the last month)	
Pain killers	
Vaginally administered prescription or preparations	

Please list any other medications or supplements you are currently taking:

_____

_____

_____

J) DIETARY REQUIREMENTS

Are you allergic to any foods? ** Y / N

_____

_____

_____

Do you have special dietary requirements? ** Y/N

_____

_____

_____

** At the end of each experimental session you will be provided with a meal.

Appendix II - iv Participant Consent Form

MASSEY UNIVERSITY
COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

The effect of aspirin on LPS/ endotoxin level in blood

PARTICIPANT CONSENT FORM

I have read the Information Sheet and have had the details of the study explained to me. My questions have been answered to my satisfaction, and I understand that I may ask further questions at any time.

I agree to participate in this study under the conditions set out in the Information Sheet.

Signature: _____ **Date:** _____

Full Name - printed _____

Appendix II - v Confidentiality Agreement

MASSEY UNIVERSITY
COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

The effect of aspirin on LPS/ endotoxin level in blood

CONFIDENTIALITY AGREEMENT

- to be completed by the person taking blood samples -

I ..... (Full Name - printed)

agree to keep confidential all information concerning the project '*The effect of aspirin on LPS/ endotoxin level in blood*'.

I will not retain or copy any information involving the project.

Signature: _____ **Date:** _____

APPENDIX III DOCUMENTS – INTRALIPID STUDY

Appendix III - i MUHEC: Southern A – Approval Application 14/11

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA

20 March 2014

Anne Gnauck
IFNHH
PN452

Dear Anne

Re: HEC: Southern A Application – 14/11
Do high fat drinks influence the permeability of the gut and the passage of bacterial products into the body?

Thank you for your letter dated 19 March 2014.

On behalf of the Massey University Human Ethics Committee: Southern A I am pleased to advise you that the ethics of your application are now approved. Approval is for three years. If this project has not been completed within three years from the date of this letter, reapproval must be requested.

If the nature, content, location, procedures or personnel of your approved application change, please advise the Secretary of the Committee.

Yours sincerely

B Finch.

Dr Brian Finch, Chair
Massey University Human Ethics Committee: Southern A

cc Prof Roger Lentle
IFNHH
PN452

Prof Richard Archer, HoI
IFNHH
PN452

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA

13 May 2014

Anne Gnauck
IFNHH
PN452

Dear Anne

Re: HEC: Southern A Application – 14/11
Do high fat drinks influence the permeability of the gut and the passage of bacterial products into the body?

Thank you for your letter dated 12 May 2014 outlining the change you wish to make to the above application.

- Amendment approved for the alteration in the timing of the administration of lactulose-mannitol solution.

If the nature, content, location, procedures or personnel of your approved application change, please advise the Secretary of the Committee. If over time, more than one request to change the application is received, the Chair may request a new application.

Yours sincerely

Dr Brian Finch, Chair
Massey University Human Ethics Committee: Southern A

cc Prof Roger Lentle
IFNHH
PN452

Prof Richard Archer, HoI
IFNHH
PN452

Appendix III - ii Information Sheet – Control group

MASSEY UNIVERSITY
COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

Do high fat drinks influence the permeability of the gut and the passage of bacterial products into the body?

I would like to invite you to participate in my project. Below are important information to tell you more about my research. Thank you for your interest.

INFORMATION SHEET
- Control group -

Who am I?

I am Anne Gnauck, undertaking research in the Gut Physiology group for my PhD. I am studying the impact of diet on gut permeability and the entry of lipopolysaccharide (LPS) / endotoxin into the bloodstream in human subjects. Our work group is part of the Institute of Food, Nutrition and Human Health at Massey University.

Why am I doing this project?

Our work group is studying the effect of diet on the absorption of gut bacteria and their products from the small intestine into the bloodstream. Bugs that live in your gut constantly release bacterial components such as lipopolysaccharides (LPS)/ endotoxin. This LPS/endotoxin can activate your immune system and can cause sickness. It seems that diet can influence the uptake of LPS/ endotoxin from the gut into the bloodstream.

My current project focuses on the effect of a high fat drink on gut permeability and the uptake of LPS/ endotoxin from the gut into the bloodstream in healthy subjects (control group) and patients diagnosed with Crohn's disease (Crohn's disease group). Persons with Crohn's disease have chronic inflammation in the wall of their small intestine therefore it is possible that the absorption of LPS/endotoxin from the gut into the bloodstream is increased.

We will measure the permeability of your gut using simple ingestible sugars that are excreted in your urine after you consume either a drink of water or a high fat drink. We will also determine the concentration of bacterial LPS/ endotoxin in your blood over a period of five hours. In addition, we will measure the level of the acute phase protein LPS-binding protein (LBP) in your blood to assess the immune response of your body to the LPS/ endotoxin exposure.

Would you like to participate?

I would like to invite women aged between 20 and 40 years with a body height between 160 to 170 cm and a body mass between 55 and 75 kg.

To be suitable to join my project you should NOT:

- Have a history of allergic reactions to soya oil, egg, peanuts and sweeteners like acesulfame K and aspartame.
- Have a history of inability to digest fat
- Have dietary excesses or particularities e.g. vegetarian, food allergies
- Be a smoker or tobacco chewer

- Be pregnant or lactating
- Take vitamin/ mineral supplements
- Take non-steroidal anti-inflammatory drugs
- Have a history of diabetes, kidney disorder, cancer, Crohn's disease, ulcerative colitis or irritable bowel disease
- Have a recent history of abdominal pain, nausea, vomiting, diarrhea, passage of blood and mucus in your bowel movement
- Have an aversion to venipuncture, difficult veins, a history of bleeding disorder or be on anticoagulant therapy

If you are interested in joining the study you will be asked to come for the health screening during office hours to the Human Nutrition Lab on the main campus in Palmerston North. The health assessment will take about 30 min to complete and includes questions about your medical history and your dietary habits. If you pass the health check we will also measure your body height and body mass.

I will orally inform you on the day of the health screening session if you are accepted as participant for the study. Appointments for the experimental session will be agreed per email.

What you have to do to join the project?

If you chose to participate you will be asked to attend the Human Nutrition Lab on the main campus in Palmerston North at 8 am for two sessions one week apart. Each experimental session will take 5 to 5.5 hours during which you will be expected to remain in the laboratory (videos, magazines and access to the internet will be available). You will be asked to record all food and drinks you consume on the day before each experimental session. You will be also asked not to consume any alcoholic drinks 24 hours prior each experimental session and not to do any exercise on the day prior the experimental session and on the morning of the experimental session. You will be asked not to eat anything after 8 pm and not to drink anymore after 10 pm on the night before the experimental session. You will be also asked to refrain from urinating after 6 am on the day of the experimental session. You will be asked not to eat any food during the study period but a meal will be provided at the end of each experimental session. You will receive either a control drink (water) or a high fat drink containing around 50 g fat (467 kcal or 1950 kJ) and some sugar solution (lactulose and mannitol) and water at each experimental session.

The following is scheduled for both experimental sessions:

8 am *(0 h)*	• You will be asked to empty your bladder in the urine collection container provided *(you will do this in a toilet in our facility)*. • An intravenous cannula will be inserted into the vein of your non-dominant arm (normally left) and a blood sample of 12 ml will be taken. • You will then receive either a drink of flavoured water (250 ml) or a flavoured drink of intralipid® (250 ml), a fat solution based on soya oil, what you have to drink immediately.
10:00 am *(+ 2 h)*	• A blood sample of 12 ml will be taken. • In connection, you will receive a further 100 ml solution which contains 10 g lactulose and 5 g mannitol followed immediately by 300 ml of water.
11:00 am *(+ 3 h)*	• Another blood sample of 12 ml will be taken.

12:00 pm *(+ 4 h)*	• A further blood sample of 12 ml will be taken.
1:00 pm *(+ 5 h)*	• The last blood sample of 12 ml will be taken and the intravenous cannula will be removed. • You will be asked to empty your bladder in the urine collection container provided *(you will do this in a toilet in our facility).* • You will receive a meal.

Note: If you have to urinate in between the urine collection points, an additional urine collection container will be provided.

Some question you may want to answer:

- ***Is taking blood samples through an intravenous cannula harmful or painful?***

The cannula inserted into the vein in your arm and could cause slight discomfort.

Note: If you have a history of difficult veins or aversion to venipuncture you will not be able to participate in the project.

- ***Does the consumption of the high fat drink have any side effects?***

The consumption of the high fat drink is harmless but this amount of fat may cause stomach and gut pain as well as fatty stool.

- ***What we are going to measure in your urine and blood?***

We will measure the amount of lactulose and mannitol excretion in your urine using High-performance liquid chromatography (HPLC) to assess the permeability of your gut. We will measure your blood lipids. We will also measure the bacterial LPS/endotoxin concentration and the concentration of the acute phase protein LBP in your blood using a LPS-specific enzyme-linked immunosorbent assay (ELISA), the Limulus amebocyte lysate-test and a LBP-specific ELISA, respectively.

- ***Who will see the information about you?***

When you join the trial you will be given a unique personal identifier code and thereafter all information will be filed with this. The list with the codes will be stored in a locked filing cabinet accessed by the research team only. You will receive a summary of anonymised results of the study. The outcome of the study will be published as peer reviewed article in a scientific journal and/ or be presented to academic societies.

- ***Can I receive a summary of my personal data?***

One hypothesis is that the level of LPS/ endotoxin is correlated with illness were too much fat is consumed i.e. such as obesity. However, this is currently unproven. Thus we will not be providing you with your personal LPS/endotoxin and LBP results as it currently has no value as a health predictor.

- ***Who is funding this project?***

The project is funded by Massey University.

Compensation of Injury

If physical injury results from your participation in this project, you should visit a treatment provider to make a claim to ACC as soon as possible. ACC cover and entitlement are not automatic and your claim will be assessed by ACC in accordance with the Accident Compensation Act 2001. If your claim is accepted, ACC must inform you of your entitlement, and must help you access those entitlements. Entitlements may include, but not be limited to, treatment costs, travel costs for rehabilitation, loss or earning, and/ or lump sum for permanent impairment. Compensation for mental trauma may also be included, but only if this is incurred as a result of physical injury.

If your ACC claim is not accepted you should immediately contact the researcher. The researcher will initiate processes to ensure you receive compensation equivalent to that to which you would have been entitled had ACC accepted your claim from Massey University.

Will you get any financial compensation?

You will receive a $ 100 voucher at the end of each experimental session (a total of $ 200 for the two experimental sessions).

What are your rights?

You are under no obligation to accept this invitation. If you decide to participate, you have the right to:

- Decline to answer any particular question;
- Withdraw from the study at any time;
- Ask any questions about the study at any time during participation;
- Provide information on the understanding that your name will not be used unless you give permission to the researcher;
- Be given access to a summary of the project findings when it is concluded.

If you are interested in participating please contact Anne Gnauck who will answer your questions.

Anne Gnauck
IFNHH
Massey University
Riddet Road
Palmerston North 4474

Phone **06 356 9099 ext. 81386**
Mobile **021 1507654**
Email **A.Gnauck@massey.ac.nz**

or

Roger G. Lentle
IFNHH
Massey University
Riddet Road
Palmerston North 4474

Phone 06 356 9099 ext. 81420
Email R.G.Lentle@massey.ac.nz

This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application 14/11.
If you have any concerns about the conduct of this research, please contact
Dr Brian Finch, Chair, Massey University Human Ethics Committee: Southern A,
telephone 06 350 5799 x 84459, email humanethicsoutha@massey.ac.nz.

Appendix III - iii Information Sheet – Crohn's disease group

MASSEY UNIVERSITY
COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

Do high fat drinks influence the permeability of the gut and the passage of bacterial products into the body?

I would like to invite you to participate in my project. Below are important information to tell you more about my research. Thank you for your interest.

INFORMATION SHEET

- Crohn's disease group -

Who am I?

I am Anne Gnauck, undertaking research in the Gut Physiology group for my PhD. I am studying the impact of diet on gut permeability and the entry of lipopolysaccharide (LPS) / endotoxin into the bloodstream in human subjects. Our work group is part of the Institute of Food, Nutrition and Human Health at Massey University.

Why am I doing this project?

Our work group is studying the effect of diet on the absorption of gut bacteria and their products from the small intestine into the bloodstream. Bugs that live in your gut constantly release bacterial components such as lipopolysaccharides (LPS)/ endotoxin. This LPS/endotoxin can activate your immune system and can cause sickness. It seems that diet can influence the uptake of LPS/ endotoxin from the gut into the bloodstream. It is possible that in persons with a chronically inflamed gut e.g. with Crohn's disease an increased entry of LPS/endotoxin from the gut into the bloodstream takes place.

My current project focuses on the effect of a high fat drink on gut permeability and the uptake of LPS/ endotoxin from the gut into the bloodstream in healthy subjects (control group) and patients diagnosed with Crohn's disease (Crohn's disease group). It is possible that the absorption of LPS/endotoxin from the gut into the bloodstream is increased in patients with CD because of the chronic inflammation of the intestine. We will measure the permeability of your gut using simple ingestible sugars that are excreted in your urine after you consume either a drink of water or a high fat drink. We will also determine the concentration of bacterial LPS/ endotoxin in your blood over a period of six hours. In addition, we will measure the level of the acute phase protein LPS-binding protein (LBP) in your blood to assess the immune response of your body to the LPS/ endotoxin exposure.

Would you like to participate?

I would like to invite women aged between 20 and 40 years who are diagnosed with Crohn's disease at least 6 months ago and are currently in remission state.

To be suitable to join my project you should NOT:

- Have a history of allergic reactions to soya oil, egg, peanuts and sweeteners like acesulfame K and aspartame
- Have a history of inability to digest fat

- Have dietary excesses or particularities e.g. food allergies
- Be a heavy smoker or tobacco chewer
- Be pregnant or lactating
- Take vitamin/ mineral supplements
- Have a history of diabetes, kidney disorder and cancer
- Have an aversion to venipuncture, difficult veins, a history of bleeding disorder or be on anticoagulant therapy

Note: You will be allowed to take your regular medication during the period of study but you will be asked not to take your medications during the experimental session. You can take any medication related to your Crohn's disease in the morning 1 hour prior the experimental session (before 7 am) and after the experimental session at 1 pm.

If you are interested in joining the study you will be asked to come for the health screening during office hours to the Human Nutrition Lab on the main campus in Palmerston North. The health assessment will take about 30 min to complete and includes questions about your medical history and your dietary habits. If you pass the health check we will also measure your body height and body mass.

I will orally inform you on the day of the health screening session if you are accepted as participant for the study. Appointments for the experimental session will be agreed per email.

What you have to do to join the project?

If you chose to participate you will be asked to attend the Human Nutrition Lab on the main campus in Palmerston North at 8 am for two sessions one week apart. Each experimental session will take 5 to 5.5 hours during which you will be expected to remain in the laboratory (videos, magazines and access to the internet will be available). You will be asked to record all food and drinks you consume on the day before each experimental session. You will be also asked not to consume any alcoholic drinks 24 hours prior each experimental session and not to do any exercise on the day prior the experimental session and on the morning of the experimental session. You will be asked not to eat anything after 8 pm and not to drink anymore after 10 pm on the night before the experimental session. You will be also asked to refrain from urinating after 6 am on the day of the experimental session. You will be asked not to eat any food during the study period but a meal will be provided at the end of each experimental session. You will receive either a control drink (water) or a high fat drink containing around 50 g fat (476 kcal or 1950 kJ) and some sugar solution (lactulose and mannitol) and water at each experimental session.

The following is scheduled for both experimental sessions:

8 am *(0 h)*	• You will be asked to empty your bladder in the urine collection container provided *(you will do this in a toilet in our facility).* • An intravenous cannula will be inserted into the vein of your non-dominant arm (normally left) and a blood sample of 12 ml will be taken. • You will then receive either a drink of flavoured water (250 ml) or a flavoured drink of intralipid® (250 ml), a fat solution based on soya oil, what you have to drink immediately.
10:00 am *(+ 2 h)*	• A blood sample of 12 ml will be taken. • In connection, you will receive a further 100 ml solution which contains10 g lactulose and 5 g mannitol followed immediately by 300 ml of water.

11:00 am *(+ 3 h)*	• Another blood sample of 12 ml will be taken.
12:00 pm *(+ 4 h)*	• A further blood sample of 12 ml will be taken.
1:00 pm *(+ 5 h)*	• The last blood sample of 12 ml will be taken and the intravenous cannula will be removed. • You will be asked to empty your bladder in the urine collection container provided *(you will do this in a toilet in our facility).* • You will receive a meal.

Note: If you have to urinate in between the urine collection points, an additional urine collection container will be provided.

Some question you may want to answer:

- ***Is taking blood samples through an intravenous cannula harmful or painful?***

The cannula inserted into the vein in your arm and could cause slight discomfort.

Note: If you have a history of difficult veins or aversion to venipuncture you will not be able to participate in the project.

- ***Does the consumption of the high fat drink have any side effects?***

The consumption of the high fat drink is harmless but this amount of fat may cause stomach and gut pain as well as fatty stool.

- ***What we are going to measure in your urine and blood?***

We will measure the amount of lactulose and mannitol excretion in your urine using High-performance liquid chromatography (HPLC) to assess the permeability of your gut. We will measure your blood lipids. We will also measure the bacterial LPS/endotoxin concentration and the concentration of the acute phase protein LBP in your blood using a LPS-specific enzyme-linked immunosorbent assay (ELISA), the Limulus amebocyte lysate-test and a LBP-specific ELISA, respectively.

- ***Who will see the information about you?***

When you join the trial you will be given a unique personal identifier code and thereafter all information will be filed with this. The list with the codes will be stored in a locked filing cabinet accessed by the research team only. You will receive a summary of anonymised results of the study. The outcome of the study will be published as peer reviewed article in a scientific journal and/ or be presented to academic societies.

- ***Can I receive a summary of my personal data?***

One hypothesis is that the level of LPS/ endotoxin is correlated with illness were too much fat is consumed i.e. such as obesity. However, this is currently unproven. Thus we will not be providing you with your personal LPS/endotoxin and LBP results as it currently has no value as a health predictor.

- ***Who is funding this project?***

The project is funded by Massey University.

Compensation of Injury

If physical injury results from your participation in this project, you should visit a treatment provider to make a claim to ACC as soon as possible. ACC cover and entitlement are not automatic and your claim will be assessed by ACC in accordance with the Accident Compensation Act 2001. If your claim is accepted, ACC must inform you of your entitlement, and must help you access those entitlements. Entitlements may include, but not be limited to, treatment costs, travel costs for rehabilitation, loss or earning, and/ or lump sum for permanent impairment. Compensation for mental trauma may also be included, but only if this is incurred as a result of physical injury.

If your ACC claim is not accepted you should immediately contact the researcher. The researcher will initiate processes to ensure you receive compensation equivalent to that to which you would have been entitled had ACC accepted your claim from Massey University.

Will you get any financial compensation?

You will receive a $ 100 voucher at the end of each experimental session (a total of $ 200 for the two experimental sessions).

What are your rights?

You are under no obligation to accept this invitation. If you decide to participate, you have the right to:

- Decline to answer any particular question;
- Withdraw from the study at any time;
- Ask any questions about the study at any time during participation;
- Provide information on the understanding that your name will not be used unless you give permission to the researcher;
- Be given access to a summary of the project findings when it is concluded.

If you are interested in participating please contact Anne Gnauck who will answer your questions.

Anne Gnauck
IFNHH
Massey University
Riddet Road
Palmerston North 4474

Phone **06 356 9099 ext. 81386**
Mobile **021 1507654**
Email A.Gnauck@massey.ac.nz

or

Roger G. Lentle
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Massey University
Riddet Road
Palmerston North 4474

Phone 06 356 9099 ext. 81420
Email R.G.Lentle@massey.ac.nz

This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application 14/11.
If you have any concerns about the conduct of this research, please contact
Dr Brian Finch, Chair, Massey University Human Ethics Committee: Southern A,
telephone 06 350 5799 x 84459, email humanethicsoutha@massey.ac.nz.

Appendix III - iv Health Screening Questionnaire – Control group

MASSEY UNIVERSITY
COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

Do high fat drinks influence the permeability of the gut and the passage of bacterial products into the body?

HEALTH SCREENING QUESTIONNAIRE

– Control group –

for

First name: _____ Birthday: _____

Surname: _____ age: _____ years

Phone: _____ Email: _____

Address: _____

Body height: ____ m Body weight: ____ kg

A) **GENERAL HEALTH**

Please answer the following questions with a tick or cross in the YES or NO box.

	YES	NO
Do you suffer from heartburn, indigestion or have a history of any other digestive disease other than appendicitis?		
Do you suffer from diabetes, persistent sugar in the urine or thyroid disease like goitre or have hormonal trouble?		
Do you have any kidney disorders e.g. renal stones, proteinuria (protein in urine) or suffer from uremia or recurrent urinary tract infections?		
Do you have any liver disorders?		
Have you had any major abdominal surgery in the last 3 months?		
Have you been told by a health professional i.e. doctor that your immune system is impaired?		
Have you had any immunization in the last 3 months i.e. influenza?		
Have you had a gut infection in the last 3 months?		
Do you have any bleeding from the bowel or any blood in the urine in the last 3 months?		
Have you had any chronic or recent constipation (in the last 3 months)?		
Have you had diarrhoea in the last 1 month?		
Do you currently have nausea, vomiting?		
Have you currently had mucus in stools?		
Have you had any urinary tract infections in the last 3 months?		
Are your periods regular? Please provide details e.g. last day of your last period and the number of days between successive periods *(we need this information as no experimental session will be scheduled on the days of your period)* _____		
Are you pregnant or lactating?		

B) **TAKING BLOOD SAMPLE** – ***An intravenous cannula will be inserted into your vein of the non-dominant arm for each experimental session. The cannula will be removed at the end of each experimental session. A total of 5 blood samples of 12 ml each will be taken at each experimental session.***

Have you any aversion to injections? Y / N

Have you experienced difficulties with the taking of blood samples? Y / N
If so, please give details.

_____

_____

Do you have any history of significant pain with venipuncture? Y / N
If so, please give details.

_____

_____

Do you have any bleeding disorders or are you taking anticoagulant? Y/N
If so, please give details.

_____

_____

C) **URINATION** – ***during each experimental session you will be asked to provide a specimen of urine at three time points (there is a toilet in our facility and you are assured privacy for urinating)***

Do you have any difficulty in urinating that would prevent you from providing the required specimen? Y /N
If so, please give details.

_____

_____

D) **VAGINAL DISCHARGE** – ***Vaginal discharge during the experiment will interfere with the test.***

Do you have any current condition that causes you to have an abnormal vaginal discharge? *(asked as this will interfere with the test)* Provision for paper towels for use by you prior to you providing the specimen will be made. Y / N

_____

_____

E) **ALLERGIES** – ***as part of this study you will be asked to take a drink of intralipid®***

The intralipid® drink contains proteins from soya, egg and peanuts as well as sweeteners like acesulfame K and aspartame. Are you allergic to any of these components? Y / N

Do you have a history of the inability to digest fat? Y /N
If so, please give details.

_____
_____

F) **LIFESTYLE HABITS**

Do you smoke (cigarettes, pipe tobacco, or any other herbs)? Y / N

How many standard drinks containing alcohol do you usually consume on a week day?
Please state the kind of drink and amount e.g. 3 cans/pints of beer (330ml), one glass of wine etc.
_____
_____

How many standard alcoholic drinks to you drink on an average weekend?
Please state the kind of drink and amount e.g. 3 cans/pints of beer (330ml), one glass of wine etc.
_____
_____

Have you ever been told you have an alcohol consumption rate that is on the higher side? Y / N
If so, please give details.
_____
_____

Are you vegetarian or do you follow a restricted diet? Y / N
Please indicate the kind of diet

_____

G) SUPPLEMENTATION

Do you take any of the following supplements?
Please answer the following questions with a tick or cross in the YES or NO box.

	YES	NO
Vitamin supplements		
Mineral supplements		
Iron supplements *i.e. pills against anaemia*		
Extra calcium		
Fish oil		
Other health foods		

H) MEDICATION

Do you take any of the following medication?
Please answer the following questions with a tick or cross in the YES or NO box.

	YES	NO
Pills for gastric ulcer or stomach disorder		
Blood thinning pills e.g. warfarin or aspirin		
Probiotic or prebiotic supplements e.g. lactulose, fibre supplements		
Immuno-suppressants or steroids (oral, spray or cream)		
Antibiotics (within the last month)		
Pain killers		
Vaginally administered prescription or preparations		

Please list any other medications or supplements you are currently taking:

_____

_____

_____

I) DIETARY REQUIREMENTS

Are you allergic to any foods? ** Y / N

_____

_____

Do you have special dietary requirements? ** Y/N

_____

_____

** *At the end of each experimental session you will be provided with a meal.*

Appendix III - v Health Screening Questionnaire – Crohn's disease group

MASSEY UNIVERSITY
COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

Do high fat drinks influence the permeability of the gut and the passage of bacterial products into the body?

HEALTH SCREENING QUESTIONNAIRE

– Crohn's disease group –

for

First name: _____ Birthday: _____

Surname: _____ age: _____ years

Phone: _____ Email: _____

Address: _____

Body height: _____ m Body weight: _____ kg

A) GENERAL HEALTH

Please answer the following questions with a tick or cross in the YES or NO box.

	YES	NO
Do you suffer from heartburn, indigestion or have a history of any other digestive disease other than appendicitis?		
Do you suffer from diabetes, persistent sugar in the urine or thyroid disease like goitre or have hormonal trouble?		
Do you have any kidney disorders e.g. renal stones, proteinuria (protein in urine) or suffer from uremia or recurrent urinary tract infections?		
Do you have any liver disorders?		
Have you been told by a health professional i.e. doctor that your immune system is impaired?		
Have you had any immunization in the last 3 months i.e. influenza?		
Have you had a gut infection in the last 3 months?		
Have you had any chronic or recent constipation (in the last 3 months)?		
Have you had diarrhoea in the last 1 month?		
Do you currently have nausea, vomiting?		
Have you had any urinary tract infections in the last 3 months?		
Are your periods regular? Please provide details e.g. last day of your last period and the number of days between successive periods *(we need this information as no experimental session will be scheduled on the days of your period)* _____		
Are you pregnant or lactating?		

B) **HISTORY OF CROHN'S DISEASE**

Is there a family history of Crohn's disease? Y / N

Please provide information if other family members are diagnosed with Crohn's disease.

_____

When were you diagnosed with Crohn's disease? _____

When did you have the first symptoms? _____

Do you know which region of your gut is affected by your Crohn's disease? Y / N

If so, please give details.

_____

Have you had any operations for your Crohn's disease? Y / N

If so, please give details.

_____

_____

Have you had any other major abdominal surgery in the last 3 months? Y / N

If so, please give details.

_____

_____

Is your Crohn's disease under control? Y / N

Please give details about when was your last relapse and how long was it.

_____

_____

Are you taking any medication for your Crohn's disease at the moment? Y / N

Please provide information about type and dose if you are on medication.

_____

_____

Have you recently had to increase the dosage of any of your drugs? Y /N

If so, please give details.

_____

_____

C) <u>TAKING BLOOD SAMPLES</u> – ***An intravenous cannula will be inserted into your vein of the non-dominant arm for each experimental session. The cannula will be removed at the end of each experimental session. A total of 5 blood samples of 12 ml each will be taken at each experimental session.***

Have you any aversion to injections? Y / N

Have you experienced difficulties with the taking of blood samples? Y / N
If so, please give details.

_____

_____

Do you have any history of significant pain with venipuncture? Y / N
If so, please give details.

_____

_____

Do you have any bleeding disorders or are you taking anticoagulant? Y / N
If so, please give details.

_____

_____

D) <u>URINATION</u> – ***during each experimental session you will be asked to provide a specimen of urine at three time points (there is a toilet in our facility and you are assured privacy for urinating)***

Do you have any difficulty in urinating that would prevent you from providing the required specimen? Y /N
If so, please give details.

_____

_____

E) <u>VAGINAL DISCHARGE</u> – ***Vaginal discharge during the experiment will interfere with the test.***

Do you have any current condition that causes you to have an abnormal vaginal discharge? *(asked as this will interfere with the test)* Provision for paper towels for use by you prior to you providing the specimen will be made. Y / N

_____

_____

F) **ALLERGIES** – ***as part of this study you will be asked to take a drink of intralipid®***

The intralipid® drink contains proteins from soya, egg and peanuts as well as sweeteners like acesulfame K and aspartame. Are you allergic to any of these components? Y / N

Do you have a history of the inability to digest fat? Y /N
If so, please give details.

_____

_____

G) **LIFESTYLE HABITS**

Do you smoke (cigarettes, pipe tobacco, or any other herbs)? Y / N

If so, how many cigarettes do you smoke per day?

_____

If not, did you smoke in the past? Y / N

_____

How many standard drinks containing alcohol do you usually consume on a week day?
Please state the kind of drink and amount e.g. 3 cans/pints of beer (330ml), one glass of wine etc.

_____

_____

How many standard alcoholic drinks to you drink on an average weekend?
Please state the kind of drink and amount e.g. 3 cans/pints of beer (330ml), one glass of wine etc.

_____

_____

Have you ever been told you have an alcohol consumption rate that is on the higher side? Y / N
If so, please give details.

_____

_____

Are you vegetarian or do you follow a restricted diet? Y / N
Please indicate the kind of diet

_____

H) SUPPLEMENTATION

Do you take any of the following supplements?
Please answer the following questions with a tick or cross in the YES or NO box.

	YES	NO
Vitamin supplements		
Mineral supplements		
Iron supplements *i.e. pills against anaemia*		
Extra calcium		
Fish oil		
Other health foods		

I) MEDICATION

Do you take any of the following medications?
Please answer the following questions with a tick or cross in the YES or NO box.

	YES	NO
Pills for gastric ulcer or stomach disorder		
Blood thinning pills e.g. warfarin or aspirin		
Probiotic or prebiotic supplements e.g. lactulose, fibre supplements		
Immuno-suppressants or steroids (oral, spray or cream)		
Antibiotics (within the last month)		
Pain killers		
Vaginally administered prescription or preparations		

Please list any other medications or supplements you are currently taking *(other medications than medications for the treatment of Crohn's disease)*:

_____

_____

_____

J) DIETARY REQUIREMENTS

Are you allergic to any foods? ** Y / N

_____

_____

Do you have special dietary requirements? ** Y/N

_____

_____

** *At the end of each experimental session you will be provided with a meal.*

Appendix III - vi Food Record Protocol

Do high fat drinks influence the permeability of the gut and the passage of bacterial products into the body?

FOOD RECORD PROTOCOL

For

Name: _____

Date: _____

time	Food and drinks consumed	quantity

time	Food and drinks consumed	quantity

Appendix III - vii Participant Consent Form

MASSEY UNIVERSITY
COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

Do high fat drinks influence the permeability of the gut and the passage of bacterial products into the body?

PARTICIPANT CONSENT FORM

I have read the Information Sheet and have had the details of the study explained to me. My questions have been answered to my satisfaction, and I understand that I may ask further questions at any time.

I agree to participate in this study under the conditions set out in the Information Sheet.

Signature: _____ **Date:** _____

Full Name - printed _____

Appendix III - viii Confidentiality Agreement

MASSEY UNIVERSITY
COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

Do high fat drinks influence the permeability of the gut and the passage of bacterial products into the body?

CONFIDENTIALITY AGREEMENT

- to be completed by the person taking blood samples -

I ..... (Full Name - printed)

agree to keep confidential all information concerning the project '*Do high fat drinks influence the permeability of the gut and the passage of bacterial products into the body?*'.

I will not retain or copy any information involving the project.

Signature: _____ **Date:** _____

Te Kunenga ki Pūrehuroa

Institute of Food, Nutrition and Human Health
Private Bag 11222, Palmerston North 4442, New Zealand T 06 356 9099 www.massey.ac.nz

APPENDIX IV DOCUMENTS – CROHN'S DISEASE STUDY

Appendix IV - i MUHEC: Southern A – Approval Application 15/18

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA

22 April 2015

Anne Gnauck
School of Food and Nutrition
PN452

Dear Anne

Re: HEC: Southern A Application – 15/18
Is the permeability of the gut in persons with Crohn's disease accompanied by greater absorption of bacterial endotoxin?

Thank you for your letter dated 22 April 2015.

On behalf of the Massey University Human Ethics Committee: Southern A I am pleased to advise you that the ethics of your application are now approved. Approval is for three years. If this project has not been completed within three years from the date of this letter, reapproval must be requested.

If the nature, content, location, procedures or personnel of your approved application change, please advise the Secretary of the Committee.

Yours sincerely

Mr Jeremy Hubbard, Chair
Massey University Human Ethics Committee: Southern A

cc Prof Roger Lentle
School of Food & Nutrition
PN452

Prof Harjinder Singh, HoS
School of Food & Nutrition
PN452

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA

3 June 2015

Anne Gnauck
School of Food and Nutrition
PN452

Dear Anne

Re: HEC: Southern A Application – 15/18
Is the permeability of the gut in persons with Crohn's disease accompanied by greater absorption of bacterial endotoxin?

Thank you for your letter dated 3 June 2015 outlining the changes you wish to make to the above application.

The changes have been approved and noted, as follows:

- To collect 15ml blood samples and faecal samples to determine C-reactive protein in blood and faecal calprotectin in control group and those with confirmed Crohn's disease.

If the nature, content, location, procedures or personnel of your approved application change, please advise the Secretary of the Committee. If over time, more than one request to change the application is received, the Chair may request a new application.

Yours sincerely

Mr Jeremy Hubbard, Chair
Massey University Human Ethics Committee: Southern A

cc Prof Roger Lentle
School of Food & Nutrition
PN452

Prof Harjinder Singh, HoS
School of Food & Nutrition
PN452

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA

20 July 2015

Anne Gnauck
School of Food and Nutrition
PN452

Dear Anne

Re: HEC: Southern A Application – 15/18
Is the permeability of the gut in persons with Crohn's disease accompanied by greater absorption of bacterial endotoxin?

Thank you for your letter dated 8 July 2015 outlining the changes you wish to make to the above application.

The change, to extend the recruiting area to the Wellington region, has been approved and noted.

If the nature, content, location, procedures or personnel of your approved application change, please advise the Secretary of the Committee. If over time, more than one request to change the application is received, the Chair may request a new application.

Yours sincerely

Mr Jeremy Hubbard, Chair
Massey University Human Ethics Committee: Southern A

cc Prof Roger Lentle
School of Food & Nutrition
PN452

Prof Harjinder Singh, HoS
School of Food & Nutrition
PN452

Appendix IV - ii Information Sheet – Control group

Version 1 For participants from the Palmerston North area

Is the permeability of the gut in persons with Crohn's disease accompanied by greater absorption of bacterial endotoxin?

I would like to invite you to participate in my project. Below are important information to tell you more about my research. Thank you for your interest.

INFORMATION SHEET

- Control group -

Who am I?

I am Anne Gnauck, undertaking research in the Gut Physiology group of Professor Roger G. Lentle for my PhD. I am studying how readily your gut absorbs chemicals e.g. sugars and toxins that are made by the bacteria that are in your intestine. Our work group is part of the School of Food and Nutrition at Massey University.

Why am I doing this project?

Bugs that live in your gut constantly release bacterial components such as endotoxin. The amount of endotoxin from gut bacteria that are absorbed and enter the bloodstream is important as excessive quantities can activate your immune system and can cause sickness. It seems that a reduced barrier function of the gut can allow greater amounts of endotoxin to move from the gut into the bloodstream. People with Crohn's disease have inflammation in the wall of their small intestine which may facilitate the absorption of endotoxin.

My current project focuses on whether people with Crohn's disease have greater levels of endotoxin in their bloodstream.

We will measure the permeability of your gut using two simple ingestible sugars (mannitol and lactulose) that are excreted in your urine after they have been absorbed. We will also determine the concentration of endotoxin in your fasted blood. We will measure the level of the acute phase protein LBP which is a protein that is in your blood and absorbs any endotoxin that gets into the circulation.

Would you like to participate?

I would like to invite women aged between 20 and 60 years.

To be suitable to join my project you should NOT:

- Have dietary excesses or particularities e.g. food allergies
- Drink more than one alcoholic standard drink per day (or seven alcoholic standard drinks per week)
- Be a smoker or tobacco chewer
- Be pregnant or lactating
- Take vitamin/ mineral supplements
- Take non-steroidal anti-inflammatory drugs

- Have a history of diabetes, kidney disorder, cancer, Crohn's disease, ulcerative colitis or irritable bowel disease
- Have a recent history of abdominal pain, nausea, vomiting, diarrhea, passage of blood and mucus in your bowel movement
- Have an aversion to venipuncture (blood taken from the vein of your arm), difficult veins, a history of bleeding disorder or be on anti-coagulant therapy (medication that prevents the clotting of your blood)

If you are interested in joining the study you will be asked to come for the health screening during office hours to the Human Nutrition Lab on the main campus in Palmerston North. The health assessment will take about 30 min to complete and includes questions about your medical history and your dietary habits. If you pass the health check we will also measure your body height and body mass.

I will inform you on the day of the health screening session if you are accepted as participant for the study. Appointments for the experimental session will be agreed per email.

What you have to do to join the project?

If you chose to participate you will be asked to attend the Human Nutrition Lab on the main campus in Palmerston North at 8 am for the experimental session. The experimental session will take 3 to 3.5 hours during which time you will be expected to stay in the laboratory (videos, magazines and access to the internet will be available). You will be asked to provide a fecal sample. You can do this either in a toilet in our facility on the day of experimental session or in your private toilet on the day prior the experimental session. You will be given a fecal collection container when you attend the health screening session. You will be asked not to consume any alcoholic drinks 24 hours prior each experimental session and not to do any exercise on the day prior the experimental session or on the morning of the experimental session. You will be asked not to eat anything after 8 pm and not to drink anymore after 10 pm on the night before the experimental session. You will be also asked to refrain from urinating after 6 am on the day of the experimental session. You will be asked not to eat any food during the study period but a meal will be provided at the end of each experimental session. You will be given a sugar solution (lactulose and mannitol) to drink and some water to drink during the experimental session. The lactulose-mannitol drink tastes sweet and has no aftertaste.

The following is scheduled for the experimental session:

8 am *(0 h)*	• You will be asked to empty your bladder in the urine collection container provided *(you will do this in a toilet in our facility).* • A blood sample of 15 ml will be taken. • You will receive a 100 ml solution which contains 10 g lactulose and 5 g mannitol followed immediately by 300 ml of water.
9:30 am *(+ 1.5 h)*	• You will be asked to empty your bladder in the urine collection container provided.
11:00 am *(+ 3 h)*	• You will be asked to empty your bladder in the urine collection container provided. • You will receive a meal.

Note: If you have to urinate in between the urine collection points, an additional urine collection container will be provided.

Some question you may want to answer:

- ***Is taking blood samples harmful or painful?***

Blood will be taken from the vein in your arm and could cause slight discomfort on rare occasions.

Note: If you have a history of difficult veins or aversion to venipuncture you will not be able to participate in the project.

- ***What we are going to measure in your urine and blood?***

We will measure the amount of lactulose and mannitol excretion in your urine using High-performance liquid chromatography (HPLC) and use these to assess the permeability of your gut. We will measure the calprotectin concentration in your fecal to assess the degree of inflammation in your gut. We will also measure the bacterial endotoxin concentration, the concentration of the acute phase protein LBP and CRP in your blood which rises when your gut is inflamed.

- ***Who will see the information about you?***

When you join the trial you will be given a unique personal identifier code and thereafter all information will be filed with this. The list with the codes will be stored in a locked filing cabinet accessed by the research team only. You will receive a summary of anonymised results of the study. The outcome of the study will be published article in a scientific journal and/ or be presented to academic societies. Your name or identity will not be disclosed in any of these publications.

- ***Can I receive a summary of my personal data?***

Should you have abnormal values of fecal calprotectin and of CRP in blood we will inform you and will provide a copy of your results to give to your doctor. It is possible that the level of endotoxin and LBP in your blood increases when you are ill e.g. with active Crohn's disease. However, this is currently unproven. Thus we will not be providing you with your personal results as it currently has no value as a health predictor.

- ***Who is funding this project?***

The project is funded by Massey University.

Compensation of Injury

If physical injury results from your participation in this project, you should visit a treatment provider to make a claim to ACC as soon as possible. ACC cover and entitlement are not automatic and your claim will be assessed by ACC in accordance with the Accident Compensation Act 2001. If your claim is accepted, ACC must inform you of your entitlement, and must help you access those entitlements. Entitlements may include, but not be limited to, treatment costs, travel costs for rehabilitation, loss or earning, and/ or lump sum for permanent impairment. Compensation for mental trauma may also be included, but only if this is incurred as a result of physical injury.

If your ACC claim is not accepted you should immediately contact the researcher. The researcher will initiate processes to ensure you receive compensation equivalent to that to which you would have been entitled had ACC accepted your claim from Massey University.

Will you get any financial compensation?

You will receive a $50 voucher at the end of the experimental session.

What are your rights?

You are under no obligation to accept this invitation. If you decide to participate, you have the right to:

- Decline to answer any particular question;
- Withdraw from the study at any time;
- Ask any questions about the study at any time during participation;
- Provide information on the understanding that your name will not be used unless you give permission to the researcher;
- Be given access to a summary of the project findings when it is concluded.

If you are interested in participating please contact Anne Gnauck who will answer your questions.

Anne Gnauck
School of Food and Nutrition
Massey University
Riddet Road
Palmerston North 4474

Phone **06 356 9099 extn. 84537**
Mobile **021 1507654**
Email A.Gnauck@massey.ac.nz

or

Roger G. Lentle
School of Food and Nutrition
Massey University
Riddet Road
Palmerston North 4474

Phone 06 356 9099 extn. 81420
Email R.G.Lentle@massey.ac.nz

This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application 15/18.
If you have any concerns about the conduct of this research, please contact
Mr Jeremy Hubbard, Acting Chair, Massey University Human Ethics Committee: Southern A,
telephone 04 801 5799 x 63487, email humanethicsoutha@massey.ac.nz.

Version 2 For participants from the Wellington area

MASSEY UNIVERSITY
COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

Is the permeability of the gut in persons with Crohn's disease accompanied by greater absorption of bacterial endotoxin?

I would like to invite you to participate in my project. Below are important information to tell you more about my research. Thank you for your interest.

INFORMATION SHEET

- Control group -

Who am I?

I am Anne Gnauck, undertaking research in the Gut Physiology group of Professor Roger G. Lentle for my PhD. I am studying how readily your gut absorbs chemicals e.g. sugars and toxins that are made by the bacteria that are in your intestine. Our work group is part of the School of Food and Nutrition at Massey University.

Why am I doing this project?

Bugs that live in your gut constantly release bacterial components such as endotoxin. The amount of endotoxin from gut bacteria that are absorbed and enter the bloodstream is important as excessive quantities can activate your immune system and can cause sickness. It seems that a reduced barrier function of the gut can allow greater amounts of endotoxin to move from the gut into the bloodstream. People with Crohn's disease have inflammation in the wall of their small intestine which may facilitate the absorption of endotoxin.

My current project focuses on whether people with Crohn's disease have greater levels of endotoxin in their bloodstream.

We will measure the permeability of your gut using two simple ingestible sugars (mannitol and lactulose) that are excreted in your urine after they have been absorbed. We will also determine the concentration of endotoxin in your fasted blood. We will measure the level of the acute phase protein LBP which is a protein that is in your blood and absorbs any endotoxin that gets into the circulation.

Would you like to participate?

I would like to invite women aged between 20 and 60 years.

To be suitable to join my project you should NOT:

- Have dietary excesses or particularities e.g. food allergies
- Drink more than one alcoholic standard drink per day (or seven alcoholic standard drinks per week)
- Be a smoker or tobacco chewer
- Be pregnant or lactating
- Take vitamin/ mineral supplements
- Take non-steroidal anti-inflammatory drugs

- Have a history of diabetes, kidney disorder, cancer, Crohn's disease, ulcerative colitis or irritable bowel disease
- Have a recent history of abdominal pain, nausea, vomiting, diarrhea, passage of blood and mucus in your bowel movement
- Have an aversion to venipuncture (blood taken from the vein of your arm), difficult veins, a history of bleeding disorder or be on anti-coagulant therapy (medication that prevents the clotting of your blood)

If you are interested in joining the study you will be asked to come for the health screening during office hours to the Sport and Exercise Lab on the campus in Wellington. The health assessment will take about 30 min to complete and includes questions about your medical history and your dietary habits. If you pass the health check we will also measure your body height and body mass.

I will inform you on the day of the health screening session if you are accepted as participant for the study. Appointments for the experimental session will be agreed per email.

What you have to do to join the project?

If you chose to participate you will be asked to attend the Sport and Exercise Lab on the campus in Wellington at 9 am for the experimental session. The experimental session will take 3 to 3.5 hours during which time you will be expected to stay in the laboratory (videos, magazines and access to the internet will be available). You will be asked to provide a fecal sample. You can do this either in a toilet in our facility on the day of experimental session or in your private toilet on the day prior the experimental session. You will be given a fecal collection container when you attend the health screening session. You will be asked not to consume any alcoholic drinks 24 hours prior each experimental session and not to do any exercise on the day prior the experimental session or on the morning of the experimental session. You will be asked not to eat anything after 8 pm and not to drink anymore after 10 pm on the night before the experimental session. You will be also asked to refrain from urinating after 6 am on the day of the experimental session. You will be asked not to eat any food during the study period but a meal will be provided at the end of each experimental session. You will be given a sugar solution (lactulose and mannitol) to drink and some water to drink during the experimental session. The lactulose-mannitol drink tastes sweet and has no aftertaste.

The following is scheduled for the experimental session:

9 am *(0 h)*	• You will be asked to empty your bladder in the urine collection container provided *(you will do this in a toilet in our facility).* • A blood sample of 15 ml will be taken. • You will receive a 100 ml solution which contains 10 g lactulose and 5 g mannitol followed immediately by 300 ml of water.
10:30 am *(+ 1.5 h)*	• You will be asked to empty your bladder in the urine collection container provided.
12:00 am *(+ 3 h)*	• You will be asked to empty your bladder in the urine collection container provided. • You will receive a meal.

Note: If you have to urinate in between the urine collection points, an additional urine collection container will be provided.

Some question you may want to answer:

- ***Is taking blood samples harmful or painful?***

Blood will be taken from the vein in your arm and could cause slight discomfort on rare occasions.

Note: If you have a history of difficult veins or aversion to venipuncture you will not be able to participate in the project.

- ***What we are going to measure in your urine and blood?***

We will measure the amount of lactulose and mannitol excretion in your urine using High-performance liquid chromatography (HPLC) and use these to assess the permeability of your gut. We will measure the calprotectin concentration in your fecal to assess the degree of inflammation in your gut. We will also measure the bacterial endotoxin concentration, the concentration of the acute phase protein LBP and CRP in your blood which rises when your gut is inflamed.

- ***Who will see the information about you?***

When you join the trial you will be given a unique personal identifier code and thereafter all information will be filed with this. The list with the codes will be stored in a locked filing cabinet accessed by the research team only. You will receive a summary of anonymised results of the study. The outcome of the study will be published article in a scientific journal and/ or be presented to academic societies. Your name or identity will not be disclosed in any of these publications.

- ***Can I receive a summary of my personal data?***

Should you have abnormal values of fecal calprotectin and of CRP in blood we will inform you and will provide a copy of your results to give to your doctor. It is possible that the level of endotoxin and LBP in your blood increases when you are ill e.g. with active Crohn's disease. However, this is currently unproven. Thus we will not be providing you with your personal results as it currently has no value as a health predictor.

- ***Who is funding this project?***

The project is funded by Massey University.

Compensation of Injury

If physical injury results from your participation in this project, you should visit a treatment provider to make a claim to ACC as soon as possible. ACC cover and entitlement are not automatic and your claim will be assessed by ACC in accordance with the Accident Compensation Act 2001. If your claim is accepted, ACC must inform you of your entitlement, and must help you access those entitlements. Entitlements may include, but not be limited to, treatment costs, travel costs for rehabilitation, loss or earning, and/ or lump sum for permanent impairment. Compensation for mental trauma may also be included, but only if this is incurred as a result of physical injury.

If your ACC claim is not accepted you should immediately contact the researcher. The researcher will initiate processes to ensure you receive compensation equivalent to that to which you would have been entitled had ACC accepted your claim from Massey University.

Will you get any financial compensation?

You will receive a $50 voucher at the end of the experimental session.

What are your rights?

You are under no obligation to accept this invitation. If you decide to participate, you have the right to:

- Decline to answer any particular question;
- Withdraw from the study at any time;
- Ask any questions about the study at any time during participation;
- Provide information on the understanding that your name will not be used unless you give permission to the researcher;
- Be given access to a summary of the project findings when it is concluded.

If you are interested in participating please contact Anne Gnauck who will answer your questions.

Anne Gnauck
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This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application 15/18.
If you have any concerns about the conduct of this research, please contact
Mr Jeremy Hubbard, Acting Chair, Massey University Human Ethics Committee: Southern A,
telephone 04 801 5799 x 63487, email humanethicsoutha@massey.ac.nz.

Appendix IV - iii Information Sheet – Crohn's disease group

Version 1 For participants from the Palmerston North area

MASSEY UNIVERSITY
COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

Is the permeability of the gut in persons with Crohn's disease accompanied by greater absorption of bacterial endotoxin?

I would like to invite you to participate in my project. Below are important information to tell you more about my research. Thank you for your interest.

INFORMATION SHEET
- Crohn's disease group -

Who am I?

I am Anne Gnauck, undertaking research in the Gut Physiology group of Professor Roger G. Lentle for my PhD. I am studying how readily your gut absorbs chemicals e.g. sugars and toxins that are made by the bacteria that are in your intestine. Our work group is part of the School of Food and Nutrition at Massey University.

Why am I doing this project?

The amount of endotoxin from gut bacteria that are absorbed and enter the bloodstream is important as excessive quantities can activate your immune system and can cause sickness. It is possible that more endotoxin is absorbed in persons with a chronically inflamed gut e.g. with Crohn's disease.

My current project focuses on whether active or inactive Crohn's disease increase absorption of endotoxin from the gut into the bloodstream.

We will measure the permeability of your gut using two simple ingestible sugars (mannitol and lactulose) that are excreted in your urine after they have been absorbed. We will also determine the concentration of endotoxin in your fasted blood. We will measure the level of acute phase protein LBP which is a protein that is in your blood and absorbs any endotoxin that gets into the circulation.

Would you like to participate?

I would like to invite women aged between 20 and 60 years who are diagnosed with Crohn's disease at least 6 months ago and who are either in remission (having a Harvey-Bradshaw Index (HBI) below 5) or who have a mild to moderate disease (having HBI between 5 to 16).

To be suitable to join my project you should NOT:

- Have dietary excesses or particularities e.g. food allergies
- Drink more than one alcoholic standard drink per day (or seven alcoholic standard drinks per week)
- Be a heavy smoker or tobacco chewer
- Be pregnant or lactating
- Take vitamin/ mineral supplements
- Have a history of diabetes, kidney disorder and cancer

- Have an aversion to venipuncture (blood taken from the vein of your arm), difficult veins, a history of bleeding disorder or be on anticoagulant therapy (medication that prevents the clotting of your blood)

Note: You will be allowed to take your regular medication during the period of study but you will be asked to postpone taking your medications until after the experimental session. You can take any medication related to your Crohn's disease in the morning 1 hour prior the experimental session (before 7 am) and after the experimental session at 11:00 am.

If you are interested in joining the study you will be asked to come for the health screening during office hours to the Human Nutrition Lab on the main campus in Palmerston North. The health assessment will take about 30 min to complete and includes questions about your medical history and your dietary habits. If you pass the health check we will also measure your body height and body mass.

I will inform you on the day of the health screening session if you are accepted as participant for the study. Appointments for the experimental session will be agreed per email.

What you have to do to join the project?

If you chose to participate you will be asked to attend the Human Nutrition Lab on the main campus in Palmerston North at 8 am for the experimental session. The experimental session will take 3 to 3.5 hours during which time you will be expected to stay in the laboratory (videos, magazines and access to the internet will be available). You will be asked to provide a fecal sample. You can do this either in a toilet in our facility on the day of experimental session or in your private toilet on the day prior the experimental session. You will be given a fecal collection container when you attend the health screening session. You will be asked not to consume any alcoholic drinks 24 hours prior each experimental session and not to do any exercise on the day prior the experimental session or on the morning of the experimental session. You will be asked not to eat anything after 8 pm and not to drink anymore after 10 pm on the night before the experimental session. You will be also asked to refrain from urinating after 6 am on the day of the experimental session. You will be asked not to eat any food during the study period but a meal will be provided at the end of each experimental session. You will be given a sugar solution (lactulose and mannitol) to drink and some water to drink during the experimental session. The lactulose-mannitol drink tastes sweet and has no aftertaste.

The following is scheduled for the experimental session:

8 am *(0 h)*	• You will be asked to empty your bladder in the urine collection container provided *(you will do this in a toilet in our facility).* • A blood sample of 15 ml will be taken. • You will receive a 100 ml solution which contains 10 g lactulose and 5 g mannitol followed immediately by 300 ml of water.
9:30 am *(+ 1.5 h)*	• You will be asked to empty your bladder in the urine collection container provided.
11:00 am *(+ 3 h)*	• You will be asked to empty your bladder in the urine collection container provided. • You will receive a meal.

Note: If you have to urinate in between the urine collection points, an additional urine collection container will be provided.

Some question you may want to answer:

- ***Is taking blood samples harmful or painful?***

Blood will be taken from the vein in your arm and could cause slight discomfort on rare occasions.

Note: If you have a history of difficult veins or aversion to venipuncture you will not be able to participate in the project.

- ***What we are going to measure in your urine and blood?***

We will measure the amount of lactulose and mannitol excreted in your urine by High-performance liquid chromatography (HPLC) and use these to assess the permeability of your gut. We will measure the calprotectin concentration in your fecal to assess the degree of inflammation in your gut. We will also measure the bacterial endotoxin concentration, the concentration of the acute phase protein LBP and CRP in your blood which rises when your gut is inflamed.

- ***Who will see the information about you?***

When you join the trial you will be given a unique personal identifier code and thereafter all information will be filed with this. The list with the codes will be stored in a locked filing cabinet accessed by the research team only. You will receive a summary of anonymised results of the study. The outcome of the study will be in a scientific journal and/ or be presented to academic societies. Your name or identity will not be disclosed in any of these publications.

- ***Can I receive a summary of my personal data?***

Should you have abnormal values of fecal calprotectin and of CRP in blood we will inform you and will provide a copy of your results to give to your doctor. It is possible that the level of endotoxin and LBP in your blood increases when you are ill e.g. with active Crohn's disease. However, this is currently unproven. Thus we will not be providing you with your personal results as it currently has no value as a health predictor.

- ***Who is funding this project?***

The project is funded by Massey University.

Compensation of Injury

If physical injury results from your participation in this project, you should visit a treatment provider to make a claim to ACC as soon as possible. ACC cover and entitlement are not automatic and your claim will be assessed by ACC in accordance with the Accident Compensation Act 2001. If your claim is accepted, ACC must inform you of your entitlement, and must help you access those entitlements. Entitlements may include, but not be limited to, treatment costs, travel costs for rehabilitation, loss or earning, and/ or lump sum for permanent impairment. Compensation for mental trauma may also be included, but only if this is incurred as a result of physical injury.

If your ACC claim is not accepted you should immediately contact the researcher. The researcher will initiate processes to ensure you receive compensation equivalent to that to which you would have been entitled had ACC accepted your claim from Massey University.

Will you get any financial compensation?

You will receive a $50 voucher at the end of the experimental session.

What are your rights?

You are under no obligation to accept this invitation. If you decide to participate, you have the right to:

- Decline to answer any particular question;
- Withdraw from the study at any time;
- Ask any questions about the study at any time during participation;
- Provide information on the understanding that your name will not be used unless you give permission to the researcher;
- Be given access to a summary of the project findings when it is concluded.

If you are interested in participating please contact Anne Gnauck who will answer your questions.

Anne Gnauck
School of Food and Nutrition
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Riddet Road
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Roger G. Lentle
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Riddet Road
Palmerston North 4474

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Email R.G.Lentle@massey.ac.nz

This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application 15/18.
If you have any concerns about the conduct of this research, please contact
Mr Jeremy Hubbard, Acting Chair, Massey University Human Ethics Committee: Southern A,
telephone 04 801 5799 x 63487, email humanethicsoutha@massey.ac.nz.

Version 2 For participants from the Wellington area

MASSEY UNIVERSITY
COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

Is the permeability of the gut in persons with Crohn's disease accompanied by greater absorption of bacterial endotoxin?

I would like to invite you to participate in my project. Below are important information to tell you more about my research. Thank you for your interest.

INFORMATION SHEET
- Crohn's disease group -

Who am I?

I am Anne Gnauck, undertaking research in the Gut Physiology group of Professor Roger G. Lentle for my PhD. I am studying how readily your gut absorbs chemicals e.g. sugars and toxins that are made by the bacteria that are in your intestine. Our work group is part of the School of Food and Nutrition at Massey University.

Why am I doing this project?

The amount of endotoxin from gut bacteria that are absorbed and enter the bloodstream is important as excessive quantities can activate your immune system and can cause sickness. It is possible that more endotoxin is absorbed in persons with a chronically inflamed gut e.g. with Crohn's disease.

My current project focuses on whether active or inactive Crohn's disease increase absorption of endotoxin from the gut into the bloodstream.

We will measure the permeability of your gut using two simple ingestible sugars (mannitol and lactulose) that are excreted in your urine after they have been absorbed. We will also determine the concentration of endotoxin in your fasted blood. We will measure the level of acute phase protein LBP which is a protein that is in your blood and absorbs any endotoxin that gets into the circulation.

Would you like to participate?

I would like to invite women aged between 20 and 60 years who are diagnosed with Crohn's disease at least 6 months ago and who are either in remission (having a Harvey-Bradshaw Index (HBI) below 5) or who have a mild to moderate disease (having HBI between 5 to 16).

To be suitable to join my project you should NOT:

- Have dietary excesses or particularities e.g. food allergies
- Drink more than one alcoholic standard drink per day (or seven alcoholic standard drinks per week)
- Be a heavy smoker or tobacco chewer
- Be pregnant or lactating
- Take vitamin/ mineral supplements
- Have a history of diabetes, kidney disorder and cancer

- Have an aversion to venipuncture (blood taken from the vein of your arm), difficult veins, a history of bleeding disorder or be on anticoagulant therapy (medication that prevents the clotting of your blood)

Note: You will be allowed to take your regular medication during the period of study but you will be asked to postpone taking your medications until after the experimental session. You can take any medication related to your Crohn's disease in the morning 1 hour prior the experimental session (before 7 am) and after the experimental session at 11:00 am.

If you are interested in joining the study you will be asked to come for the health screening during office hours to the Sport and Exercise Lab on the campus in Wellington. The health assessment will take about 30 min to complete and includes questions about your medical history and your dietary habits. If you pass the health check we will also measure your body height and body mass.

I will inform you on the day of the health screening session if you are accepted as participant for the study. Appointments for the experimental session will be agreed per email.

What you have to do to join the project?

If you chose to participate you will be asked to attend the Sport and Exercise Lab on the campus in Wellington at 9 am for the experimental session. The experimental session will take 3 to 3.5 hours during which time you will be expected to stay in the laboratory (videos, magazines and access to the internet will be available). You will be asked to provide a fecal sample. You can do this either in a toilet in our facility on the day of experimental session or in your private toilet on the day prior the experimental session. You will be given a fecal collection container when you attend the health screening session. You will be asked not to consume any alcoholic drinks 24 hours prior each experimental session and not to do any exercise on the day prior the experimental session or on the morning of the experimental session. You will be asked not to eat anything after 8 pm and not to drink anymore after 10 pm on the night before the experimental session. You will be also asked to refrain from urinating after 6 am on the day of the experimental session. You will be asked not to eat any food during the study period but a meal will be provided at the end of each experimental session. You will be given a sugar solution (lactulose and mannitol) to drink and some water to drink during the experimental session. The lactulose-mannitol drink tastes sweet and has no aftertaste.

The following is scheduled for the experimental session:

9 am *(0 h)*	• You will be asked to empty your bladder in the urine collection container provided *(you will do this in a toilet in our facility).* • A blood sample of 15 ml will be taken. • You will receive a 100 ml solution which contains 10 g lactulose and 5 g mannitol followed immediately by 300 ml of water.
10:30 am *(+ 1.5 h)*	• You will be asked to empty your bladder in the urine collection container provided.
12:00 am *(+ 3 h)*	• You will be asked to empty your bladder in the urine collection container provided. • You will receive a meal.

Note: If you have to urinate in between the urine collection points, an additional urine collection container will be provided.

Some question you may want to answer:

- ***Is taking blood samples harmful or painful?***

Blood will be taken from the vein in your arm and could cause slight discomfort on rare occasions.

Note: If you have a history of difficult veins or aversion to venipuncture you will not be able to participate in the project.

- ***What we are going to measure in your urine and blood?***

We will measure the amount of lactulose and mannitol excreted in your urine by High-performance liquid chromatography (HPLC) and use these to assess the permeability of your gut. We will measure the calprotectin concentration in your fecal to assess the degree of inflammation in your gut. We will also measure the bacterial endotoxin concentration, the concentration of the acute phase protein LBP and CRP in your blood which rises when your gut is inflamed.

- ***Who will see the information about you?***

When you join the trial you will be given a unique personal identifier code and thereafter all information will be filed with this. The list with the codes will be stored in a locked filing cabinet accessed by the research team only. You will receive a summary of anonymised results of the study. The outcome of the study will be in a scientific journal and/ or be presented to academic societies. Your name or identity will not be disclosed in any of these publications.

- ***Can I receive a summary of my personal data?***

Should you have abnormal values of fecal calprotectin and of CRP in blood we will inform you and will provide a copy of your results to give to your doctor. It is possible that the level of endotoxin and LBP in your blood increases when you are ill e.g. with active Crohn's disease. However, this is currently unproven. Thus we will not be providing you with your personal results as it currently has no value as a health predictor.

- ***Who is funding this project?***

The project is funded by Massey University.

Compensation of Injury

If physical injury results from your participation in this project, you should visit a treatment provider to make a claim to ACC as soon as possible. ACC cover and entitlement are not automatic and your claim will be assessed by ACC in accordance with the Accident Compensation Act 2001. If your claim is accepted, ACC must inform you of your entitlement, and must help you access those entitlements. Entitlements may include, but not be limited to, treatment costs, travel costs for rehabilitation, loss or earning, and/ or lump sum for permanent impairment. Compensation for mental trauma may also be included, but only if this is incurred as a result of physical injury.

If your ACC claim is not accepted you should immediately contact the researcher. The researcher will initiate processes to ensure you receive compensation equivalent to that to which you would have been entitled had ACC accepted your claim from Massey University.

Will you get any financial compensation?

You will receive a $50 voucher at the end of the experimental session.

What are your rights?

You are under no obligation to accept this invitation. If you decide to participate, you have the right to:

- Decline to answer any particular question;
- Withdraw from the study at any time;
- Ask any questions about the study at any time during participation;
- Provide information on the understanding that your name will not be used unless you give permission to the researcher;
- Be given access to a summary of the project findings when it is concluded.

If you are interested in participating please contact Anne Gnauck who will answer your questions.

Anne Gnauck
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This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application 15/18.
If you have any concerns about the conduct of this research, please contact
Mr Jeremy Hubbard, Acting Chair, Massey University Human Ethics Committee: Southern A,
telephone 04 801 5799 x 63487, email humanethicsoutha@massey.ac.nz.

Appendix IV - iv Health Screening Questionnaire - Control group

MASSEY UNIVERSITY
COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

Is the permeability of the gut in persons with Crohn's disease accompanied by greater absorption of bacterial endotoxin?

HEALTH SCREENING QUESTIONNAIRE

– Control group –

for

First name: _____ Birthday: _____

Surname: _____ age: _____ years

Phone: _____ Email: _____

Address: _____

Body height: _____ m Body weight: _____ kg

A) GENERAL HEALTH

Please answer the following questions with a tick or cross in the YES or NO box.

	YES	NO
Do you suffer from heartburn, indigestion or have a history of any other digestive disease other than appendicitis?		
Do you suffer from diabetes, persistent sugar in the urine or thyroid disease like goitre or have hormonal trouble?		
Do you have any kidney disorders e.g. renal stones, proteinuria (protein in urine) or suffer from uremia or recurrent urinary tract infections?		
Do you have any liver disorders?		
Have you had any major abdominal surgery in the last 3 months?		
Have you been told by a health professional i.e. doctor that your immune system is impaired?		
Have you had any immunization in the last 3 months i.e. influenza?		
Have you had a gut infection in the last 3 months?		
Do you have any bleeding from the bowel or any blood in the urine in the last 3 months?		
Have you had any chronic or recent constipation (in the last 3 months)?		
Have you had diarrhoea in the last 1 month?		
Do you currently have nausea, vomiting?		
Have you currently had mucus in stools?		
Have you had any urinary tract infections in the last 3 months?		
Are you still menstruating?		
Are your periods regular? Please provide details e.g. last day of your last period and the number of days between successive periods *(we need this information as no experimental session will be scheduled on the days of your period)* _____		
Are you pregnant or lactating?		

B) **TAKING BLOOD SAMPLE** – ***during the experimental session you will be asked for one 10 ml blood sample.***

Have you any aversion to injections? Y / N

Have you been told you have veins that are difficult to get blood from? Y / N
If so, please give details.

_____

_____

Do you have any bleeding disorders or are you taking anticoagulant? Y/N
If so, please give details.

_____

_____

C) **URINATION** – ***during the experimental session you will be asked to provide a specimen of urine at three time points (there is a clean toilet in our facility and you are assured privacy for urinating)***

Do you have any difficulty in urinating or problems that would prevent you from providing three specimens over a period of 3 hours? Y /N
If so, please give details.

_____

_____

D) **VAGINAL DISCHARGE** – ***Vaginal discharge during the experiment will interfere with the test.***

Do you have any current condition that causes you to have a vaginal discharge? *(asked as this will interfere with the test)* Provision for paper towels for use by you prior to you providing the specimen will be made. Y / N

_____

_____

E) LIFESTYLE HABITS

Do you smoke (cigarettes, pipe tobacco, or any other herbs)? Y / N

How many standard drinks containing alcohol do you usually consume on a week day?*
Please state the kind of drink and amount e.g. 3 cans/pints of beer (330ml), one glass of wine etc.

_____

_____

How many standard alcoholic drinks to you drink on an average weekend?*
Please state the kind of drink and amount e.g. 3 cans/pints of beer (330ml), one glass of wine etc.

_____

_____

Have you ever been told you have an alcohol consumption rate that is on the higher side? Y / N
If so, please give details.

_____

_____

**Note: A high consumption of alcohol can affect the permeability of your gut. Hence, you will be excluded from the study if you consume more than one alcoholic standard per day or more than seven alcoholic standard drinks per week.*

Are you vegetarian or do you follow a restricted diet? Y / N
Please indicate the kind of diet

_____

_____

F) SUPPLEMENTATION

Do you take any of the following supplements?
Please answer the following questions with a tick or cross in the YES or NO box.

	YES	NO
Vitamin supplements		
Mineral supplements		
Iron supplements *i.e. pills for anaemia*		
Extra calcium		
Fish oil		
Other health foods		

G) **MEDICATION**

Do you take any of the following medication?
Please answer the following questions with a tick or cross in the YES or NO box.

	YES	NO
Pills for gastric ulcer or stomach disorder		
Blood thinning pills e.g. warfarin or aspirin		
Probiotic or prebiotic supplements e.g. lactulose, fibre supplements		
Immuno-suppressants or steroids (oral, spray or cream)		
Antibiotics (within the last month)		
Pain killers		
Vaginally administered prescription or preparations		

Please list any other medications or supplements you are currently taking:

_____

_____

_____

H) **DIETARY REQUIREMENTS** – ***At the end of the experimental session you will be provided with a meal.***

Are you allergic to any foods? Y / N
If so, please give details.

_____

_____

Do you have special dietary requirements? Y/N
If so, please give details.

_____

_____

Appendix IV - v Health Screening Questionnaire – Crohn's disease group

MASSEY UNIVERSITY
COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

Is the permeability of the gut in persons with Crohn's disease accompanied by greater absorption of bacterial endotoxin?

HEALTH SCREENING QUESTIONNAIRE

– Crohn's disease group –

for

First name: _____ Birthday: _____

Surname: _____ age: _____ years

Phone: _____ Email: _____

Address: _____

Body height: ____ m Body weight: ____ kg

A) GENERAL HEALTH

Please answer the following questions with a tick or cross in the YES or NO box.

	YES	NO
Do you suffer from heartburn, indigestion or have a history of any other digestive disease other than appendicitis?		
Do you suffer from diabetes, persistent sugar in the urine or thyroid disease like goitre or have hormonal trouble?		
Do you have any kidney disorders e.g. renal stones, proteinuria (protein in urine) or suffer from uremia or recurrent urinary tract infections?		
Do you have any liver disorders?		
Have you been told by a health professional i.e. doctor that your immune system is impaired?		
Have you had any immunization in the last 3 months i.e. influenza?		
Have you had a gut infection in the last 3 months?		
Have you had any chronic or recent constipation (in the last 3 months)?		
Have you had diarrhoea in the last 1 month?		
Do you currently have nausea, vomiting?		
Have you had any urinary tract infections in the last 3 months?		
Are you still menstruating?		
Are your periods regular? Please provide details e.g. last day of your last period and the number of days between successive periods *(we need this information as no experimental session will be scheduled on the days of your period)* _____		
Are you pregnant or lactating?		

B) **HISTORY OF CROHN'S DISEASE**

Do any of your relatives have Crohn's disease? Y / N

If so, please provide information.

_____

_____

When were you diagnosed with Crohn's disease? _____

When did you have the first symptoms? _____

Do you know which regions of your gut are affected by your Crohn's disease? Y / N

If so, please give details.

_____

_____

Do you know any of the medical characteristics of your Crohn's disease ? Y / N

If so, please give details e.g. non-stricturing and non-penetrating, stricturing, penetrating.

_____

_____

Have you had any operations for your Crohn's disease? Y / N

If so, please give details.

_____

_____

Have you had any other major abdominal surgery in the last 3 months? Y / N

If so, please give details.

_____

_____

Is your Crohn's disease currently under control? Y / N

Please give details about when was your last relapse and how long was it.

_____

_____

Are you taking any medication for your Crohn's disease at the moment? Y / N

If so, please list below.

Date started	Name of the medicine	Dose of tablets	Now many times a day/week

C) <u>TAKING BLOOD SAMPLES</u> – ***during the experimental session you will be asked for one 10 ml blood sample.***

Have you any aversion to injections? Y / N

Have you been told you have veins that are difficult to get blood from? Y / N
If so, please give details.

_____

Do you have any bleeding disorders or are you taking anticoagulant? Y / N
If so, please give details.

_____

D) <u>URINATION</u> – ***during the experimental session you will be asked to provide a specimen of urine at three time points (there is a clean toilet in our facility and you are assured privacy for urinating)***

Do you have any difficulty in urinating or problems that would prevent you from providing three specimens over a time period of 3 hours? Y /N
If so, please give details.

_____

E) <u>VAGINAL DISCHARGE</u> – ***Vaginal discharge during the experiment will interfere with the test.***

Do you have any current condition that causes you to have a vaginal discharge? *(asked as this will interfere with the test)* Provision for paper towels for use by you prior to you providing the specimen will be made. Y / N
If so, please give details.

_____

F) **LIFESTYLE HABITS**

Do you smoke (cigarettes, pipe tobacco, or any other herbs)? Y / N

If so, how many cigarettes do you smoke per day?

_____

If not, did you smoke in the past? Y / N

_____

How many standard drinks containing alcohol do you usually consume on a week day?*
Please state the kind of drink and amount e.g. 3 cans/pints of beer (330ml), one glass of wine etc.

_____

_____

How many standard alcoholic drinks to you drink on an average weekend?*
Please state the kind of drink and amount e.g. 3 cans/pints of beer (330ml), one glass of wine etc.

_____

_____

Have you ever been told you have an alcohol consumption rate that is on the higher side?* Y / N
If so, please give details.

_____

_____

**Note: A high consumption of alcohol can affect the permeability of your gut. Hence, you will be excluded from the study if you consume more than one alcoholic standard per day or more than seven alcoholic standard drinks per week.*

Are you vegetarian or do you follow a restricted diet? Y / N
Please indicate the kind of diet

_____

_____

G) SUPPLEMENTATION

Do you take any of the following supplements?
Please answer the following questions with a tick or cross in the YES or NO box.

	YES	NO
Vitamin supplements		
Mineral supplements		
Iron supplements *i.e. pills for anaemia*		
Extra calcium		
Fish oil		
Other health foods		

H) MEDICATION

Do you take any of the following medications?
Please answer the following questions with a tick or cross in the YES or NO box.

	YES	NO
Pills for gastric ulcer or stomach disorder		
Blood thinning pills e.g. warfarin or aspirin		
Probiotic or prebiotic supplements e.g. lactulose, fibre supplements		
Immuno-suppressants or steroids (oral, spray or cream)		
Antibiotics (within the last month)		
Pain killers		
Vaginally administered prescription or preparations		

Please list any other medications or supplements you are currently taking *(other medications than medications for the treatment of Crohn's disease)*:

_____

_____

_____

I) DIETARY REQUIREMENTS – *at the end of the experimental session you will be provided with a meal.*

Are you allergic to any foods? Y / N
If so, please give details.

_____

_____

Do you have special dietary requirements? Y/N
If so, please give details.

_____

_____

Appendix IV - vi Assessment of Disease Activity by Harvey - Bradshaw Index

MASSEY UNIVERSITY
COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

Is the permeability of the gut in persons with Crohn's disease accompanied by greater absorption of bacterial endotoxin?

ASSESSMENT OF DISEASE ACTIVITY

– Crohn's disease group –

for

First name: _____ Birthday: _____

Surname: _____

Date of HBI calculation: _____

HARVEY-BRADSHAW INDEX (HBI)

	Score	participants rating
General well-being (yesterday)	0 = very well 1 = slightly below average 2 = poor 3 = very poor 4 = terrible	
Abdominal pain (yesterday)	0 = none 1 = mild 2 = moderate 3 = serve	
Number of liquid stools per day (yesterday)		
Abdominal mass	0 = none 1 = dubious 2 = definite 3 = tender	
Complications	☐ None ☐ Arthralgia ☐ Uveitis ☐ Erythema nodosum ☐ Aphthous ulcers ☐ *Pyoderma gangrenosum* ☐ Anal fissure ☐ New fistula ☐ Abscess	

HBI score _____

Remission	*< 5*
Mild disease	*5 - 7*
Moderate disease	*8 - 16*
Serve disease	*> 16*

Appendix IV - vii Participant Consent Form

MASSEY UNIVERSITY
COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

Is the permeability of the gut in persons with Crohn's disease accompanied by greater absorption of bacterial endotoxin?

PARTICIPANT CONSENT FORM

I have read the Information Sheet and have had the details of the study explained to me. My questions have been answered to my satisfaction, and I understand that I may ask further questions at any time.

I agree to participate in this study under the conditions set out in the Information Sheet.

Signature: _____ **Date:** _____

Full Name - printed _____

Appendix IV - viii Confidentiality Agreement

MASSEY UNIVERSITY
COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

Is the permeability of the gut in persons with Crohn's disease accompanied by greater absorption of bacterial endotoxin?

CONFIDENTIALITY AGREEMENT

- to be completed by the person taking blood samples -

I ..... (Full Name - printed)

agree to keep confidential all information concerning the project *'Is the permeability of the gut in persons with Crohn's disease accompanied by greater absorption of bacterial endotoxin?*.

I will not retain or copy any information involving the project.

Signature: _____ **Date:** _____

APPENDIX V REVIEW PAPER – LIPOPOLYSACCHARIDES

INTERNATIONAL REVIEWS OF IMMUNOLOGY
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REVIEW

The characteristics and function of bacterial lipopolysaccharides and their endotoxic potential in humans

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ABSTRACT

Cross-talk between enteral microbiota and human host is essential for the development and maintenance of the human gastrointestinal and systemic immune systems. The presence of lipopolysaccharides (LPS) lysed from the cell membrane of Gram-negative bacteria in the gut lumen is thought to promote the development of a balanced gut immune response whilst the entry of the same LPS into systemic circulation may lead to a deleterious pro-inflammatory systemic immune response.

Recent data suggest that chronically low levels of circulating LPS may be associated with the development of metabolic diseases such as insulin resistance, type 2 diabetes, atherosclerosis and cardiovascular disease. This review focuses on the cross-talk between enteral commensal bacteria and the human immune system via LPS. We explain the structural characterisation of the LPS molecule and its function in the bacteria. We then examine how LPS is recognised by various elements of the human immune system and the signalling pathways that are activated by the structure of the LPS molecule and the effect of various concentrations. Further, we discuss the sequelae of this signalling in the gut-associated and systemic immune systems i.e. the neutralisation of LPS and the development of tolerance to LPS.

ARTICLE HISTORY
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KEYWORDS:
Endotoxin; lipopolysaccharides; LPS-induced immune response; LPS recognition

Introduction

The humans intestine is inhabited by 10^{14} individual bacteria, a number that is ten times greater than the total number of cells in the human body. It is estimated that 500 to 1,000 bacterial species, predominately from the phyla Bacteroidetes and Firmicutes, colonize the gastrointestinal tract. Minor species of gut-colonizing bacteria including those from the phylum Proteobacteria notably from the genera Escherichia, Salmonella, Vibrio and Heliobacter, and from the phyla Verrucomicrobia, Actinobacteria (notably the genus Bifidobacterium), Fusobacteria and Cyanobacteria [1]. Under normal physiological conditions humans live in a symbiotic relationship with these bacteria. The human gut-associated lymphoid tissue (GALT) is fine-tuned by "cross-talk" between the host and commensal enteral bacteria promoting tolerance of their presence and their products as well controlling the composition of the mucosal biofilm [1].

Cross-talk between the enteral microbiota and the human host is essential for the development and maintenance of the human gastrointestinal and systemic immune system [1]. The

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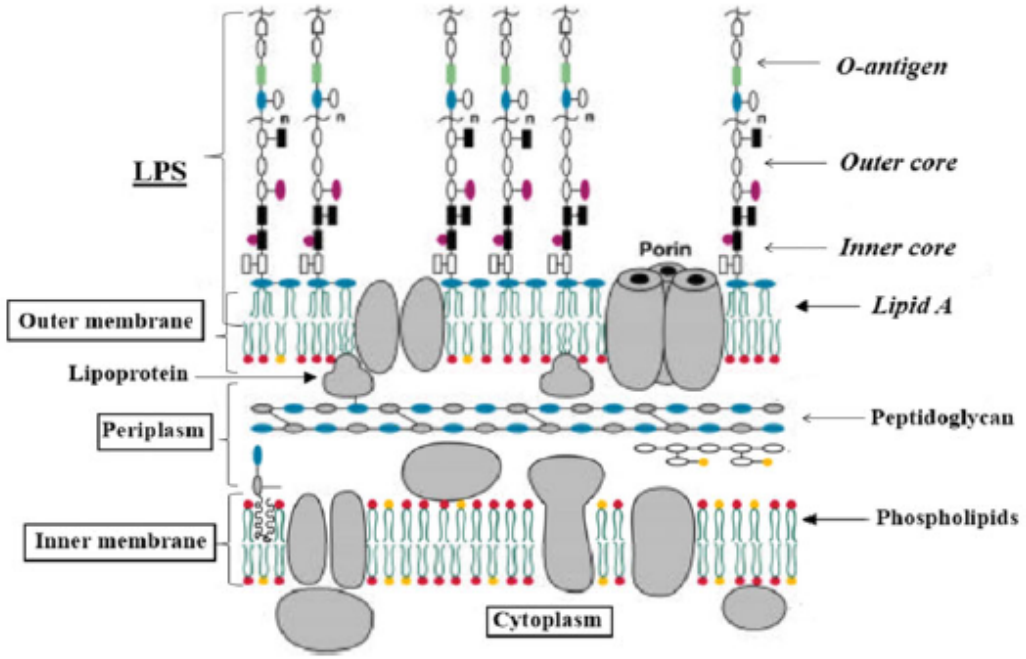

Figure 1. Model of the inner and outer membrane of Gram-negative bacterium *E. coli* K-12. Showing how lipid A anchors the LPS molecule in the outer layer of the outer membrane (modified Figure 1 from [10]).

presence of the bacterial cell membrane component lipopolysaccharide (LPS) from Gram-negative bacteria in the lumen is thought to aid in the development of a balanced immune system as it may influence the maturation of regulatory T cells in the GALT [2] and facilitate the destruction of invading organisms. Conversely, high concentrations of LPS in the systemic circulation can lead to septic shock which is clearly deleterious to the host [3]. Further, recent data suggest that persistent low levels of circulating LPS may be associated with the development of metabolic diseases such as insulin resistance, type 2 diabetes, atherosclerosis and cardiovascular disease [4–6]. Together these findings indicate that both the location and the concentration of LPS determine whether the subsequent immune response is beneficial or harmful to the host.

The structure of the outer membrane of gram-negative bacteria

The cellular structure of Gram-negative bacteria such as species of the genus Bacteroides, Escherichia and Salmonella differs from that of Gram-positive bacteria such as Lactobacilli and Clostridia [1]. In addition to the (inner) cytoplasmic membrane and the peptidoglycan layer, Gram-negative bacteria possess a secondary outer membrane (Figure 1). Again, Gram-negative bacteria have a significantly thinner peptidoglycan layer than do Gram-positive bacteria. The inner membrane of Gram-negative bacteria is, like most biological membranes, a symmetric phospholipid bilayer. Conversely the outer membrane is inherently asymmetric comprising an inner phospholipid component and an outer lipopolysaccharide (LPS) component. Hence, the outer membrane differs both chemically and structurally from that of other cell membranes [7].

The outer layer of the outer membrane of *Escherichia coli* and that of other enteric bacteria contains around 10^6 molecules of LPS that comprise three quarters of the surface of the bacterial cell. Hence, LPS constitutes the bulk of the outer layer of these organisms [8]. The LPS

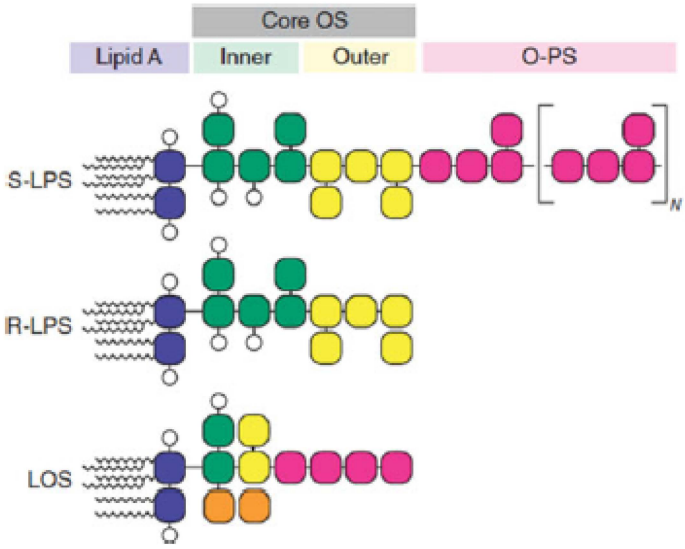

Figure 2. Schematic overview of the structural components of lipopolysaccharide (LPS) and lipooligosaccharide (LOS). Smooth-LPS (S-LPS) consists of lipid A, a core oligosaccharide (OS) and an O-polysaccharide (O-PS)/ O-antigen. Rough-LPS (R-LPS) lacks O-PS and in some cases also parts of the core OS. LOS lacks O-PS but has a oligosaccharide extension attached to its core OS (Figure 1 from [8]).

component is thought to regulate the level of permeability of the outer membrane in response to changes in the environment and thus enables Gram-negative bacteria to survive in a diverse range of conditions and to cope with a range of chemical and physical stressors that include antimicrobial molecules, changes in temperature, pH and in oxygen concentration [7].

Some Bacteroides species i.e. *Bacteroides fragilis* are able to express zwitterionic polysaccharide moieties such as polysaccharide A (PSA) in the outer layers of their outer membranes and form an outer capsule [9].

Characterisation of lipopolysaccharide (LPS)

The LPS molecule consists of lipid A, a core oligosaccharide (OS) and an O-antigen/ O-polysaccharide (O-PS) [10]. The lipid A component is linked to OS in the inner core and serves to anchor the LPS molecule within the outer layer. The OS of the outer core binds to O-antigen (Figure 1 and Figure 2) [10].

LPS molecules are classified as smooth(S)- or rough(R)-LPS according to the presence and characteristics of the core OS and the O-antigen. LPS molecules in species of Enterobacteriaceae, Pseudomonadaceae, Pasteurellaceae, and Vibrionaceae contain all three structural components [10] and are called S-LPS [8]. Bacteria with defects in the mechanism by which the O-antigen or the core OS are assembled, produce truncated LPS molecules, termed R-LPS (Figure 2). LPS molecules extracted from S-LPS producing bacteria are heterogeneous with respect to their O-antigen chain length and relative proportion of R-LPS.

A broad range of pathogenic Gram-negative bacteria including mucosal pathogens, lack the O-antigen component but still possess oligosaccharide extensions to their inner core OS forming structures that are termed lipooligosaccharides (LOS) (Figure 2) [8]. LPS molecules from a range of Gram-negative bacteria contain similar lipid A and inner core OS components but show a wide variation in the outer core OS and in the O-antigen components [8].

Lipid A. Lipid A, a glucosamine-based phospholipid, anchors the LPS molecule within the hydrophobic outer layer of the outer cell membrane in most Gram-negative bacteria species

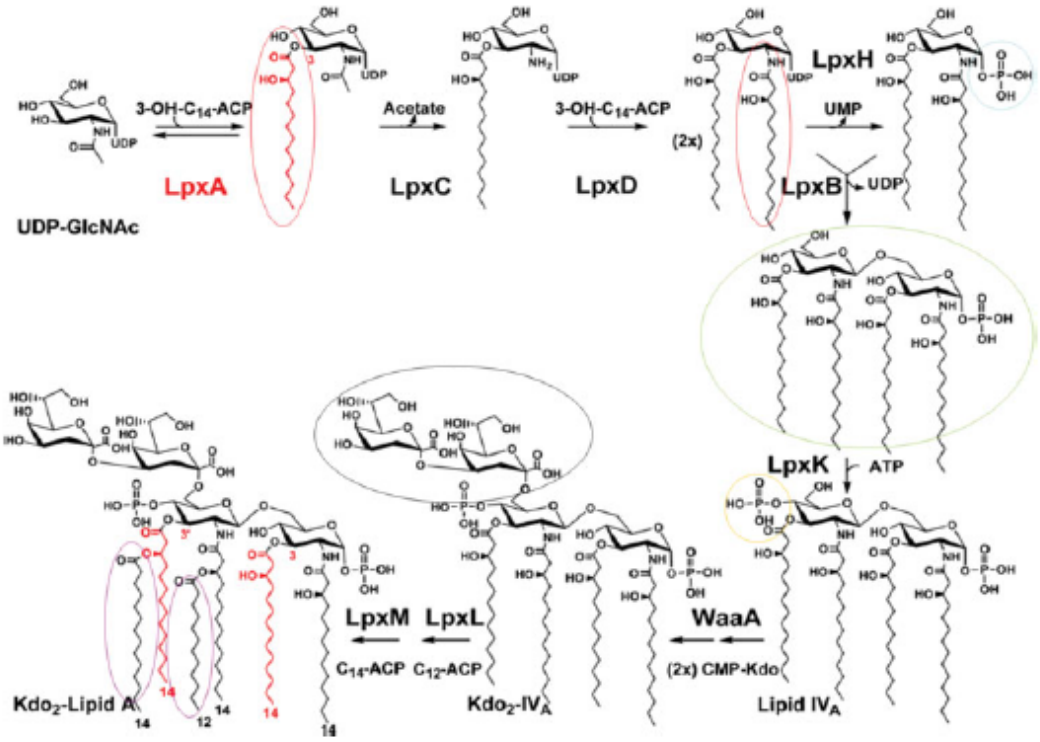

Figure 3. Structure and biosynthesis of Kdo2-lipid A in *E. coli* K-12. During the initial stage of synthesis, two fatty acids are added to UDP-GlcNAc (red circles) leading to the formation of UDP-2,3-diacyl-GlcN which then is dephosphorylated at position 1 (blue circle). The resulting UMP-2,3-diacyl-GlcN is then linked to another UDP-2,3-diacyl-GlcN so that a molecule with 4 primary acyl chains and one phosphate group is formed (green circle). A second phosphate group is then added at position 4′ (orange circle) to form the precursor lipid A termed lipid IV_A. Lipid IV_A is subsequently linked with two units of the saccharide KDO at position 5′ (black circle) to form the basic tetra-acylated lipid A –KDO complex. During modification of lipid A subsequent up to two secondary fatty acids can be linked to the primary fatty acids at position R3′ and R2′ (pink circle) to form penta- or hexa-acylated lipid A (modified Figure 2 from [10]).

(Figure 1) [10]. Lipid A is initially synthesized in the cytoplasm and at the interface between the cytoplasm and the inner layer of the (inner) cytoplasmic membrane [7]. Biosynthesis of the LPS molecules commences with the linkage of a precursor lipid A with four acyl chains, termed lipid IV_A, to two units of the saccharide KDO (see Figure 3 for more details). Further saccharide units are added to this basic structure to form the lipid A-OS complex [10]. The lipid A-OS complex is then transported to the outer layer of the cytoplasmic membrane [11] where it is linked to the O-antigen [7]. In general, one lipid A –core OS unit anchors one O-antigen [8]. Additional species-specific lipid A modifications can occur in the outer layer of the cytoplasmic membrane or in the inner layer of the outer membrane before the molecule is finally transported to the outer layer of the outer membrane [11]. The formation of the KDO - lipid A complex is required for bacterial growth.

Core oligosaccharide. The biosynthesis of oligosaccharide residues takes place in the cytoplasm. The core oligosaccharide (OS) of S-LPS consist of two segments—the inner and the outer core OS. Most Gram-negative bacteria form an inner core composed of 3-Deoxy-D-manno-oct-2-ulosonic acid (KDO) and l-glycero-d-manno-heptose residues that are linked at position 5′ of lipid A. The outer core forms the attachment site to the O-antigen [7] and consists mainly of hexose units but there are considerable variations in the component saccharides.

O-antigen (O-polysaccharide). The O-antigen and the O-polysaccharide (O-PS), respectively, are constructed of two to six saccharide units [7]. The simplest O-antigens consist of repeating disaccharide units in which the component monosaccharide units are linked by single glycosidic linkages. More complex O-antigens consist, either of larger homopolysaccharide repeats that are linked by a specific sequence of different glycosidic linkages, or of heteropolysaccharide units interspaced with saccharide and nonsaccharide moieties such as CO-acetyl groups and amino acids [8]. Hence, variations in constituent saccharide units, in the type of linkage between saccharide units and the complete or partial substitution of saccharide units by nonsugar moieties, result in a diverse range of O-antigens.

Biosynthesis of the repeating oligosaccharide units of the O-antigen precursors occurs in the inner layer of the cytoplasmic membrane. The completed O-antigen precursors are subsequently transported to the outer layer of the cytoplasmic membrane where they are polymerized to form an O-antigen. Concomitantly, the O-antigen is linked to the lipid A- OS complex (see above) in the interface between the cytoplasmic membrane and the periplasm. The mature LPS molecule is then transported into the newly assembled outer membrane via a series of cassette transporters [7].

The function of the lipopolysaccharide in gram-negative bacteria

The structural properties of the LPS molecule and its high concentration in the outer layer of the outer membrane reflect its function in maintaining the integrity of the cell membrane and in mediating interactions of the organism with its environment [8].

The barrier function

The inner part of the LPS molecule, containing lipid A and the inner core OS, is responsible for maintaining the barrier function of the outer cell membrane [8]. The LPS molecules are predominantly bound together by electrostatic interactions between phosphate groups of the component lipid A moieties and magnesium ions located in the outer membrane. Further, in the hydrophilic region of LPS, the core OS, there is hydrogen-bonding between the sugars of the core OS in adjacent LPS molecules, whilst the hydrophobic regions, the lipid A moiety within LPS molecules, interact hydrophobically with those in adjacent LPS molecules. These various interactions generate a compact arrangement of LPS molecules within the outer layer of the outer cell membrane [7].

A repertoire of structurally different lipid A's is required to enable the integrity of the membrane of the bacteria cell to be maintained under differing growth conditions [8]. The modification of lipid A by incorporation of suitable secondary acyl chains allows bacteria to "adapt" to such environmental variations as change in pH and temperature and the presence of antimicrobial peptides [11]. The acylation pattern is species-specific and varies with respect to both the numbers of acyl chains and the length (and degree of saturation) of fatty acids [12]. Thus, for example the lipid A of LPS from *Escherichia coli* has a hexa-acylated subunit bearing two phosphate groups and most commensal intestinal bacteria are able to produce and incorporate 3-hydroxymyrisate or 3-hydroxylaureate as primary acyl chains with laureate, myristate or both as secondary acyl chains in order to enhance their resistance to antimicrobial peptides produced by the host [12]. The presence of the secondary acyl chains renders the outer membrane of *E. coli* impermeable to bile acids enabling the organism to colonize the gut. In contrast, species of Gram-negative bacteria that can live in water such as *Pseudomonas aeruginosa* mainly synthesize lipid A with a single secondary acyl chain (five acyl chains in total). Hence their outer membrane is more permeable to bile acid and to host antimicrobial peptides

so that they are unable to survive in the lumen of the gut [12]. A number of pathogens such as *Yersinia pestis* are able to alter the acylation patterns of their lipid A moieties in response to temperature changes so as to maintain the membrane integrity whilst they colonize the host [13]. Hence, the ability to modify their lipid A moieties allows Gram-negative bacteria to survive under a range of different conditions [10].

The protection of the organism

The outer part of LPS, containing the outer core OS and O-antigen, varies greatly in structure [8]. The O-antigen extends outwards from the cell surface to form a protective shield [10] so that its glycan chain can influence the extent of the surrounding unstirred layer and thus limit the access of molecules in the general environment to the base of the outer membrane. The O-antigen can also act as a binding site for bacteriophages and for products of the hosts immune system [7]. Long chain O-antigens may also contribute to the virulence of human pathogens [10] as they often contain virulence factors [8].

The structure of O-antigens varies widely, according to environmental situations even within a species. Hence, for example *E. coli* has around 170 different O-serotypes [8] that can modulate the properties of the outer membrane of the organism and its interaction with the hosts immune system.

The endotoxic potential of LPS in humans

The lipid A component of LPS is the ligand of the human pattern recognition receptor Toll-like receptor 4 (TLR4) that is expressed by immune and other cells. Hence, the lipid A components of the LPS molecule are responsible for the induction of an innate immune response in both immune and other cells [8,10]. However, the pro-inflammatory potential of lipid A, also referred to as its endotoxicity, varies between Gram-negative bacterial species according to its structure [8,14–17]. Again, the magnitude of the inflammatory response to a particular LPS can vary with the dose that is received by the host [18].

The recognition of LPS by the TLR4/MD-2 complex

The Toll-like receptor 4 and its pattern of expression. The Toll-like receptor 4 (TLR4) is a transmembrane protein containing extracellular leucine-rich repeats (LRR), a transmembrane domain and an intracellular Toll/ IL-1 receptor (TIR) domain [19]. TLR4 is predominantly expressed by endothelial cells [20] and immune cells such as the macrophages and lymphocytes in peripheral blood [21] and in lower concentration by immune cells within the lamina propria [21–23]. TLR4 can also be expressed in small quantities by enterocytes in the small intestine and colonic mucosa of healthy humans [22,23].

However, TLR4 alone cannot recognise LPS. Hence, it must associate with the myeloid differentiation factor 2 (MD-2) before it can induce an immune response to a LPS challenge. The MD-2 can either be a membrane-bound (m), as occurs in immune cells, or in a soluble (s) form. Nonimmune cells such as epithelial and endothelial cells can express sMD-2 receptors [8]. Both forms of MD-2 can associate with TLR4 to form a heterodimer that initiates a complex, ligand specific, signalling after binding with LPS [24]. However, these cells cannot by themselves mediate a response to LPS as it must be presented in the appropriate molecular context (see below).

The transport route of LPS to the TLR4/MD-2 complex. LPS cannot directly bind to the TLR4/MD-2 complex, LPS must be correctly "presented" to the receptor complex. Both,

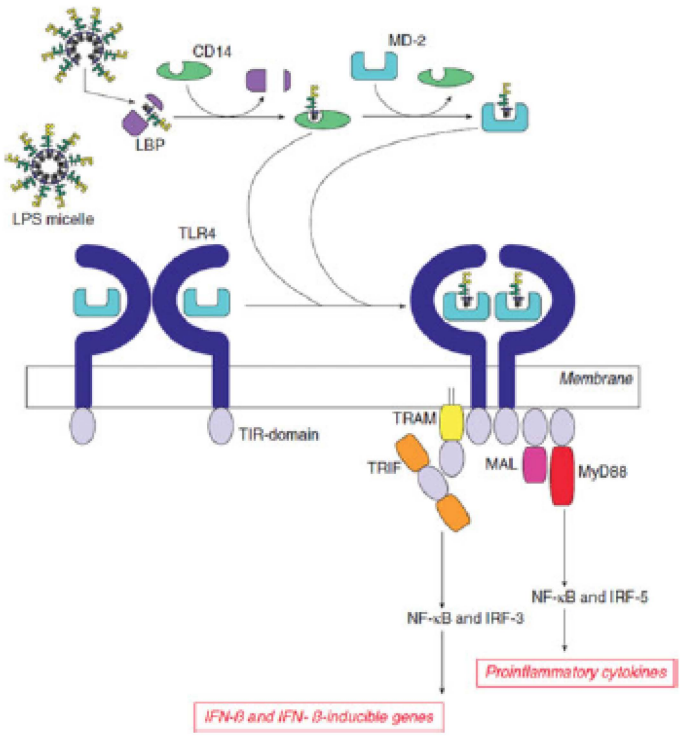

Figure 4. Schematic steps involved in the recognition of intercellular LPS. LPS-binding protein (LBP) sequesters single LPS molecules from the LPS aggregate and transfers it to CD14. CD14 acts to transfer the bound LPS to membrane-bound or to soluble MD-2. LPS-loaded MD-2 induces the TLR4 to dimerize and activates the TLR4-mediated signalling cascade. TLR4 activation either induce a MyD88-dependent or TRIF–dependent pro-inflammatory immune response (Figure 8 from [8]).

membrane-bound (m) and soluble (s) forms of CD14 have the capacity to bind and correctly present LPS molecules to both membrane-bound and soluble forms of MD-2. Immune cells such as macrophages express mCD14 so they can "directly" interact with LPS. In contrast, in nonimmune cells, i.e. endothelial and epithelial cells, sCD14 must present LPS to sMD-2 which then interacts with the sMD-2 receptors on the surface of nonimmune cells [8]. However, both forms of CD14 have normally a low affinity for LPS. However, this affinity is dramatically increased in the presence of LPS-binding protein (LBP) [25].

LBP can bind with a single LPS molecule either from an LPS aggregate or from fatty micelles [8] and present them to m/sCD14 (Figure 4) [25]. Hence, the LBP-mediated transport of LPS to m/sCD14 is important in the induction of the LPS-dependent immune response [26]. LBP can also mediate the binding of LPS to high-density lipoproteins (HDL) whereupon it can no longer induce an immune response in circulating immune cells [27]. Hence, LBP-mediated binding of LPS to HDL somehow neutralises its pro-inflammatory potential (discussed below). Thus LPS presentation by LBP can either promote or reduce the pro-inflammatory potential of LPS. In this respect it is hypothesised that during the first 10 minutes of exposure LBP mediates the transport of LPS to CD14 and after this it is more likely to present LPS to HDL [27].

Interactions between the LPS and the TLR4/ MD-2 complex. In contrast to nonspecific binding of LPS by LBP and CD14, both forms of MD-2 are able to interact specifically with LPS molecules based on the configuration of their acyl chains and their allied phosphate groups in

the contained lipid A [8]. Hence, the structural form of the lipid A moiety, notably the presence of six acyl chains, governs the interaction with MD-2 and the subsequent change in its conformation [19,24].

The hydrophobic pocket of MD-2 is able to accommodate four acyl chains. The presence of a fifth acyl chain induces a structural change in MD-2 that allows it also to be accommodated in the pocket, but a sixth acyl chain cannot be accommodated and must remain on the surface of the MD-2 protein [19,24]. This projecting sixth acyl chain interacts with a specific phenylalanine residue on the TLR4 monomer [24]. The presence of a sixth acyl chain, whilst not inducing accommodation in the pocket, induces structural changes in the F126 loop of MD-2 that promotes the hydrophilic interaction of MD-2 with TLR4. The latter structural change allows the TLR4/MD-2 complex to interact with another TLR4 monomer to form a TLR4 dimer (Figure 4). Meanwhile, the structural change induced by the fifth acyl chain that leads to its accommodation in the pocket, allows for ionic interaction between the phosphate groups and the positively charged residues of two TLR4 monomers which further facilitates the dimerization of the TLR4/MD-2 complex [24]. It is noteworthy that, hydrogen bonding between the phosphate groups of the lipid A unit and the TLR4 dimer can only occur when the lipid A contains two phosphate groups [19].

In contrast, the accommodation of a tetra-acylated lipid A in the pocket leaves the F126 loop of MD-2 exposed to the surrounding solvent [24]. The tetra-acylated form of lipid A is not acylated with a secondary fatty acid at position R3′. Hence, the exposure of the loop enables the establishment of hydrogen bonds between the 3-OH of R3′ of lipid A (Figure 3) and the F126 loop of MD-2 [19]. The differing types of interactions of LPS with MD-2 and TLR4 according to the form of the contained lipid A can influence the character of the subsequent TLR4 downstream signalling [19,24].

Interactions between LPS and the TLR4/MD-2 complex can also be mediated by carbohydrate moieties within the inner core of LPS but they do not have such a powerful promoting function as those described above [24].

TLR4-mediated signalling pathways. The ligand bound TLR4/MD-2 complex ultimately activates both the MyD88 (myeloid differentiation factor 88)-dependent and the TRIF (TIR-domain-containing adaptor inducing interferon-β)-dependent signalling pathways. As these two pathways have overlapping functions, fine tuning of their relative contributions is necessary to avoid an excessive pro-inflammatory response. As both pathways are only activated by the TLR4 receptor, the modulation of the activation of the signalling cascades is critical for the homeostasis of the intestinal and the systemic immunity [28].

In general, the binding of LPS to the TLR4/MD-2 complex allows for interactions between the intracellular TIR domain of the TLR4 receptor and the intracellular TIR domain-containing adapter molecules such as MAL (MyD88 adaptor-like protein) and TRAM (TRIF-related adaptor protein). The TLR4-bound adapter molecule MAL then recruits MyD88 which subsequently activates the MyD88-dependent pathway [29]. The dissociation of MAL from the TLR4 receptor, followed by its degradation, enables the translocation of the TLR4/TRAM complex from the cell membrane into endosomes situated within the cytoplasm. This internalisation of the TLR4/TRAM complex induces the recruitment of TRIF and activates the TRIF-dependent signalling cascade (Figure 5) [30].

A cascade of molecules is involved in the downstream signalling from both TLR4 signalling pathways. The signalling cascade of the MyD88-dependent pathway includes the following steps. Firstly, IRAK4 (interleukin-1 receptor-associated kinase 4) is recruited to the TLR4-associated MyD88 and subsequently initiates the phosphorylation of IRAK1 [29].

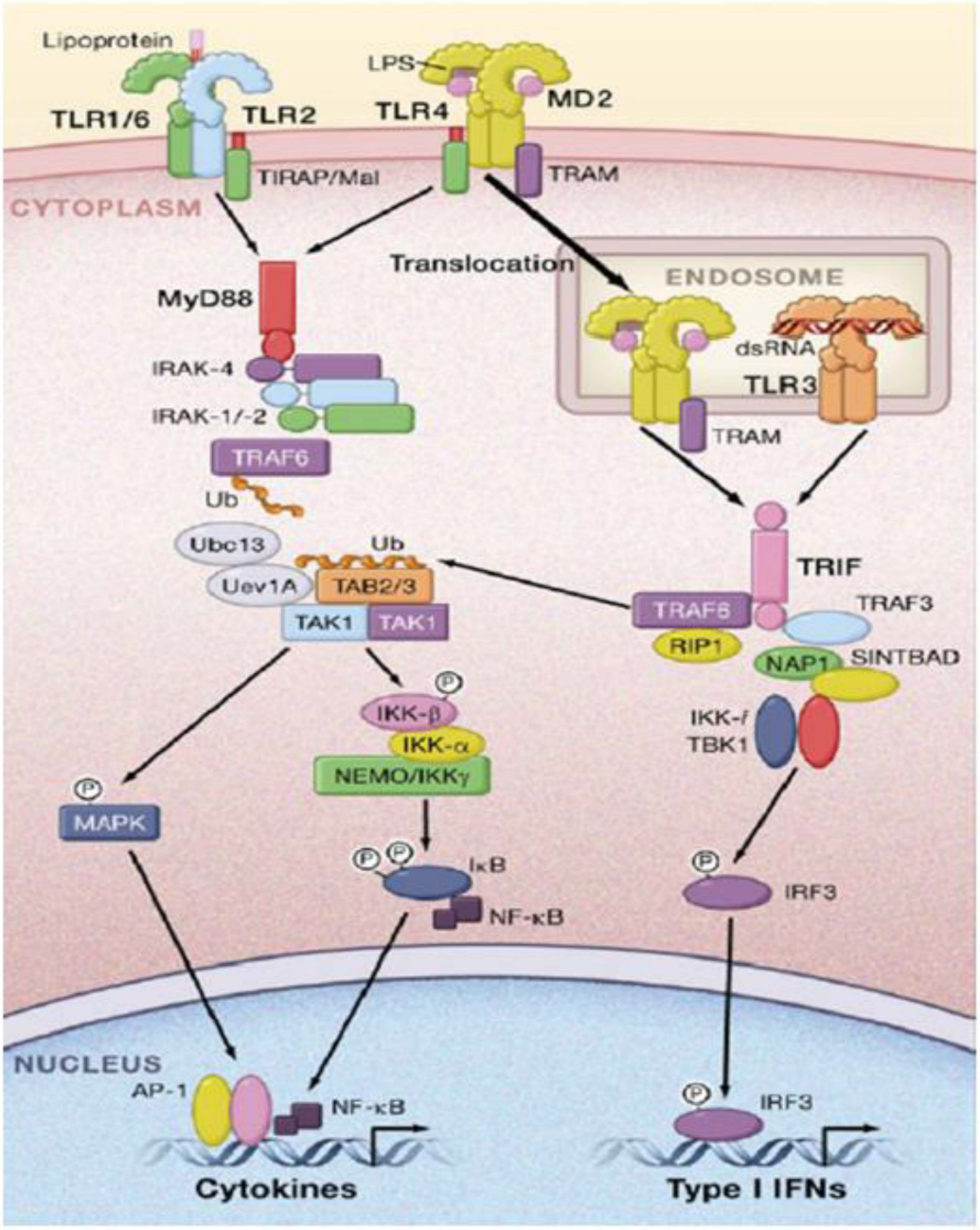

Figure 5. TLR4-mediated signalling pathways. The interaction between LPS and the TLR4/MD-2 complex at the cell membrane activates MyD88-dependent downstream signalling, leading to the activation of the transcription factors NF-$_{\kappa}$B and AP-1. Both transcription factors mediate the production of pro-inflammatory cytokines. The internalisation of the TLR4 receptor induces the TRIF-dependent pathway which activates the transcription factor IRF3 and the expression of the IFNβ and type I interferon-inducible genes and/or induces the delayed activation of the transcription factors NF-$_{\kappa}$B and AP-1 (Figure 1 from [31]).

IRAK-1 then induces the TRAF6-mediated downstream signalling cascade that results either in the activation and translocation of the transcription factor NF-κB (nuclear factor κB), and/or the activation of the transcription factor activator protein (AP)-1 by the MAPK

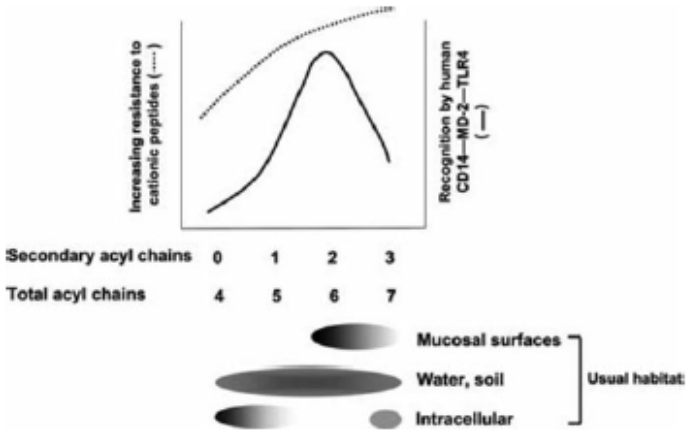

Figure 6. The relationship between the amount of secondary acyl chains, the resistance against host antibacterial peptides and the TLR4 recognition as well as the habitat of Gram-negative bacteria. Hexa-acylated lipid A bearing four primary and two secondary acyl chains and is the most endotoxic form of lipid A as it induces the greatest production of the pro-inflammatory cytokine TNF-α. Gram-negative bacteria use hexa-acylated lipid A to form highly impermeable outer membrane that has a high resistance to host released antimicrobial peptides and is therefore well adapted to the condition within the lumen of the gut (Figure 1 from [12]).

(mitogen-activated protein kinase) signalling cascade (Figure 5) [28]. Both transcription factors can promote the expression of pro-inflammatory cytokines such as tumour necrosis factor (TNF)α and interleukin (IL)-1β and mediate a pro-inflammatory response to the LPS challenge [31].

The TRIF-dependent pathway can induce a delayed activation of the NF-κB and the MAPK pathway via TRAF6 downstream signalling, and/or support the TRAF3-mediated activation and translocation of the transcription factor IRF3 (interferon regulatory factor 3) which in turn stimulates the gene expression of interferon (INF) β and type I interferon-inducible genes (Figure 5). The IRF3-mediated pathway is essential for the clearance of microorganisms by host antiviral and antibacterial peptides [28].

The structure of lipid a determines the endotoxicity of the LPS molecule

As described above, the structure of the lipid A moiety influences the character of the subsequent interaction with the TLR4/MD-2 complex [19,24]. Since LPS molecules with a hexa-acylated lipid A subunit induce a stronger pro-inflammatory immune response than do those with four acyl chains (Figure 6), it is assumed that dimerization of TLR4/MD-2 is required before the activation of the TLR4 downstream signalling pathways can take place [19].

As most pathogenic Gram-negative bacteria including *Heliobacter pylori*, *Yersinia pestis*, *Francisella tularensis*, *Chlamydia trachomatis*, and *Legionella pneumophilla* express LPS molecules that contain tetra-acylated lipid A and induce a reduced TLR4-mediated pro-inflammatory immune response in comparison to that from hexa-acylated LPS produced by commensal organisms such as nonpathogenic *E. coli*, it may be that lipid A modification enables pathogens to evade the recognition by the TLR4/MD-2 complex and avoid the stimulation of the hosts pro-inflammatory immune system [3,8,12]. However, it remains to be seen why the host should evolve an immune response that activates a pro-inflammatory response to enteral commensal bacteria but not to pathogens.

Evidence suggests that although tetra- and hexa-acylated LPS interact differently with MD-2 and TLR4 [19,24] both can initiate TLR4-mediated signalling pathways. However, both

types of LPS molecules can induce other feedback loops in host cells depending on their structural differences [16]. For example, both tetra- and hexa-acylated LPS molecules activate the expression of cell surface activation markers in dendritic cells (DCs) in a similar manner when given at physiologic high doses (100 ng/mL) to either mice or humans. However, the outcomes of this activation differs in the two species; 30 min after the stimulation with tetra-acyl LPS there is stronger NF-κB translocation and activation of the TLR4-dependent signalling in mouse DCs and macrophages than occurs after stimulation with hexa-acyl LPS. The stronger activation of the NF-κB pathway by tetra-acylated LPS also leads to a greater upregulation in gene expression of TNF-α and IL-12, for up to 4 hours post-stimulus. However, there is a concomitant increase in the post-transcriptional degradation of the two pro-inflammatory cytokines which results in a reduction in the release of TNF-α and IL-12. The latter process accounts for the weaker pro-inflammatory immune response induced by tetra-acylated LPS compared to that from hexa-acylated LPS [16] and not because tetra-acylated LPS has not been inefficiently detected by the immune system per se.

Not only does the acylation pattern of the lipid A moiety affect the activation of the TLR4 signalling but also the number of associated phosphate groups. Hexa-acylated lipid A moieties with two phosphate groups strongly activate both the MyD88-dependent and the TRIF-dependent pathways whereas hexa-acylated lipid A moieties with a single phosphate group strongly activate the TRIF-dependent pathway but activate the MyD88-dependent pathway only weakly. This pattern of activation, termed "TRIF bias" is associated with less endotoxicity [28] as it ultimately induces the expression of antiviral and antibacterial peptides that are involved in the clearance of bacteria rather than the induction of inflammation.

Again the site at which the LPS is phosphorylated influences the immune response. Hence, penta-acylated, 1-monophosphorylated LPS moieties from the enteral commensal *Bacteroides thetaiotaomicron,* induce stronger NF-κB activation than does LPS from pathogenic *Porphyromonas gingivalis* which is phosphorylated at position 4′ [32].

Hence, the structure of lipid A influences the balance in activation of the MyD88-dependent and TRIF-dependent pathways and the extent of the ultimate inflammatory response [19,24]. All in all, LPS molecules bearing a lipid A subunit with four primary and two secondary acyl chains (hexa-acylated lipid A) along with two phosphate groups are highly endotoxic as they induce the greatest expression of the pro-inflammatory cytokine TNF-α. Less endotoxic LPS molecules that do not contain these moieties promote another immune response [16] and may compete with other more endotoxic forms at the sites that activate the immune response [8].

The effect of the total dose of LPS on TRL4-signalling pathways

Not only the structure of the lipid A moieties but also the size of the dose of LPS, dictate the levels of activation of the MyD88-dependent and TRIF-dependent signalling cascades [18; see Table 1].

The concentration of LPS extracted from *E. coli* that is necessary to induce a strong pro-inflammatory immune response i.e. production of TNF-α, *in-vitro* is around 10 ng/mL [33]. High physiological concentrations of LPS from *E. coli*, i.e. 10–100 ng/mL, are known to induce both the MyD88-dependent and the TRIF-dependent pathways and to robustly upregulate TLR4-mediated production of IL-6 and TNF-α [34,35] by macrophages [33,36]. The subsequent expression of anti-inflammatory cytokines such as IL-10 and AP-20 together with other pro-tolerant mechanisms are thought to mediate a negative feedback loop which then down-regulates the initial pro-inflammatory immune response [34].

Table 1. Overview of the immune response to LPS depending on its structure and dose.

	Initial immune response	Subsequent effect	Tolerance to LPS
Endotoxic LPS			
High dose	–robust production of pro-inflammatory IL-6, TNF-α and IL-1β –enhanced production of IRAK-1 → *strong initial pro-inflammatory immune response*	–delayed production of anti-inflammatory IL-10 –immediate degradation of IRAK-1 –expression of inhibitors of the MyD88-dependent signalling cascade i.e. MyD88s, SHIP-1, IRAK-M → *downregulation of the initial pro-inflammatory immune response* –maturation of tolergenic macrophages → *improved bacterial clearance* –increased levels of serum LBP → *enhanced neutralisation of circulating LPS*	YES
Low dose	–weak production of TNF-α –production of ROS	–fails to induce production of anti-inflammatory IL-10 –fails to induce the expression of inhibitors of the MyD88-dependent pathway → *prolonged mild pro-inflammatory immune response* –maturation of nontolergenic macrophages → *stronger pro-inflammatory immune response to the second LPS challenge*	NO
Less endotoxic LPS			
High dose	–weaker production of TNF-α and IL-12 TRIF bias induced by monoacylated LPS: –fails to express active IL-1β → *reduced pro-inflammatory immune response* –increased release of anti-bacterial peptides → *enhanced bacterial clearance*	TRIF bias induced by monoacylated LPS: –reduced antigen presenting function of DCs –reduced production of pro-inflammatory cytokines by T cells → *more a pro-tolerant than pro-inflammatory immune response*	NO

In contrast to the response to such high concentrations of LPS, challenge with "subclinical" levels of *E. coli* LPS (< 10 ng/mL) induces a weak pro-inflammatory response that fails to activate the MyD88-dependent PI3K signalling cascade that is necessary to activate the anti-inflammatory feedback loops [18] and to induce the expression of the anti-inflammatory cytokine IL-10 in murine macrophages [37]. Instead in murine macrophages at least, low concentrations of LPS induce the translocation of the TLR4 complex into mitochondria where it upregulates the production of mitochondrial reactive oxygen species (ROS) [18]. These ROS stabilize activating transcription factor (ATF)2 and reduce the pro-inflammatory immune response. The failure of low concentrations of LPS to induce this secondary phase of negative feedback is likely responsible for the continued expression of low levels of pro-inflammatory mediators [18]. At all events the activation of the alternative TLR4-dependent signalling cascade that results from challenge with low concentrations of endotoxic LPS induces a continuing low grade rather than the large-but-short acute inflammatory response induced by high concentrations of LPS.

The Immune response to Intact gram-negative bacteria

As noted hitherto, the endotoxic potential of LPS is determined by the lipid A that it contains. Since lipid A is incorporated into the outer layer of the outer cell membrane to anchor the LPS molecule in the membrane, it is not directly exposed to the environment [10]. It has therefore been assumed that only solubilised LPS and not LPS components in the outer membrane can act as endotoxins [8,10]. Hence, the immunomodulatory effects of LPS have been generally evaluated using purified solubilised LPS [33–41]. However, more recent studies indicate that entire Gram-negative bacteria can also activate the hosts immune response [2,42]. Again, formalin-killed bacteria in which the LPS remains bound to the bacterial cell membrane have a similar immunomodulatory effect to that of soluble LPS [43,44]. Hence, it seems that lipid A within the bacterial membrane can be recognised *in-situ* by the host immune system.

The magnitude of the immune response to LPS *in-situ* may differ from that to soluble LPS. Hence, recent data indicates that the stimulation of HT-29 intestinal epithelial cells with soluble LPS derived from *E. coli* induces less expression of genes related to pro-inflammatory immune response and to barrier function than does stimulation with live whole *E. coli* [45]. Thus it appears that the complex cross-talk between intestinal epithelial cells and live commensal bacteria may involve a number of bacterial components other than LPS. However, whether the greater expression of pro-inflammatory genes leads to upregulation of the secretion of cytokines has not been investigated. Further, the proportion of increased effect on pro-inflammatory gene expression that results from lysis of the whole live or formalin killed bacteria within the cell medium has not been determined.

The relative importance of other bacterial species or their products has been demonstrated in a study in which Caco-2 cells were interposed between a layer of luminal bacteria and an underlying layer of DCs. In this model, the co-stimulation by *E. coli* (or its LPS) and intact live lactic acid producing bacteria prevented the production of TNF-α by DCs [46].

Variation in the response to LPS according to cell type

The response to LPS varies according to the type of cell that is exposed to it. Intestinal mucosal cells such as enterocytes are hyporesponsive to LPS in the lumen in that high concentrations do not promote the production of pro-inflammatory cytokines [47]. Nevertheless, the presence of LPS in the lumen can activate mechanisms that reduce the endotoxic potential of LPS

[42] and minimize its penetration into gut-associated-lymphoid tissue (GALT) and the systemic circulation [48].

In contrast, high concentrations of LPS in the systemic circulation lead to hypotension, coagulopathy and organ failure, a potentially fatal condition termed septic shock. Septic shock results from a gross and unregulated pro-inflammatory immune activation in macrophages and vascular endothelial cells [3]. Somewhat lower concentrations of circulating LPS can induce moderate fever, stimulate the immune system and promote microbial killing. Subclinical levels of LPS in the circulation may be associated with prolonged mild or subclinical inflammation [18]. Hence, the pro-inflammatory or endotoxic potential of LPS is determined by the site at which LPS recognition takes place and its concentration. As discussed hitherto, the endotoxicity of LPS at a particular site also depends on the chemical structure of the LPS molecule [8]. Typical mediators of these endotoxin-induced pro-inflammatory reactions are tumour necrosis factor (TNF)-α and various interleukins (IL) such as IL-1β, IL-6 and IL-12 [36,49–51].

Reactions of mucosal cells to luminal LPS

The intestine comprises the largest surface at which there is interaction between the host and the environment [1]. It is thought that LPS is continually released into the gut lumen from on-going lysis of contained Gram-negative commensal bacteria. Such lysis is mediated by defensive secretions such as antimicrobial peptides and bile acids. Small amounts of LPS are also released during the process of bacterial division [49]. About 1 g of membrane-unbound LPS is contained within the lumen of intestine at any given time. The host has developed a finely tuned monitory and response system to modulate the population of normal commensal enteral microbiota as well as invading pathogens and their secretions e.g. LPS [42,48].

The hyporesponsiveness of enterocytes on exposure to the luminal LPS

The pattern of expression of the TLR4/MD-2 complex by the intestinal epithelium. The induction of an immune response to LPS in intestinal epithelial cells (IECs) depends on their ability to recognise it. In general, the LPS receptor TLR4 is expressed at low levels by small intestinal and colonic enterocytes in healthy humans [22,23]. The TLR4 receptor is also expressed by mature crypt cells [21] i.e. Paneth cells [52] but this expression is reduced or halted at least in the colon as epithelial cells mature and move toward the lumen [21]. The TLR4 co-adapter molecule MD-2 is not expressed in mature colonic crypt cells of healthy humans [22] nor in colonic HT-29 crypt cell lines [20]. The general hyporesponsiveness of mature IECs is likely based on the downregulation of the TLR4/ MD-2 receptor complex [21]. The treatment of HT-29 cells with the T cell-derived pro-inflammatory cytokines IFN-γ and TNF-α, upregulates the expression of TLR4 mRNA in these cells [20,40]. Further, treatment with INF-γ is able to induce the expression of the MD-2 gene [20]. Subsequent stimulation of these cells with LPS then induces the secretion of the pro-inflammatory cytokine IL-8 [20,40]. Together these findings suggest that the expression of TLR4 and MD-2 by mature IECs as well as the TLR4-mediated immune response is dynamically regulated in response to the surrounding cytokine profile.

This mechanism likely explains why patients with inflammatory bowel diseases (IBD) show an elevated expression of the TLR4/MD-2 complex [53]. However, whilst the expression of TLR4 is increased compared with that of normal subjects in the apical surface of enterocytes of subjects with Crohn's disease, its expression is relatively increased at the basolateral surface of granular cells of subjects with ulcerative colitis [23]. The increase in TLR4 expression at either of the two sites in patients with IBD in combination with an altered pattern of expression of

other TLRs [23] may explain their enhanced responsiveness to bacteria and their products including LPS [53] generating a vicious circle that results in protracted inflammation of the intestine.

The development of tolerance to intestinal LPS. Stimulation of naïve human intestinal cell lines such as HT-29 cells with bacterial products including LPS, reduces the expression of TLR4 [54], indicating that, on first contact with bacterial LPS, TLR4 receptor production is downregulated.

Whilst both primary adult and foetal murine enterocytes express the TLR4/MD-2 complex in their cytoplasm, adult IECs do not generate a pro-inflammatory immune response on exposure to LPS whereas foetal IECs do produce such a response. Hence, the age-dependent tolerance in IECs to LPS is not the result of a simple downregulation the production of the TLR4/MD-2 complex. It seems that exposure of murine neonatal IECs to LPS during vaginal delivery induces a TLR4-dependent NF-κB activation followed by a decrease in levels of the protein IRAK-1 via a post-transcriptional mechanism. This reduction in the level of IRAK-1 is not seen in neonatal IECs taken from mice born by caesarean section [47]. Further, there is no alteration in the levels of IRAK-1 phosphorylation inhibitors such as Toll-interacting protein (Tollip) and sMyD88 in these cells. Since the downregulation of IRAK-1 must then inhibit any downstream signalling via the TLR4 receptor (see above) it seems that the post-natal downregulation of IRAK-1 expression in IECs is solely responsible for the development of tolerance to luminal LPS by enterocytes [47].

In contrast to IECs obtained from adult murine intestine, intestinal cell lines which have not undergone post-natal priming with LPS, develop tolerance after subsequent stimulation with LPS through the overexpression of Tollip [55]. Tollip interacts with IRAK-1 and inhibits its phosphorylation by IRAK-4 subsequently supressing TLR4-mediated downstream signalling [56].

Hence a number of pathways can induce tolerance to LPS by intestinal epithelial cells depending on the timing of first contact with this agent. Such tolerance, induced either by reduced expression of the TLR4/MD-2 complex or by inhibition of TLR4 downstream signalling, may avoid an overstimulation of the host immune system by luminal LPS and facilitate the postnatal microbial colonization of the gut.

The Innate Immune response to colonizing luminal commensal bacteria

Dendritic cell responses to luminal LPS exposure. Hyporesponsiveness of adult enterocytes to luminal LPS could inhibit antigen-presentation to T cells in the submucosa and maintain general tolerance to this material in the intestinal lumen [21–23]. However, luminal bacterial antigens such as LPS are also scavenged by Microfold (M)-cells that cover the luminal surfaces of Payer's patches (PP). These cells transport luminal antigens, including LPS, into the sub-epithelial dome where they are sampled by dendritic cells (DCs) or destroyed by macrophages [57]. However, macrophages in the GALT seem to be hyporesponsive to the immune challenge with LPS [47]. Hence, the immune response in the GALT to LPS is likely to be mediated solely by DCs. This immune response can be boosted by a subclass of intestinal DCs that can extend their processes into the intestinal lumen, a process which allows them to collect antigenic material directly from the lumen [46].

Interestingly, in a two layer transwell model comprising Caco-2 cells and underlying immature DCs, only the direct application of Gram-negative bacteria such as *E. coli* (or its LPS) was able to induce the maturation of DCs and the subsequent production of low levels of TNF-α via TLR4. However, the DCs that lie beneath Caco-2 enterocytes had a different pattern

of expression of surface activation markers and expression of cytokines compared to those that were in direct contact with Gram-negative bacteria [46]. Hence, it seems that molecules expressed by enterocytes influence the immune response of the underlying DCs to luminal bacteria.

Again, the co-stimulation with either *E. coli Nissle*, or its LPS, and lactic acid bacteria (LAB) prevented maturation of underlying TNF-α producing DCs in the same transwell model but this effect was observed only with live and not with killed LAB [46]. Hence, the cytokine profiles in underlying DCs that are induced by luminal LPS can be modified in the presence of other bacterial components.

The secretion of antimicrobial peptides by enterocytes and paneth cells. Presentation of LPS to lymphocytes by DCs can lead to the maturation of Immunoglobulin (Ig) A-producing B cells [58]. The IgA secreted by these cells is subsequently taken up via the basal regions of enterocytes and released into the lumen. This IgA, termed secretory(s)IgA, combines with epitopes of bacteria within the lumen 'smothering' them without inducing a pro-inflammatory response [58]. Interestingly, Gram-negative Bacteroides species appear to be more efficient in activating such IgA secretion than are Gram-positive Lactobacillus species [59]. The lower IgA-responsiveness to Lactobacillus species may allow these and other Gram-positive bacteria species of the genus Clostridium and Enterococcus to colonize the epithelial surfaces and the mucus layer, whilst colonisation by Gram-negative bacteria of the genus Bacteroides and the family of Enterobacteriaceae including Escherichia and Salmonella species is restricted to the intestinal lumen [1]. The differences in the species of commensal bacteria that inhabit the apical surfaces of enterocytes from those that exist solely within the lumen may also limit the exposure of M-cells and of subclasses of DCs, respectively, to certain types of LPS and consequently limit the recognition of certain types of Gram-negative bacteria or their LPS by intestinal cells.

Crypt cells such as Paneth cells which express TLR4 on their luminal surfaces [52] release antimicrobial molecules such as α-defensin and ß-defensin into the lumen in response to stimulation of either of their luminal surfaces with LPS or of their basal surfaces with pro-inflammatory cytokines such as TNF-α and IL-1ß [57]. Hence, they mediate the clearance of bacteria from the lumen rather than inducing a pro-inflammatory immune response.

Intestinal alkaline phosphatase and the reduction of the endotoxic potential of luminal LPS. The process of bacterial killing releases and solubilises a significant quantity of bacterial components including LPS into the lumen [60]. Enterocytes are thought to expresses intestinal alkaline phosphatase (IAP) in order to detoxify these components thus avoiding the establishment of florid pro-inflammatory reactions in the intestinal mucosal region [42,60].

IAP is secreted by the brush border and is either membrane-bound [61] or contained within brush border membrane vesicles that are released into the lumen from the microvilli of enterocytes [62]. Further, IAP may also be released into systemic circulation [63,64]. Although IAP is predominantly secreted in the duodenum, in the mouse at least, it does not lose its activity as it moves distally through the small and the large intestine [65].

Treatment with enteropathogenic *E. coli* and *Shigella* induces the secretion of IAP in Caco-2_{BBE} cells [62]. Hence, the presence of luminal Gram-negative bacteria or their LPS seems to induce a signalling cascade that increases IAP activity and subsequently elevates the modification of luminal LPS [48]. The expression of IAP may be also associated with the release of

sIgA and antimicrobial peptides. It has been proposed that IAP reduces the toxicity of LPS by dephosphorylation [42,48,60]. Hence, for the example the modification of LPS by calf-IAP (cIAP) renders it nonendotoxic to zebrafish [42]. IAP has a high affinity for LPS molecules bearing two phosphate groups but not for those with monophosphorylated lipid A moieties [66]. However, the enzymatic activity of IAP varies with substrate and pH [61,67,68]. Hence, it is currently unclear whether the dephosphorylation of various bacterial components that is observed *in-vitro,* occurs under physiological conditions. Nevertheless, dephosphorylation of LPS by IAP seems to be involved in the development of immune tolerance to gut-colonizing bacteria and LPS, as in zebrafish that lack IAP there is a much greater infiltration of the lamina propria by neutrophils than occurs in wild type zebrafish [42].

Interestingly, oral challenge of Iap-knockout (KO) mice that had been chronically exposed to LPS from commensal bacteria, with *Salmonella typhimurium* was able to induce an enteral tolerance of LPS but this did not occur in wild type mice. In contrast, the systemic immune response that occurs during salmonellosis was more intense in Iap-KO mice than in wild type mice [69].

Bates *et al* found that, in a germ-free zebrafish model, the expression of IAP was activated by MyD88-depedent signalling only in the presence of intact, live Gram-negative bacteria or after challenge with LPS [42]. Hence, the secretion of IAP and the subsequent reduction in the endotoxicity of LPS may be part of a TLR4-mediated delayed negative feedback loop [70] and may be a first line of defence preventing the penetration of endotoxic LPS into the GALT and the systemic circulation [48].

However, as mentioned hitherto, mature human enterocytes and Caco-2 cells only express very low levels of TLR4 receptors on their luminal surfaces and are generally hyporesponsive to the stimulation by luminal LPS [21–23,40]. Thus it seems unlikely that IAP expression by human enterocytes, *in-vivo* or in Caco-2 cells, is directly induced by the enterocyte TLR4-signalling cascade. It remains unclear how the expression of IAP is regulated in the human intestine.

Other studies have shown that IAP activity and the rates of detoxification of LPS are reduced in starved mice compared to those in fed mice [65] and that the consumption of fat appears to increase the secretion of IAP [64]. Hence, the expression of IAP could also be modulated by diet.

Interactions between circulating LPS and the hosts immune system

In spite of the high concentrations of LPS that exist within the intestinal lumen under normal physiological conditions, only small amounts are translocated into the systemic circulation [71]. However, LPS can also be released within immune cells during their killing of bacteria [10]. The systemic immune system responds to the presence of LPS in the systemic circulation with mechanisms that include a pro-inflammatory response, the modification of the pro-inflammatory properties of LPS and their neutralisation.

Neutralisation of systemic LPS by LBP-mediated mechanisms. The site at which exposure to LPS occurs influences the manner in which LPS is cleared from that site. Within the vascular system LPS forms aggregates (micelles) that are sequestered by LPS-binding protein (LBP), an acute phase reactant produced by hepatocytes [72]. Subsequently, the LPS is transferred from LBP to mCD14 and sCD14 [25] and also to HDL and other serum lipoproteins e.g. very low density lipoprotein (VLDL) and chylomicrons [27,73]. As described hitherto, the recognition of LPS via the TLR4/MD-2 complex in both immune and nonimmune cells is mediated by

CD14 [8]. The recognition process activates a broad range of host defence mechanisms. However, the LPS that is bound to HDL or other serum lipoproteins does not induce a systemic immune response and hence is said to be "neutralised."

It seems the transport of LPS to CD14 is mediated mainly by LBP during the first 10 minutes after the LPS challenge whereas it is bound mainly to HDL after this [27]. When LPS is incorporated into HDL via LBP *in-vitro*, more than 50% of the dose is neutralised within 40 min [27], a figure which corresponds with data obtained from an *in-vivo* rabbit model in which around 50% of an intravenous LPS dose is removed from the plasma within 30 min [74]. The capability of HDL to "neutralise" circulating LPS, and to reduce the secretion of pro-inflammatory cytokines depends on the total dose of LPS, the concentration of LBP in the serum and the relative proportions of serum lipoproteins such as HDL, VLDL and chylomicrons [27,73,75–78]. LPS-loaded lipoproteins are subsequently transported to the liver [74] where LPS molecules undergo modification. LPS molecules that are not rapidly neutralised and transported to the liver, may be modified by immune cells (see below). It is noteworthy that LBP production by the liver is increased by elevated circulating levels of IL-1β, IL-6 and TNF-α [72] after LPS challenge. Hence, serum LBP levels are increased by feedback regulation to ensure adequate neutralisation of LPS. Thus, the levels of LBP in the serum are elevated in patients with sepsis compared with those of healthy control subjects [26,79].

Whilst the system of neutralisation of LPS by HDL or other serum lipoproteins is important, it is noteworthy that more than 70% of a dose of LPS injected into subcutaneous tissues is reported to be modified by immune cells at the putative site of infection before any of the monomers of LPS undergo transport via the lymphatic system to the liver [80].

Detoxification of LPS. On arrival in the liver, LPS is deacylated by the lipase acyloxyacyl hydrolase (AOAH) in hepatic Kupffer cells. AOAH mediates the deacylation of the two secondary acyl chains of the lipid A moiety and thus reduces the endotoxic potential of LPS molecules. However, detoxification of LPS by the liver is a relatively protracted process that takes several days [81] compared with the rapid, LBP-mediated, neutralisation of LPS in the circulation which takes only minutes to hours [27,74].

Immune cells such as neutrophils, macrophages and DCs also express AOAH [80] and thus can reduce the endotoxic potential of LPS molecules that remain within the circulation [74] or in subcutaneous tissues [80]. This AOAH-mediated deacylation by immune cells can occur either extracellularly [82] or intracellularly following either the phagocytosis of the organism or the absorption of LPS [83]. It seems that the production of AOAH is independent of the TLR4-mediated pathway since TLR4-deficient macrophages can take up LPS and deacylate it intracellularly without inducing a pro-inflammatory immune response [84]. Nevertheless, the deacylation of LPS in TLR4 deficient mice occurs at lower rates than in wild type mice [80]. In rabbits the intravenous injection of LPS augments plasma AOAH activity [82] indicating that LPS-induced signalling may enhance its production.

Regardless of the site at which it operates, AOAH is generally more effective in deacylating hexa-acylated lipid A than less acylated moieties [80]. Deacylation by AOAH appears to mitigate the immune response as AOAH-mediated modification of LPS molecules limits the production of IgM and IgG3 by B cells after LPS challenge [80,85]. Hence, AOAH is an important host defence mechanism in reducing the endotoxic potential of LPS.

The development of temporary systemic LPS tolerance following exposure to high concentrations of endotoxic LPS.

Whilst LBP-mediated neutralisation of circulating LPS molecules occurs relative rapidly and the detoxification of LPS by AOAH is an efficient process, both in the circulation and

in tissues, both mechanisms do not prevent the development of an initial pro-inflammatory immune response [81,86].

As discussed above, the dosage of LPS influences the pattern of immune response. A single high intravenous dose of highly endotoxic *E. coli* LPS (>10 ng/mL) induces a strong pro-inflammatory response with expression of TNF-α, IL-1β and IL-6 [36,51,87]. A subsequent similar dose induces a somewhat reduced pro-inflammatory immune response [33,36,38,39,88–91]. This phenomenon is termed systemic LPS or endotoxin tolerance. Systemic LPS tolerance is temporary and is associated with the action of elements of the innate immune system such as antigen presenting cells. Systemic tolerance to LPS during infection lasts from 5 to 10 days [86]. Whilst a single low dose of LPS (<10 ng/mL) fails to mediate LPS tolerance [36,37] it serves to enhance production of the pro-inflammatory cytokines TNF-α and IL-6 after further dose, an action termed LPS priming [33,36].

The effect of LPS dose on the development of tolerance. The reason that different intravenous doses of LPS induce different mid-term immune responses is related to the nature of the primary immune response to the first challenge. As noted hitherto, initial high doses of LPS (>10 ng/mL) induce the rapid TLR4-mediated expression of pro-inflammatory cytokines [50,51,87] as well as delaying the production of the anti-inflammatory cytokine IL-10, which latter then downregulates the pro-inflammatory immune response [37]. Hence in humans, the immune response to high concentrations of LPS comprises an initial pro-inflammatory phase that takes up to 2 hours and a subsequent anti-inflammatory stage that lasts from 2 to 4 hours [35].

The pro-inflammatory cytokines that are produced during the initial phase of the high dose response influence the maturation of macrophages. LPS tolerant macrophages have the characteristic phenotype of IL-12^{low}, IL-23^{low}, and IL-10^{high} that is typical of M1 macrophages. The pro-inflammatory immune response of these macrophages is suppressed after subsequent dose of LPS [41].

In humans the initial high dose of LPS does not appear to influence the expression of the anti-inflammatory cytokine TGFβ but a subsequent dose leads to an increase in its expression whilst those of TNF-α, IL-6 and IL-10 are reduced [35]. Hence, it seems that IL-10 is necessary for the development of tolerance whilst TGFβ is required for its maintenance.

In contrast, the administration of low doses of endotoxic LPS (<10 ng/mL) does not induce the expression of IL-10, presumably as it fails to induce the delayed anti-inflammatory feedback loop or to initiate the maturation of the LPS tolerant macrophages that are observed after high doses of LPS [37]. Rather, the maturation of non-LPS-tolerant M2 macrophages with the different cytokine phenotype of IL-12^{high}, IL-23^{high}, and IL-10^{low} is supported [37]. These non-LPS-tolerant M2-macrophages demonstrate increased production of TNF-α after subsequent challenge with LPS [37]. Further in murine macrophages, low doses of endotoxic LPS seem to induce the translocation of TLR4 complexes from the cell membrane into the mitochondria where they enhance the production of reactive oxygen species (ROS). These ROS then stabilize the transcription factor ATF2 which supports a prolonged mild pro-inflammatory immune response [18].

In summary, systemic tolerance of LPS is induced by an IL-10-mediated negative feedback loop that commences after initial exposure to high levels of endotoxic LPS. The ambient concentration of LPS which determines whether pro-tolerant or pro-inflammatory macrophages mature, is assumed to be around 10 ng/mL [33,41].

Ancillary mechanisms in the development of tolerance to LPS. A number of other mechanisms may contribute to the downregulation of the TLR4 pathway in addition to the IL-10-mediated negative feedback loop. A reduction in the expression of the LPS receptor TLR4 on the surfaces of murine peritoneal macrophages was observed one hour after initial challenge with a high dose of LPS, and the responsiveness of these cells to subsequent LPS challenge was reduced in a pattern similar to that described hitherto [38]. However, the reduced expression of TLR4 on the surfaces of peritoneal macrophages was thought to result from the internalisation of TLR4 complexes [92] as part of the TRIF-dependent pathway [31]. In contrast, no changes in the surface expression of TLR4 were seen in human monocytes following a similar high dose challenge with LPS [88]. Hence it seems likely that the genetic expression and secretion of TLR4 receptor is better able to keep pace with internalisation in human macrophages than in mouse macrophages.

As described above, the initial steps in the MyD88-dependent pathway that are involved in the response to LPS exposure, include the recruitment of IRAK-4 to MyD88 followed by the activation (phosphorylation) of IRKA-1 [29]. Initial challenge with LPS causes enhancement of activation of IRAK-1 within 5 to 10 min in human monocytes [88] and activates both NF-κB and MAPK pathways [28]. The phosphorylated IRAK-1 is immediately degraded [93] hence IRAK-1 activity returns to baseline 20 min after the LPS challenge [88] downregulating the initial immune response.

In human monocytes, a subsequent dose of LPS does not activate IRAK-1 indicating the development of tolerance [88]. This inability to activate IRAK-1 may result from reduced recruitment of MyD88 to the TLR4 complex [88] as its smaller isoform (termed MyD88s) may compete with the larger isoform for binding with the TLR4 receptor. This spliced variant of MyD88 lacks the small intermediate domain which separates the C-terminal TIR-domain from the N-terminal death domain, so whilst MyD88s is able to link the TLR4 complex with IRAKs, it cannot then initiate the phosphorylation of IRAK-1 [94]. Hence, in isolated monocytes, the lack of the IRAKs recruitment to MyD88 [89] and the consequent inability to phosphorylate IRAK-1 [94] lead to both the inhibition of the MyD88-dependent signalling cascade and the expression of pro-inflammatory cytokines during tolerance phase [38,88]. However, the role of MyD88s *in-vivo* is still unclear. Although the MyD88s that is activated by LPS is highly expressed *in-vitro* in THP-1 monocytes [94] and in leukocytes [34], the levels are only slightly elevated in circulating monocytes obtained from healthy human subjects up to 2 hours after systemic challenge with LPS [34].

A further molecule that may be involved in the development of tolerance to LPS is SHIP (SH-2 containing inositol phosphatase), an inhibitor of the NF-κB signalling cascade. The expression of SHIP is increased via the MyD88-dependent activation of the PI3K pathway after an initial high dosage with LPS [39]. Since low concentrations of LPS fail to induce the negative feedback loop via this pathway [18] it is possible that the lack of SHIP-1 production is responsible for the failure to establish tolerance to endotoxin after exposure to low doses of LPS (ambient concentrations under 10 ng/mL). Further, the elevation in production of SHIP after the challenge of human subjects with high doses of LPS seems to be relevant to the development of LPS tolerance during the initial phase, since the production of SHIP protein is no longer evident 3 hours after stimulation with LPS [34]. However, the levels of SHIP production and the duration of LPS tolerance were correlated in mice after a challenge with high doses of LPS [39] indicating there may be species-specific variations in the mechanism by which tolerance to LPS is maintained.

The maintenance of tolerance to LPS in humans is mediated by IRAK-M. Since no IRAK-1 activity is detected in LPS tolerant monocytes [88] it seems that its activity may be modulated by other inhibitors of the response to challenge with LPS during the later phase or in maintenance of LPS tolerance [34].

IRAK-M is thought to be a negative regulator of the IRAK-1 activity as it interacts with IRAK-1 and subsequently prevents the phosphorylation of IRAK-1 by IRAK-4 [34]. Moreover, IRAK-M is exclusively expressed by LPS tolerant monocytes/ macrophages [34,41]. Since IRAK-M production in humans is induced by high ambient concentrations of TNF-α and the duration of the period of tolerance correlates with the level of IRAK-M *in-vivo* it seems likely that IRAK-M participates in the downregulation of the initial pro-inflammatory phase and in the maintenance of pro-tolerant phase, as long as pro-inflammatory mediators are present initially to support the production of IRAK-M [34].

Other mechanisms involved in the maintenance of tolerance to LPS. An increase in the concentration of circulating LBP in septic mice prevents these animals from mounting a further pro-inflammatory immune response to a subsequent lethal dose of LPS [75]. The fact that AOAH-mediated detoxification of these high levels of circulating LPS may also be involved in the regulation of LPS tolerance is indicated by the finding that AOAH-deficient mice develop prolonged tolerance to a second dose of LPS that lasts for up to 2 months compared to that in wild type mice which remain tolerant to LPS only for 5 to 10 days [86]. Further, the establishment of prolonged tolerance of LPS in AOAH-deficient mice is associated with an increase in their mortality after a second challenge with a pathogen [86]. Together these observations suggest that neutralisation and deacylation of circulating LPS are critical to the appropriate regulation of tolerance to LPS. Hence, fine tuning of the interplay between these two processes and the pattern of immune response, appear critical in the response to any LPS that is released during acute infection with Gram-negative bacteria.

The development of cross-tolerance. Dosage with pure LPS, to ambient levels over 10 ng/mL, can induce tolerance to other bacterial components that were not included in the material used for the challenge. This phenomenon is termed cross-tolerance. Hence, LPS tolerant THP-1 cells develop a cross-tolerance to lipoteichoic acid (LTA) [90] and to macrophage-activating lipoprotein (MALP)-2 [91] a product of Gram-positive bacteria. Both of these agents are ligands of the TLR2 receptor. Conversely, challenge with LTA is not able to induce a tolerance to LPS in THP-1 cells [90]. However, MALP-2 is able to induce tolerance to LPS in murine peritoneal macrophages and in THP-1 cells via reduced activation of IRAK-1 by the MyD88-dependent pathway [91,95]. A similar effect occurs in murine macrophages with the ligands of TLR5, TLR7 and TLR9, whilst double stranded RNA, the ligand of TLR3, induces tolerance to LPS via inhibition of the MyD88-independent pathway [91,96].

The benefits of tolerance to LPS. The increased production of TNF-α following an initial challenge with high doses of LPS induces the migration of polymorphonuclear neutrophils (PMN) to the site of challenge. It seems that in mice, bacterial clearance by PMN is more efficient during the phase of tolerance to LPS [97]. The phagocytic activity of human macrophages is also strongly increased during the phase of tolerance to LPS whereas their antigen presenting capability is reduced [41]. This suggests that the innate antimicrobial activity of these cells is increased, whilst their ability to mediate an adaptive immune response is inhibited. Hence, it is assumed that tolerance to LPS and cross-tolerance to other bacterial components are beneficial for the host as they prevent undue pro-inflammatory immune reaction and limit the

tissue damage during infection [86]. Nevertheless, the duration of tolerance to systemic LPS is critical to the "long-term" regulation of immune response as septic patients have an increased chance of mortality to a second challenge during this period [86].

The immune response to high concentrations of less endotoxic forms of LPS. As discussed above, the pattern of the hosts immune response to LPS is altered, not only by its concentration, but also by its molecular structure. So far we have discussed the immune response to highly endotoxic LPS. Given that several mechanisms have developed to reduce the toxicity of LPS to avoid the overproduction of pro-inflammatory mediators in response to highly endotoxic LPS [86] it follows that the immune response to high levels of less endotoxic (modified) LPS differs from that of challenges with highly endotoxic LPS (see Table 1).

The activation of macrophages depends on the structure of LPS

The maturation of murine peritoneal macrophages in response to challenge with LPS is mediated predominantly by TRIF-dependent pathways [50]. However, the less endotoxic monophosphoryl lipid A (MPL) from *E. coli* induces macrophages to produce TNF-α but not active IL-1β [51]. Given that the release of active IL-1β is concomitant with the onset of endotoxic shock [98] the lack of production of IL-1β may reflect the reduction in the endotoxicity of the LPS.

The release of active IL-1β from macrophages is regulated by two separate steps, the expression of an inactive precursor of IL-1β and the subsequent activation of this precursor. Hence, both pathways must be activated to induce the release of IL-1β [99]. Although MPL is able to induce the expression of IL-1β mRNA and its translation into the IL-1β precursor, it fails to induce the maturation of the IL-1β precursor into its active form as it does not activate the caspase1 [51] that is necessary for the formation of an inflammasome. This last step is a prerequisite to the activation and secretion of active IL-1β [99].

In spite of the reduction in the magnitude of the pro-inflammatory response to less endotoxic LPS, the presence of suitably primed mature macrophages may permit a more prompt response to any further challenge with more endotoxic forms of LPS. All this underscores the importance of luminal dephosphorylation of LPS by IAP and production of monophosphorylated LPS in preventing an unduly robust inflammatory response.

The effect of LPS structure on dendritic cell function. *In-vitro* studies with bone-narrow derived dendritic cells (DCs) and *in-vivo* studies on splenic DCs indicate that neither MyD88- nor TRIF-dependent signalling is necessary for their maturation, but that maximal release of MyD88-dependent cytokines such as TNF-α, IL-6 and IL12p40 as well as TRIF-dependent cytokine IFN-β occur on synergistic stimulation of both pathways. In contrast, IL-10 production by DCs is more influenced by MyD88-dependent signalling than by TRIF-dependent signalling [50]. These findings indicate that whilst maturation of DCs can be activated by structurally different LPS molecules, the TRIF bias that is observed after the exposure to the less endotoxic dephosphorylated LPS will induce only a weak production of pro-inflammatory TNF-α, IL-6 and IL12p40 and anti-inflammatory IL-10. However, this TRIF bias is associated with a relative increase in the production of antibacterial peptides [28] which improve bacterial clearance process. This constitutes yet another example of how modification of the LPS structure by IAP may influence the outcome of the subsequent immune response.

The effect of LPS structure on regulation of the adaptive immune response. DCs have the capacity to regulate the adaptive immune response. It seems that the MyD88-mediated pathway for maturation and production of cytokines in DCs is more critical for DC-mediated presentation of antigen to T cells than is the TRIF-dependent pathway. However, the concerted activity of both signalling pathways achieves a more efficient proliferation of T cells and production of IL-12 by the T cells. Hence, the simultaneous activation of the MyD88- and TRIF-dependent pathways that occurs after challenge with endotoxic LPS, influences both DC and T cell function [50]. Conversely, TRIF bias that is mediated by the vaccine adjuvant MPL* (monophosphoryl lipid A) includes a reduction in both antigen-presentation function by DCs and production of pro-inflammatory cytokines by T cells [100]. Whilst MPL*, like endotoxic LPS, induces the differentiation of T cells into IFN-γ producing T_{h1} cells, it does not upregulate the production of acute-phase protein SAA by the liver. Further, MPL* induces clonal expansion of CD4+ T cells but not the long-term immune response by CD4+ T cells that occurs with endotoxic LPS. The authors of the study suggest that the long-term survival of CD4+ T cells may depend on the level of expression of MyD88-dependent pro-inflammatory cytokines such as TNF-α and IL-6 [100].

Whilst both human and murine DCs mediate the maturation of naïve T cells into T_{h1} cells and also activate $CD8^+$ T cells when stimulated with tetra- and hexa-acylated LPS, stimulation with tetra-acylated LPS also induces differentiation of T_{reg} cells in humans but not in mice. This suggests that there are species-specific variations in the immune response to tetra-acylated LPS [16]. Further, in humans that AOAH-mediated deacylation favours the activation of a pro-tolerant immune response.

In sum, it is evident that the acetylation and phosphorylation patterns of lipid A in LPS affect the response of both innate and adaptive immune cells and that their deacylation/dephosphorylation can influence the subsequent immune response.

The limitations of in-vitro models

The LPS-induced production of ROS by murine macrophages seems to be TLR4-dependent [18] whereas similar ROS production by human innate immune cells seems to be TLR4-independent [101]. Again, the murine TLR4/MD-2 complex interacts differently with tetra-acylated LPS than does the human recognition complex and leads to two different immune outcomes [16,19]. Hence, there are significant species-specific variations in the immune response to LPS.

A number of *in-vitro* models do not incorporate all of the responses to challenge with LPS of which the host is capable. Hence for example, the ambient levels of circulating HDL *in-vivo* are often not considered *in-vitro*. Thus, in human subjects low levels of circulating HDL are associated with a pro-inflammatory and oxidative bias [102] as the corresponding reduction in LBP-mediated neutralisation of circulating LPS and prolongation of its half-life lead to an elevated pro-inflammatory immune response.

As discussed hitherto, IAP is secreted into the lumen to dephosphorylate highly endotoxic LPS molecules with two phosphate groups into less endotoxic LPS molecules [42,48,60] before they enter the circulation and AOAH is secreted by liver cells and immune cells to deacylate circulating endotoxic hexa-acylated LPS molecules [80–83]. Hence, secretions by the host are used to modify the structure of the lipid A moiety of LPS molecules to reduce their endotoxic potential [1]. The total concentration of LPS in the circulation to some extent reflects the permeability of the gut. Given that there is detoxification of LPS both prior to and after entry into the circulation it is likely that the ratio of unmodified to modified LPS within

the circulation will reflect the effectiveness of host in reducing the endotoxic potential of LPS molecules at a given level of permeability. In which case, *in-vitro* models may not adequately indicate the effectiveness of the process of detoxification and subsequent immune response *in-vivo* at different levels of gut permeability.

As we have seen the ratio between the more and the less endotoxic LPS molecules may influence the outcome of the immune response. The ratio may also be influenced by the ratios of the various types of normal commensal microbiota within the lumen. Hence, under normal conditions, whilst *E. coli* is the predominant Gram-negative aerobic commensal species, Gram-negative bacteria of the phyla Bacteroidetes such as *B. fragilis* are generally more numerous [1]. The latter produces greater quantities of less endotoxic penta-acylated LPS molecules with one phosphate group [14] and also expresses polysaccharide A (PSA) which together reduce the consequent pro-inflammatory immune response [9]. Hence, experiments which determine the character and magnitude of the immune response to pure LPS from *E. coli* do not describe those obtained with mixtures of types of LPS obtained from the normal mixture of species of commensal bacteria.

Consequences of the LPS-induced immune response

The human intestinal lumen is inhabited by 10^{14} bacteria, a number that is ten times greater than the total number of cells in the human body. These bacteria live in a symbiotic relation with their host. On-going interactions i.e. cross-talk between the host and commensal bacteria (and/or their chemical components) are essential for the development, maintenance and balance of the intestinal and systemic immune systems [1]. Hence, the gut-associated-lymphoid tissue (GALT) has to fulfil a critical task. On one hand the GALT must tolerate the luminal microbiota and avoid the excessive pro-inflammatory stimulation, on the other hand it must regulate the composition and the number of commensal bacteria to prevent bacterial overgrowth and to protect the host from inappropriate translocation of commensal and pathogenic bacteria and pathogens or their products into the circulation [1]. Again, the hosts immune system must react rapidly and efficiently to counteract toxicity or infection. Hence, a fine tuning of the cross-talk between commensal bacteria and the hosts' intestinal and systemic immune systems is required.

Is the acute pro-inflammatory immune response to systemic endotoxic LPS from commensal used as gatekeeper?

The fact that most pathogens express less endotoxic tetra-acylated LPS molecules may indicate that modification of lipid A by pathogens like *Yersinia pestis* and *Francisella tularensis* is a survival strategy [13]. As discussed in this review, LBP-mediated neutralisation of less endotoxic LPS molecules occurs at a much slower rate than does the neutralisation of endotoxic LPS [74]. This slower neutralisation may enable suitably adapted pathogens to survive and multiply in the host.

These findings invite the question as to why the host has developed mechanisms that are able to clear and to modify LPS from harmless commensal bacteria rather than mechanisms that provoke a pro-inflammatory immune response to pathogens? Whilst the response to LPS is mediated by innate immune system, the response to pathogens that secrete specific toxins that institute necrosis is largely within the domain of the adaptive immunity as such complex and diverse molecules that are produced by then do not trigger an innate immune system. The innate response to LPS and other markers is mediated by archaic heritable immune receptors

that can detect and respond to certain generic molecular signals that indicate the presence of microbiota. Whilst such signals may have originally been entirely defensive, subsequent co-evolution has allowed a degree of symbiosis to become established. Hence, the innate system appears to have evolved mechanism to ameliorate the pro-inflammatory response. This may provide a degree of tolerance to LPS derived from commensals within the gut lumen in order to reap the benefits of symbiosis with normal microbiota.

In spite of this specialisation in GALT, translocation of LPS from the gut lumen into the systemic circulation promotes an innate immune response whose magnitude depends on the degree of exposure and type of antigen. Since, under normal physiological conditions only small quantities of LPS can enter the circulation and the levels of circulating residues of the endotoxic *E. coli* and its LPS are reported to be promptly and significantly elevated during the early phase of an infection [1], it is conceivable that such an increase can still provide indication of degree of infection or dysbiosis.

As discussed in this review, different types of gram-negative bacteria produce different types of LPS with varying degrees of endotoxicity and the host detoxifies them at different rates [42,48,60,80–83]. Hence, the balance between the concentrations of the various types of LPS, may to some extent reflect the ability of the host to detoxify/ neutralise highly endotoxic types of LPS and ultimately determines the characteristic of the immune response.

Whilst extremely high levels of endotoxic LPS in the systemic circulation may produce an overwhelming pro-inflammatory immune response that can be fatal [3], somewhat lower levels of circulating endotoxic LPS may promote a temporary systemic tolerance to LPS. The latter is associated with an increase in the clearance of bacteria by innate immune cells from the circulation [41,97] and my limit tissue damages during infection [86]. Hence, even with exposure to high systemic levels of LPS there appear to be mechanisms that ameliorate immune response to anything but an overwhelming dose of LPS.

Do chronically low levels of LPS represent a long term health risk?

It has been hypothesised that a low grade pro-inflammatory state resulting from on-going exposure to low levels of LPS and perseverated by obesity, may induce insulin resistance and metabolic syndrome [4]. This hypothesis is supported by evidence, firstly that in human subjects, intravenous injection of 3 ng/kg LPS causes the level of TNF-α and IL-6 in plasma to increase along with their gene expression in adipose tissue [103]. Secondly, that elevation of circulating LPS during acute infections leads to a transient increased systemic insulin resistance and a consequent inhibition of the insulin signalling pathway in the adipose tissue but does not influence pancreatic β-cell function [103].

Although the continued presence of low concentrations of endotoxic LPS in the systemic circulation does not immediately lead to the onset of clinical symptoms, it does induce a prolonged low grade inflammation [4]. Hence, chronic low grade elevation of circulating LPS in a mouse model termed "metabolic endotoxemia," is correlated with an increase in subclinical inflammation, the onset of obesity and insulin resistance [4]. Metabolic endotoxemia has also been correlated with the onset of diabetes in humans [6,104].

A number of conditions associated with disorders in the absorption and disposition of LPS may contribute to susceptibility. Hence, IAP-deficient mice have chronically increased gut permeability and elevated levels of serum endotoxin levels compared with those in their wild type littermates [105]. These mice also show glucose intolerance, insulin resistance and obesity and develop type 2 diabetes when maintained on a high fat diet [105].

Diet seems a particularly potent factor in promoting conditions that are associated with chronic subclinical elevation of LPS. Hence, the feeding of a high fat (HF) diet to wild type

mice also leads to the development of metabolic endotoxemia, increased gut permeability and the development of metabolic syndrome including glucose tolerance, insulin resistance, obesity, dyslipidaemia and fatty liver [4,105–107]. Again, when mice that were maintained on a fat diet were given oral supplements with IAP, they did not develop these conditions [105]. Moreover, the same work showed that oral supplementation with IAP reversed established metabolic endotoxemia induced by a HF diet as well as glucose intolerance and insulin resistance [105]. Again, in humans, plasma endotoxin levels were positively correlated with the consumption of fatty meals [71,108–110]. Moreover, host-mediated modification of lipid A within the lumen of the gut has also been shown to have a part in maintaining intestinal barrier function so as to limit the translocation of LPS from the gut lumen into the circulation [105].

Taken together, these results suggest that both acute elevation of circulating LPS and chronic low elevation are deleterious to health. Further that a diet that is high in fat may compromise the efficient detoxification of LPS.

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Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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APPENDIX VI ORIGINAL RESEARCH PAPER – PILOT STUDY

Original Article

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Aspirin-induced increase in intestinal paracellular permeability does not affect the levels of LPS in venous blood of healthy women

Anne Gnauck, Roger G Lentle and Marlena C Kruger

Abstract

The presence of subclinical levels of LPS from Gram-negative bacteria, also referred to as endotoxin, in the circulation may induce a pro-inflammatory immune response that leads to the development of obesity and insulin resistance. Recent data indicate that high-fat meals may elevate circulating levels of LPS. However, it is currently unclear how the LPS transits from the gut lumen to the general circulation. We determined whether aspirin-induced damage of the small intestinal mucosa, evidenced by an increase in the paracellular permeability, allows greater transit of LPS into the systemic circulation. The 3-h cumulative excretion of lactulose was significantly increased after the consumption of aspirin solution relative to that after the consumption of an equal volume of water in 15 healthy women (median after aspirin 0.09% of dose vs. median after water 0.03% of dose; $P = 0.004$). Dosage with aspirin also significantly increased the lactulose : mannitol ratio (median after aspirin 0.014 vs. median after water 0.005; $P = 0.017$). However, serum LPS levels after the consumption of the aspirin solution were not significantly different from those after consumption of the control (plain water). Further, there was no correlation between body fat content and circulating levels of LPS.

Keywords

Aspirin, intestinal permeability, lactulose, lipopolysaccharide, mannitol

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Introduction

A number of publications suggest that chronically elevated levels of circulating endotoxin, also referred to as LPS, may be responsible for the pro-inflammatory status associated with obesity and insulin resistance in mice and humans.[1–3] Endotoxin is the immunoadjuvant fraction of the outer cell membrane of Gram-negative bacteria that is released on their lysis in the intestinal lumen. Plasma endotoxin levels are reported to increase post-prandially after the consumption of a high-fat (HF) meal in both healthy and morbidly obese humans.[4–8] As in a mouse model, low levels of exogenous LPS are reported to have a similar effect in promoting the development of obesity, diabetes and insulin resistance as a HF diet, it has been assumed that elevated levels of circulating LPS are the link between diet-induced inflammation and the development of metabolic syndrome.[1] Hence, it seems that the rate of translocation of bacterial endotoxin from the gut lumen into the circulation may be influenced by diet. However, the mechanism by which a HF meal could facilitate the transit of endotoxin across the intestinal barrier is unclear. One hypothesis is that the ingestion of a HF meal induces oxidative stress in mucosal cells, which leads to local inflammation and subsequent reduction in the integrity of the mucosal barrier, allowing greater quantities of endotoxin to traverse paracellular junctions.[6,9] Published data showing that both oxidative stress and the expression of pro-inflammatory cytokines are increased in circulating monocytes after the ingestion of a HF meal and that both of these effects are accompanied by elevations in the levels of

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plasma endotoxin support such a hypothesis.[5,6] Further, the observation that the consumption of orange juice with a HF meal mitigates the cellular oxidative stress, the rise in pro-inflammatory cytokine expression and the rise in plasma endotoxin level, suggests that the immune response may be influenced by the relative proportions of macronutrients.[9] A similar effect is reported in humans when HF meals are supplemented with a mixture of resveratrol and other polyphenols,[10] suggesting that secondary plant metabolites may be instrumental in this mitigation.

An alternative hypothesis for the transit of endotoxin is that either free or micelle-bound luminal endotoxins are able to cross the mucosal barrier as a result of dietary absorption by enterocytes. The transiting endotoxin may then be incorporated into chylomicrons and released from the basolateral site of enterocytes into the systemic circulation or solubilised and released into the portal venous system. The former hypothesis is supported by evidence indicating that concentrations of endotoxin in the fatty (chylomicron) fraction of plasma from humans are higher than those in the aqueous phase.[7,8,11] This mechanism is further supported by the finding that blocking the formation of chylomicrons in a mouse model prevents the dietary fat-induced increase of circulating endotoxin.[11] Note, it is also conceivable that the accumulation of chylomicrons at the basolateral membrane could subsequently increase paracellular permeability, as it is thought to influence tight junction integrity,[12] and thus could lead to LPS entry via the aqueous and not the fatty phase.

Given that the circulating component that carries the endotoxin varies according to the route by which it is absorbed, fat associated endotoxin that is absorbed transcellulary would be found in the chylomicron fraction whereas water-soluble endotoxin that is absorbed via the paracellular route would be found in the aqueous phase of the serum.

A further hypothesis, based on findings in obese mice, is that the consumption of a HF diet leads to shifts in the composition of the enteral microbiota and a change in the characteristics of the 'cross talk' between microbiota and mucosa that leads, in turn, to a reduction in tight junction integrity with consequent augmentation of paracellular absorption of endotoxin.[13] However, it seems unlikely that a single HF meal would promptly alter the composition of intestinal microbiota.

The aim of the current study was to investigate whether an augmentation in paracellular permeability is accompanied by an increase in the translocation of LPS from the lumen of the gut into aqueous components of serum of healthy subjects. The increased permeability of the gut to larger probes, i.e. lactulose (a molecule with a hydrated molecular volume of 0.262 nm^3)[14] following a single 600-mg dose of aspirin is well described in healthy female subjects.[15,16] This may result from disruption of the mechanical integrity of the enterocyte apex.[17–19] We therefore examined, using a human LPS-specific ELISA, whether, in healthy female subjects, the consumption of this dose of aspirin augmented circulating levels of LPS in the serum from systemic venous blood. This LPS ELISA, rather than the *Limulus* amoebocyte lysate (LAL) assay for endotoxin, was used as the LAL assay may be inconsistent, i.e. dose equivalence between LPS (pg/ml) and endotoxin (endotoxin units/ml) may vary between bacterial species,[20] and hence not consistently reflect the total quantity of circulating LPS. We also examined whether there was any correlation between percentage body fat and LPS levels in the systemic circulation.

Materials and methods

Subjects

Fifteen healthy female non-smokers of mixed ethnicity, aged between 20 and 37 yr (median age 28 yr), with body heights between 1.58 and 1.73 m, and body masses between 55 and 75 kg, were recruited from a fit and healthy population of female postgraduate students and university staff in Palmerston North, New Zealand. Female subjects were used so as to avoid confounding of the result by variation of gastric emptying times and body fat content with gender that could influence the recoveries of lactulose and mannitol.[21,22]

Given prior work linking obesity determined by waist and abdominal circumference and intestinal permeability to lactulose,[23] particular attention was paid to eliminating obese subjects and quantifying any effect from variations in percentage body fat. Hence, subjects with a body mass index (BMI) >30 were excluded from the study. The percentage of body fat of each subject was determined by whole-body densiometry using air displacement plethysmography in a Bod Pod.[24] Further, each subject completed a health screening questionnaire and underwent a clinical interview to exclude those with a family or personal history of gastrointestinal disorders; any endocrine or metabolic disorders, including diabetes; and hepatic disorders or with a history of recent abdominal pain, nausea, vomiting, diarrhoea or of passage of blood and mucus in stool. Subjects who had a history of current urinary tract infections, vaginal discharge, history of aspirin sensitivity or who had recently consumed pre- or probiotic products, and who consumed more than one alcoholic drink per day, were also excluded from the study.

Study protocol and design

Participants were instructed to refrain from consuming any antibiotics for at least 2 wk and from any non-steroidal anti-inflammatory drugs for at least 1 wk

prior to each experimental session. In addition, participants were instructed not to consume any alcohol for at least 3 d prior to each experimental session nor to eat any fried or fatty food, and to avoid exercise on the day prior to attending each experimental session. Participants attended the Human Nutrition Lab at Massey University Palmerston North campus after an overnight fast. On arrival, participants were instructed to empty their bladder into a container; subsequently, a 6-ml sample of venous blood was drawn for baseline measurements. Each participant then ingested either 100 ml of water (negative control) or a single dose of 600 mg soluble aspirin (Dispirin; Reckitt Benkinser Healthcare, Slough, UK) dissolved in 100 ml water.

Each of the two treatments were administered to each subject in randomised order with a 1-wk interval between treatments. One h after each treatment had been administered, each participant consumed a drink containing 10 g lactulose (duphalac; Abbott, Hoofddorp, the Netherlands) and 5 g mannitol (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 100 ml water followed immediately by a further 300 ml of water. Subjects were instructed to urinate into a single container during the subsequent 3-h period so as to bulk their urine over this period. At 3 h after the administration of the lactulose–mannitol solution, each subject was instructed to empty their bladder into the same container. A further venous blood sample was then drawn. The study protocol was approved by the Massey University Human Ethics Committee (Southern A 13/31).

Determination of urinary lactulose and mannitol

The volume of the initial urine sample and the total 3-h volume of urine were determined for each subject. A 15-ml aliquot of each sample was centrifuged at 3500 *g* at 4°C for 10 min, and the supernatant stored at –80°C pending HPLC analysis.

A 1-ml aliquot of each urine sample was diluted with an equal volume of MilliQ filtered water and desalted with 1 g Amberlite resin (Amberlite IRA-410 chloride form and Amberlite IR120 hydrogen form; Fluka Analytical, Sigma Aldrich, St. Louis, MO, USA) in a proportion of 1:1 (w/w). The mixture was then vortexed for 15 s and the supernatant filtered through a 0.2-µm (pore size) cellulose acetate filter (Micro-Analytix Pty Ltd, Auckland, New Zealand) into a 2-ml HPLC vial (Thermo Scientific, Thermo Fisher Scientific, Hemel Hempstead, UK). HPLC analysis was performed in a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) equipped with Degasser Model DGU-20 A, a Pump Model LC-20AT Prominence, an Autosampler Model SIL-20AC, a Column Oven CTO-20 A, a 250 × 4.0-mm cation exchange column (Aminex HPX87C; Bio-Rad Laboratories, Richmond, CA, USA) protected by a Micro Guard Cartridge pre-column (Bio-Rad Laboratories) on a 20-µl aliquot of filtrate from each sample.[15] Filtered MilliQ water was used as the mobile phase at a flow rate of 0.3 ml/min at 590 psi and at 60°C over 20 min for each standard of lactulose and mannitol with a concentration range from 62.5 to 500 µg/ml and over 40 min for each sample filtrate, respectively. The component sugars in each standard and each filtrate were detected with a Refractive Index Detector (Shimadzu RID-10 A).

The concentrations (µg/ml) of lactulose and mannitol in each filtrate were calculated from the areas under the curve relative to those of a series of concurrently run standards. The total quantity of each sugar (mg/ml) excreted in 3 h was then determined by multiplication of the total 3-h urine volume with the dilution factor of the subsample. The quantities of lactulose and mannitol excreted by each subject and each treatment were expressed as a percentage recovery of the administered dose (10 g and 5 g respectively). The lactulose: mannitol ratios (LMR) were determined from the percentages lactulose and mannitol excreted.

Calibration curves for the two sugars were linear up to 500 µg/ml. The accuracy of the quantification of these sugars in our laboratory by HPLC analysis has been previously described.[16] The intra-assay coefficient of variation was in the range of 0.2–7.7% for lactulose and 0.1–4.6% for mannitol.

Determination of LPS

Venous blood was collected into BD vacutainer® serum (6.0 ml; Becton Dickinson and Company, Oxford, UK) and allowed to clot at room temperature over a period of 2 h before centrifuging for 15 min at 3500 *g* at 4°C. The serum was decanted into endotoxin-free microcentrifuge tubes (Biopur® Safe-Lock micro test tubes; Eppendorf, Hamburg, Germany). Serum samples were further centrifuged at 13,201 *g* at 10°C for up to 4 h. Aliquots of each sample were stored at −20°C pending LPS analysis.

Serum LPS concentrations were determined by LPS ELISA (Human LPS ELISA kit; Cusabio Biotech Co., Ltd, Wuhan, P.R. China) according to the manufacturer's instructions. We used only endotoxin-free certified tubes (Biopur® Safe-Lock micro test tubes; Eppendorf) and pipette tips (Endosafe tips; Charles River, Chatillon-sur-Chalaronne, France) for the performance of the LPS ELISA. The assay was reported to have a detection range from 6.25 to 400 pg/ml. Standard curves with known concentrations of LPS between 6.25 and 400 pg/ml were run concurrently with the samples. The results were plotted in CurveExpert Professional version 2.02. The LPS concentration in each sample was determined by comparison with the standard curve. Serum samples spiked with known concentrations of LPS were also assayed using the same procedure to determine

whether the presence of serum proteins influenced the detection of LPS.

The LPS standard curve up to 400 pg/ml was a second-order polynomial. The intra- and inter-assay precisions for the LPS ELISA determined in our laboratories were <10%.

As part of the evaluation of the test prior to its use in this study, a series of 26 serum samples from similar healthy volunteers were each spiked with a known dose of LPS. Analysis of these samples gave values that corresponded to between 85% and 126% (median 97%) recovery of the spiked dose of LPS, indicating that no constituents in the serum sample appeared to inhibit or enhance the assay. In addition, two 'control' serum samples taken from subjects in the current study were each spiked 1:10 (v/v) with 100 pg/ml LPS. The recoveries from these samples both lay within the range of those in the preliminary evaluation.

Statistical analysis

Statistical analyses were performed in the SYSTAT version 13 (Systat Software Inc., Chicago, IL, USA). Differences between treatments in baseline LPS levels were assessed by a paired Student's *t*-test. Differences between baseline and post-treatment levels of LPS after the consumption of water (negative control) and after the consumption of aspirin were also each assessed by a paired Student's *t*-test. Differences between baseline and post-treatment levels of LPS with the negative control and with the treatment with aspirin were assessed by doubly repeated measures ANOVA. The effects of aspirin and the negative control on the excretion of lactulose and mannitol, and on LMR were each compared with a paired Student's *t*-test. Differences were considered significant at the $P < 0.05$ level.

The correlation between the baseline LPS levels taken prior to the control and those before treatment with aspirin was assessed by linear regression and Pearson correlation coefficient and the Bartlett χ^2statistic. Correlations between baseline LPS levels and the excretions of lactulose following treatment with water and following aspirin, respectively, were similarly assessed by linear regression, Pearson correlation coefficient and Bartlett χ^2statistic. Correlation was considered as significant with a *P*-value <0.05.

Bland–Altman plots of LPS levels at baseline and post-treatment following the consumption of water and following the consumption of the solution of aspirin were each assessed for correlation. Bland–Altman plots depict the variations in differences in values obtained with two treatments with the mean of the values obtained with the two treatments and hence are considered to better indicate the consistency of correlation between two parameters than does simple linear regression.[25]

Results

Body composition of subjects

The 15 female subjects were of similar body height (median 1.66 m; range 1.58–1.73 m) but their body mass (median 62.6 kg; range 59.8–74.4 kg), quantity of body fat (median 17.5 kg; range 8.3–27.8 kg), percentage of body fat (median 28.1%; range 13.5–40.7%) and BMI (median 23.1; range 21.1–27.5) varied considerably.

Variations in serum baseline LPS levels in healthy human subjects

The serum LPS concentrations required Asinh conversion by Johnson Transformation in Minitab version 16 to render them amenable to parametric statistical analysis.

The baseline levels of serum LPS varied widely between subjects. There were no significant differences on paired Student's *t*-test in the baseline levels of LPS taken prior to the two treatments. There was a significant linear regression ($df = 1, 13$; $F = 19.920$; $P = 0.001$) and the correlation coefficient between the two was significant ($df = 1$; Bartlett $\chi^2 = 10.420$; $P = 0.001$; $r^2 = 0.752$) (Figure 1). Hence, baseline LPS levels were relatively invariant within subjects.

Given the considerable range in baseline serum LPS levels between subjects, they were grouped on a basis of their magnitude (Table 1). Hence, group 1 ($n = 2$) comprised subjects with no detectable baseline LPS.

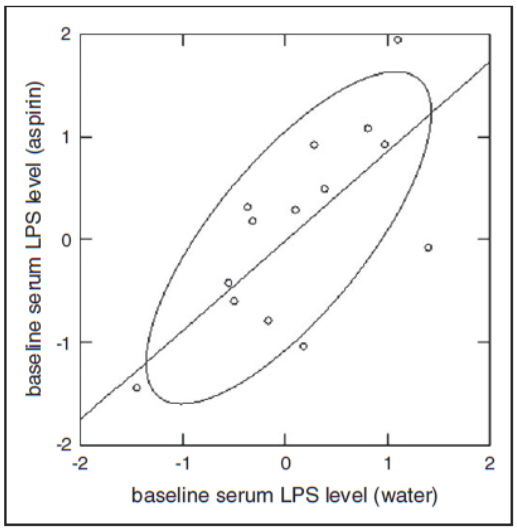

Figure 1. Correlation between successive serum baseline LPS levels from 15 subjects. There was a strong correlation between the two baseline LPS levels within subjects ($r = 0.752$; $P = 0.001$; confidence ellipse of $P = 0.6827$).

Group 2 ($n=8$; 33.03 ± 13.37 pg/ml) comprised subjects with baseline levels of LPS between 0.10 and 0.70 pg/ml, and group 3 subjects ($n=3$; 99.05 ± 12.03 pg/ml) with baseline LPS concentration over 0.70 pg/ml. Two subjects were excluded from this classification as their baseline values fell across groups.

The baseline serum LPS levels were not significantly correlated, either with body mass (kg), body fat (kg and %) or BMI (data not shown). Similarly, control LMRs were not correlated with either percentage of body fat or BMI (data not shown).

Six of 15 subjects reported that they did not consume any alcohol, seven of 15 subjects drank less than three alcoholic drinks/wk and two of 15 subjects drank between three and seven alcoholic drinks/wk. However, there were no significant differences in LPS levels and LMRs between these three groups of subjects (data not shown). No lipid fraction was detectable after centrifugation of serum samples taken from any of the subjects with either of the two treatments. Hence, the quantity of LPS contained within it could not be quantified.

Effect of aspirin on serum LPS levels

There were no significant differences on paired Student's *t*-test between pooled baseline and post-treatment LPS levels after dosage with water. Similarly there were no significant differences between pooled baseline and post-treatment LPS levels after dosage with aspirin solution. Again, there were no significant differences between the three groups in the change in LPS levels from baseline values to those after consumption of water or in the change in LPS levels from baseline values to those after consumption of aspirin solution (Table 1). Similarly, there were no significant differences between treatments on doubly repeated ANOVA of baseline levels of LPS and post-treatment levels of LPS after consumption of water and after the consumption of aspirin solution. Neither were there differences when baseline values were subtracted from the corresponding post-treatment level for aspirin and water treatment and compared by one-way ANOVA (data not shown).

Bland–Altman plots of baseline LPS levels and those after the consumption of water showed that there were no consistent differences between the two sets of readings (Figure 2A). There was also no consistent relationship between the magnitude of the results and the difference between them. Hence, with the exception of the two very high readings, the water treatment (negative control) had no discernible effect on LPS levels.

Similarly Bland–Altman plots of baseline LPS levels and those after the consumption of aspirin solution showed no consistent differences between the two readings (Figure 2B). Again, scatters of the differences between baseline LPS levels and those after treatment did not differ in any consistent manner between the water and the aspirin treatment.

The effect of aspirin on the excretion of lactulose and mannitol

The percentage of recoveries of the administered dose of mannitol were normally distributed whereas these for lactulose and the LMR required Asinh and logarithmic transformation respectively (Johnson Transformation in Minitab version 16) to render them amenable to parametric statistical analysis.

The 3-h excretion of lactulose was significantly increased after the consumption of aspirin on paired Student's *t*-test ($df=14$; $t=-3.454$; $P=0.004$) compared with that after the consumption of water [median aspirin 0.090% of dose, 25–75% interquartile range (IQR) 0.065–0.135% of dose vs. median water 0.030% of dose, 25–75% IQR 0.010–0.045% of dose] (Figure 3A). Conversely, the 3-h excretion of mannitol was not significantly different after the consumption of aspirin from that after the consumption of water (median aspirin 6.294% of dose, 25–75% IQR 5.178–7.385% of dose vs. median water 6.229% of dose, 25–75% IQR 4.553–10.036% of dose) (Figure 3B). Concomitantly, the 3-h LMR was significantly greater ($df=14$; $t=-2.716$; $P=0.017$) after the consumption of aspirin treatment compared with that after the consumption of water (median aspirin 0.014, 25–75% IQR 0.009–0.037 vs. median water 0.005, 25–75% IQR 0.002–0.009) (Figure 3C). The elevated LMR

Table 1. LPS levels at baseline and 4 h after taking water or aspirin solution.

	Water		Aspirin	
	Baseline (pg/ml)	After 4 h (pg/ml)	Baseline (pg/ml)	After 4 h (pg/ml)
Group 1 ($n=2$)[a]	< 6.25	< 6.25	< 6.25	< 6.25
Group 2 ($n=8$)[b]	33.03 ± 13.37	35.43 ± 15.89	37.34 ± 21.25	39.67 ± 20.02
Group 3 ($n=3$)[c]	99.05 ± 12.03	109.21 ± 10.18	141.26 ± 69.40	125.12 ± 79.48

[a]No LPS detectable.
[b]Baseline LPS range 0.10–0.70 pg/ml.
[c]Baseline LPS level > 0.70 pg/ml.

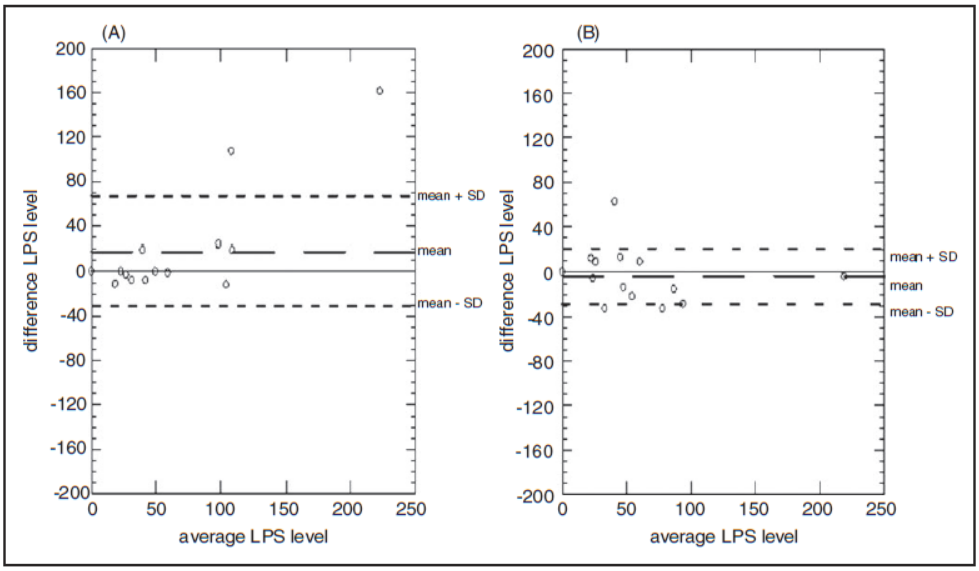

Figure 2. Bland–Altman plots of LPS levels after consumption of (A) water and of (B) aspirin solution treatment. Neither plot shows a significant tend or shift from the mean with increase in magnitude of the mean. Larger (central) dashed line shows mean; smaller dashed lines are at 1 SD from the mean.

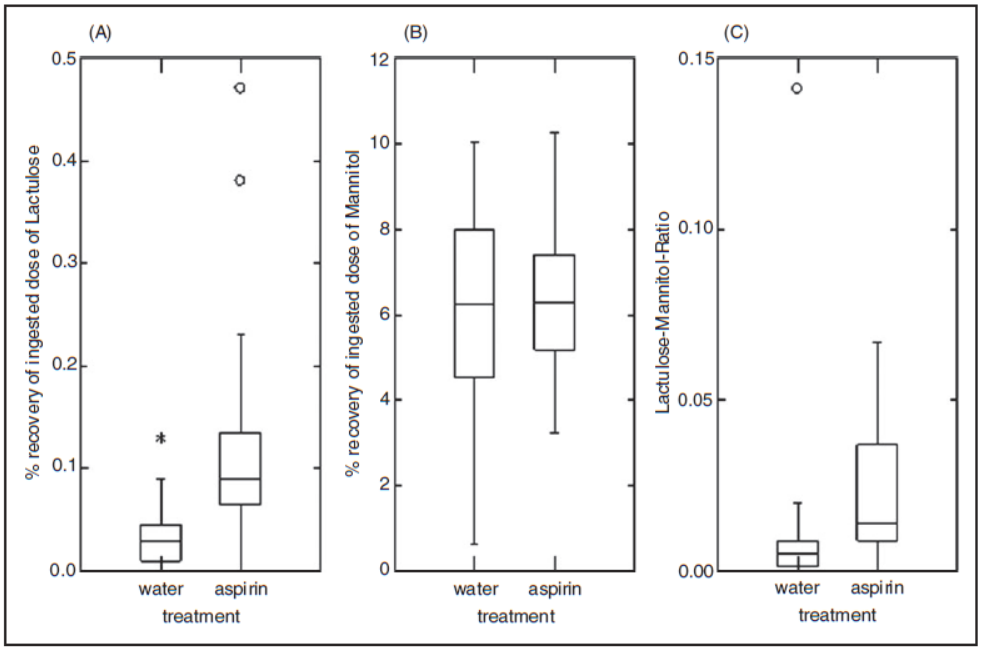

Figure 3. Box plots of percentage 3-h recovery of total dose of (A) lactulose and of (B) mannitol, and (C) change in LMR after consumption of water or of aspirin solution. The excretion of lactulose significantly increased ($P = 0.004$) after the consumption of the aspirin treatment. The excretion of mannitol after the consumption of water did not differ significantly from that after the consumption of aspirin. The LMR was significantly lower ($P = 0.017$) after consumption of aspirin compared with that after the consumption of water.
* – value above 1 SD. ° – value above 2 SD.

of one subject in the water treatment group was due to a greatly reduced excretion of mannitol.

Correlation between lactulose excretion and serum LPS concentration

There was no significant correlation within subjects between baseline serum LPS levels and the percentage of lactulose excretion after consumption of water or after consumption of aspirin solution (data not shown). There was no significant correlation between the differences between baseline and post-treatment levels of LPS with those of lactulose excretion either after consumption of water or after consumption of aspirin solution (Figure 4A). There was also no significant correlation between baseline and post-treatment values for LMR and those of LPS (Figure 4B). Whilst there were three subjects with elevated recovery of lactulose after the consumption of water, the same subject did not have elevated baseline levels of LPS (above 70 µg/ml) before the consumption of water (although three other subject did). Together these results indicate that serum baseline LPS levels were not influenced by paracellular permeability in the small intestine.

Discussion

This is the first study to determine the relationship between paracellular intestinal permeability, as measured by lactulose permeability, and circulating levels of LPS. As the levels of serum LPS that were determined after the consumption of plain water did not differ from those determined prior to the consumption of water it is evident that the consumption of the lactulose and mannitol solution had no effect on circulating levels of LPS. This result is similar to that reported with actively absorbed sugars such as Glucose.[6] Hence, our method is suitable for the evaluation of any correlation between the levels of LPS in the systemic circulation and the permeability of the gut wall.

The high degree of variation in the baseline levels of serum LPS in our sample of 15 apparently healthy female subjects fits with reports of similar levels of variation in plasma endotoxin in 201 men between the ages of 45 and 64 yr.[26] Our results, showing no correlations of serum LPS levels with either percentage of body fat or BMI, are in agreement with those of other studies.[26,27] However it is noteworthy, that the range of percentage of body fat in our subjects (13.5–40.7%) did not include morbidly obese subjects, i.e. these with BMIs >30, while other studies investigating endotoxin levels have included morbidly obese subjects with a BMIs >30.[8]

The finding that the consumption of 600 mg aspirin increases gut permeability, as determined by elevation of the urinary excretion of lactulose, is in line with previous reports.[15,16] Hence, local, aspirin-induced inflammation of the intestinal mucosa is thought to increase mucosal paracellular permeability.[15–17,19,28] However, the fact that the levels of LPS in the systemic circulation were not significantly elevated after consumption of

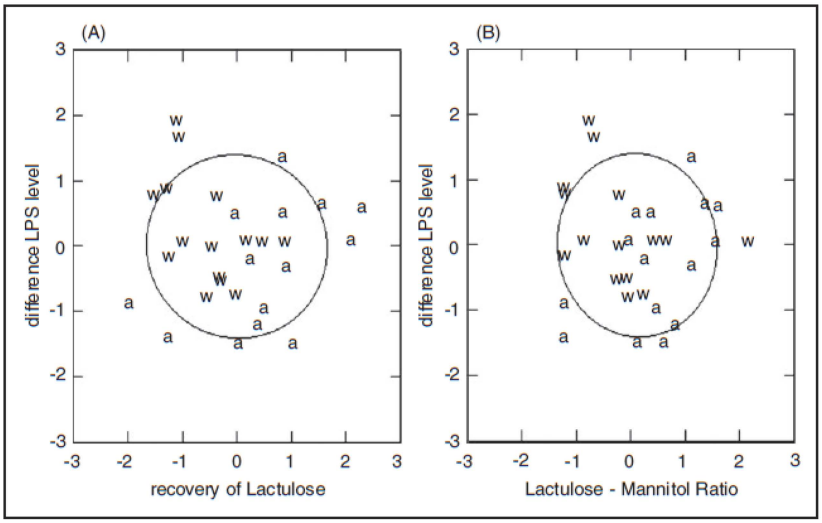

Figure 4. Correlations between percentage recovery of (A) lactulose or (B) LMR value with differences in LPS levels prior to and after the consumption of aspirin solution (a) or water (w). There was no correlation between the recovery of lactulose and the difference in LPS level over the two treatments. Similarly, the LMR did not correlate with the difference in LPS level over the two treatment (confidence ellipse of $P = 0.6827$).

aspirin indicates that such mucosal damage does not influence the ability of LPS to transit from the lumen to the systemic circulation. It is conceivable that an aspirin-induced increase in paracellular permeability did allow greater amounts of LPS to enter the portal circulation via the paracellular route but that this LPS was removed by the liver and hence did not enter the systemic circulation. The latter hypothesis is supported by the finding that significant levels of endotoxin were found in the portal venous blood but not in the systemic blood of human subjects at laparotomy,[29] and similarly by the finding that the levels of endotoxin in portal venous blood were elevated in genetically obese mice with increased intestinal permeability.[30] The systemic venous blood of human subjects with hepatic cirrhosis contains significant levels of endotoxin,[31] while that of subjects with a healthy liver does not.[29] In addition, the concentration of endotoxin in systemic venous blood is reported to be significantly correlated with the level of liver damage in human subjects with non-alcoholic liver disease.[32] Therefore, it seems likely that, in healthy subjects, any LPS in the portal venous blood will be detoxified or removed in the liver before it enters the systemic circulation.[29,30]

It is also possible that LPS is absorbed transcellularly from the gut lumen within fat micelles and incorporated into the chylomicrons.[7,11] Hence, LPS may transit with the chylomicron fraction, via the lymphatic system, to the systemic circulation and avoid detoxification in the liver.[33,34] As our subjects had been fasted overnight their venous blood did not contain a significant chylomicron fraction. Hence, further work is required to determine whether the paracellular transit of lipid associated LPS into the lymphatic and systemic circulation is elevated after the consumption of aspirin.

It is noteworthy that this study was conducted on non-smoking young healthy females and the results may not necessarily apply to males or to persons with BMIs >30. Hence, for example, the magnitude of the effect of a single dose of aspirin may differ in smokers and gut permeability may change in early pregnancy.[35] Again as the level of body fat differs in males the relation of this with LPS and with LMR may differ.

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APPENDIX VII ORIGINAL RESEARCH PAPER – METHOD DEVELOPMENT LAL ASSAY

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Research paper

The Limulus Amebocyte Lysate assay may be unsuitable for detecting endotoxin in blood of healthy female subjects

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ABSTRACT

We examined the factors that may influence the outcome of the Limulus Amebocyte Lysate (LAL) assay, when it is used for quantifying Gram-negative bacterial endotoxin, also referred to as lipopolysaccharide (LPS), in samples of human blood. We found that the method recommended by the manufacturers, based on the reaction time, was inaccurate with any type of serum samples due to the slowing of the initial phase of reaction, likely by serum proteins. We describe an alternative method that is more accurate for use with heated serum samples. Further, we found that components of fresh serum irreversibly sequester endotoxin but that this action may be largely prevented by dilution and heating, but only if this occurs prior to the addition of endotoxin. The tests also indicated that a number of types of proprietary plastic vacutainers appeared to contain significant amounts of endotoxin. However, even when appropriate blood collection containers and calculation methods were used, the levels of endotoxin in serum samples detected by LAL assay were unlikely to reflect the total quantities of endotoxin in that sample and more likely to reflect the capacity of a given serum sample to sequester endotoxin.

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1. Introduction

Endotoxin, also referred to as lipopolysaccharide (LPS), is a component of the outer cell membranes of Gram-negative bacteria that is released into the intestinal lumen on their lysis (Raetz and Whitfield, 2002; Tran and Whitfield, 2009). A number of publications suggest that elevation in the levels of circulating endotoxin may be responsible for the pro-inflammatory status associated with obesity and insulin resistance in mice (Cani et al., 2007a; Cani et al., 2007b; Kim et al., 2012) and in humans (Lassenius et al., 2011; Ghanim et al., 2009). The Limulus Amebocyte Lysate (LAL) assay has been used to determine endotoxin levels in blood samples but its detection is complicated by interference from constituents of blood and blood fractions (DuBose et al., 1980; Levin et al., 1970; Novitsky et al., 1985; Roth et al., 1990; Novitsky and Roslansky, 1985; Webster, 1980). Hence, an accurate method for the detection of endotoxin in human blood is necessary to enable the long term health risks from circulating endotoxin to be accurately assessed.

The LAL assay was originally devised in the 1960s by Frederik B. Bang and Jack Levin. Their assay, the gel-clot method, detected endotoxin based on its ability to promote clotting of the blood of the North American horseshoe crab, *Limulus polyphemus*, in the presence of endotoxin (Levin and Bang, 1964; Levin and Bang, 1968) which results in the formation of solid gel (Levin and Bang, 1968; Novitsky, 1994).

The performance of the LAL assay has subsequently been improved and two other methods, the turbidimetric and the chromogenic LAL assays, based on the same clotting cascade have been developed. The former assay determines the concentration of endotoxin based on the turbidity that develops in the presence of endotoxin and is measured at 360 nm (Fink

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et al., 1981). In the chromogenic LAL assay, the conversion of a pro-enzyme to its active form is catalysed by endotoxin which then splits p-nitroaniline (pNA) from a colourless substrate. The activation of the enzyme and the consequent release of pNA are proportional to the concentration of endotoxin in the sample (Lonza, Walkersville, MD, USA, n.d.). The release of pNA causes the solution to change from colourless to yellow (Iwanaga et al., 1978) which is quantified by absorption at 405 nm. In either the turbidimetric or the chromogenic LAL assay the endotoxin levels can be determined using either an endpoint or a kinetic method.

All of these assays have been approved by the United States Food and Drug Administration (FDA) for the determination of endotoxins in parenterally delivered drugs for humans and animals, for biological products and for medical devices. However, the LAL assays may be unsuitable for the determination of endotoxin levels in blood samples or other human fluids (Lonza, Walkersville, MD, USA, n.d.). Hence, it has been reported that the recoveries of known quantities of endotoxin, spiked to samples of whole blood, were consistently lower in the plasma or serum fractions compared to those when similar volumes of saline were spiked with the same quantities of endotoxin. Early work by Levin indicated that the recovery of endotoxin spiked into blood samples was lower in serum than in plasma (Levin et al., 1970). However, later work by Webster suggested that the process of coagulation necessary for the preparation of serum samples does not sequester endotoxin spiked into blood (Webster, 1980).

The inhibitory effects of plasma and serum on the recovery of spiked endotoxin could result either from interaction of plasma or serum proteins with the reagents used in the LAL assay or from them binding directly to endotoxin. Since the inhibitory effects of plasma and serum on the quantification of endotoxin, either by the gel clot or by the turbidimetric assay, were decreased by diluting endotoxin-spiked samples (DuBose et al., 1980; Novitsky et al., 1985; Novitsky and Roslansky, 1985) the former explanation would seem more likely. On the other hand, given that efficient neutralisation and clearance of endotoxin from the blood stream, e.g. by LPS-binding protein (LPB) and LPS-antibodies, are necessary for human survival (Hailman et al., 1994; Harris et al., 1990; Wurfel et al., 1994) it seems likely that the spiked endotoxin would be sequestered by these constituents. The latter hypothesis is supported by the finding that recoveries of spiked doses of endotoxin were decreased when the interval between the time of spiking of fresh serum with endotoxin and of the subsequent heat inactivation was increased (Novitsky et al., 1985; Novitsky and Roslansky, 1985; Webster, 1980) whereas the recovery of endotoxin that was spiked into sera that had already been heat inactivated did not decrease with increase in incubation time (Webster, 1980). Subsequent studies have shown that chloroform extraction, pH, dilution or a combination of dilution and heat inactivation of plasma or serum prior to the LAL assay all improved the recoveries of spiked endotoxin (DuBose et al., 1980; Roth et al., 1990; Novitsky and Roslansky, 1985; Webster, 1980). Hence, it appears that the sequestration of spiked endotoxin could be reversed to some extent by appropriate treatment of the serum or sample.

Since this earlier work was conducted with LAL reagent that had been prepared by the authors themselves, the aim of the current work was to determine whether the inhibition of the LAL reaction by serum samples would also occur in commercially available standardised LAL assays. In this paper we show that constituents of serum samples and some chemical components in some vacutainers may interfere with the dynamics of the chromogenic kinetic LAL assay. We also report an alternative method for calculating endotoxin content that is better suited for qualification of endotoxin in heat denatured blood fractions than the method recommended by the manufacturer. We also examine the effect of storage temperature and the duration of the heat inactivation of diluted serum samples at various temperatures on the recoveries of known quantities of endotoxin, spiked prior to sample storage, or immediately prior to assay.

2. Methodology

We used two commercially available chromogenic kinetic LAL assays, namely the Endosafe® PTS (Portable Test System) from Charles River Laboratories (Charleston, SC, USA) and the Kinetic-QCL™ LAL kit from Lonza (Walkersville, MD, USA), that are widely used for determination of endotoxin in samples other than blood. Only endotoxin free certified tubes (Biopur® Safe/Lock micro test tubes, Eppendorf AG, Germany) and pipette tips (Endosafe® tips, Charles River, France) were used in both tests and in all associated experimental procedures.

2.1. Subjects and sampling of serum samples

We used two sets of blood samples taken from a total of 18 female volunteers.

For the experiments using the Endosafe® PTS kit (see Section 2.2.2 below), fasted venous blood samples were drawn into plastic 'serum' vacutainers (6.0 ml, Ref. 367837; Becton, Dickinson and Company (BD), UK) twice from each of 15 healthy, non-obese female non-smokers aged between 20 and 37 years of age.

For the experiments using the Kinetic-QCL™ LAL kit (see Section 2.3.2 below), venous blood samples were drawn from three healthy, non-obese, non-smoking female postgraduate students aged between 27 and 32 years. In this case samples were each drawn from each volunteer into a plastic 'serum' and a glass 'serum' (10 ml, Ref. 366430; BD, USA) vacutainer after fasting. In addition blood samples were drawn into plastic 'serum' and into glass 'serum' vacutainers from the second volunteer 1.5 and 3 h after breakfast. Also four samples of postprandial venous blood were each drawn from a third volunteer into both 'plastic' and 'glass' serum vacutainers on different occasions. Both projects were approved by Massey University Human Ethic Committee Southern A (application number 12/61 and 13/31) and informed written consent was given by all volunteers.

All samples were allowed to clot for 2 h at room temperature and subsequently centrifuged at 3500 *g* at 4 °C for 15 min (Megafuge 1.0R, Heraus Instruments GmbH, Hanau, Germany). Raw serum was decanted into endotoxin free micro-centrifuge tubes. Aliquots of raw serum were either analysed on the same day or stored at either +4 °C or −80 °C pending further analysis.

2.2. Determination of endotoxin levels in serum samples using the Endosafe® PTS kit

2.2.1. Method

The Endosafe® PTS kit uses a portable reader machine and an entirely pre-formulated disposable test cartridge that contains a 'built-in' endotoxin standard curve. Two types of cartridges are available, Inhibition/Enhancement Screening (I/E Screen) cartridges and Endosafe® PTS cartridges, both of which contain four sample channels. All four channels of the I/E Screening cartridge and two of the four sample channels of the Endosafe® PTS cartridge contain known concentrations of endotoxin and are used to determine any inhibition or enhancement of the LAL reaction by the sample. In both cases, the cartridge is inserted into the Endosafe® PTS reader machine and 25 µl of each sample is pipetted into the reservoir of the appropriate sample channel. The samples are then pumped by the reader machine into channels containing LAL reagent to start the reaction. The optical densities (OD) of each sample are then determined every second at 405 nm. The sequence of reading is halted once the OD values in all four channels increase by 0.2 units above the initial value. The endotoxin concentration and/or the recoveries of spiked endotoxin in each channel (depending on the type of cartridge) are automatically calculated based on the time in seconds that it takes for the initial OD value to increase by 0.2 units. Endotoxin recoveries in the range between 50% and 200% of the spiked concentration are stated by the manufacturer to be acceptable.

Ten I/E Screen cartridges (lot 2135827) and thirty-three Endosafe® PTS cartridges with a stated detection range from 0.005 to 0.5 EU/ml (three from lot 3163540 and thirty from lot 3120564) were used for this study.

2.2.2. Experimental work

2.2.2.1. Determination of the effect of sample diluent on the recovery of spiked endotoxin. Aliquots of five serum samples from healthy female postgraduate students in plastic 'serum' vacutainers, were thawed and pooled. Aliquots of the pooled serum were then diluted 5, 10, 20, 40 and 100 fold with Endosafe® LAL reagent water (LRW). Aliquots of pooled serum were similarly diluted with Endosafe® Endotoxin-specific Buffer (ESB) and with Endosafe® Dispersing Agent (DA) (all from Charles River Laboratories, Charleston, SC, USA). In addition, one aliquot of the pooled sample was also diluted 30 fold with DA. The diluted samples were subsequently loaded onto I/E Screening cartridges for the determination of recoveries of spiked endotoxin to assess their relative inhibition and the enhancement.

2.2.2.2. Determination of the effect of combined dilution and heating of raw serum samples on the recoveries of spiked endotoxin. Aliquots of the pooled sample similarly diluted with similar diluents were each transferred into endotoxin free glass tubes (depyrogenated pack of Endosafe® 10 × 75 mm Borosilicate Glass Tubes, Charles River Laboratories, Charleston, SC, USA) and heated for 10 min at 80 °C in a heat block (Thermomixer®, Eppendorf AG, Hamburg, Germany). The heated samples were then allowed to cool before being decanted into micro-centrifuge tubes and loaded onto I/E Screening cartridges for the determination of recoveries of spiked endotoxin. The recoveries obtained with the diluted and heated sera were then compared with those obtained with diluted unheated serum to determine their relative inhibition and enhancement. The results were interpolated to find the optimum sample diluent and dilution factor i.e. recovery of spiked endotoxin that was closest to 100% with a minimum of dilution, for use in the subsequent determination of endogenous endotoxin in serum samples using Endosafe® PTS cartridges.

2.2.2.3. Determination of endogenous endotoxin in serum samples and the recoveries of endotoxin spiked into serum samples. Four thawed and 21 fresh serum samples from 15 healthy women were each diluted 1/30 with DA and heated for 10 min at 80 °C in a heating block. Heated samples were allowed to cool before being loaded onto Endosafe® PTS cartridges to assay endogenous endotoxin levels and the recoveries of spiked endotoxin.

2.3. Determination of endotoxin levels using the kinetic chromogenic LAL assay for micro-plate readers

2.3.1. Method

We used the kinetic-QCL™ LAL kit from Lonza (Walkersville, MD, USA) to perform LAL assays using a micro-plate reader. One hundred microliters of each sample or standard was pipetted into a 96-well micro-plate (flat bottomed, sterile non-pyrogenic polystyrene; Coastar® 3596, Corning Incorporated, USA) and incubated in a micro-plate reader (EL_X808_{IU}, BioTek Instruments, Inc., USA) for 10 min at 37 °C. One hundred microliters of freshly prepared LAL reagent was then added to each well. The plate was shaken for 30 s and the optical density (OD) of each sample was read at 405 nm immediately after shaking and every 30 s thereafter for a period of 98 min. Data was subsequently uploaded into Gene5™ software (BioTek Instruments, Inc., USA).

The detection range of the LAL assay was stated by the manufacturer to lie between 0.005 and 50 EU/ml. Standard curves were generated in CurveExpert Professional version 2.0.2 based on the reaction times in seconds of a series of known endotoxin concentrations between 0.00625 and 0.4 EU/ml in LRW. The reaction time was defined, as recommended by the manufacturer, as the time taken for OD to increase by 0.2 units from the initial (baseline) OD value of either the standards or the samples (Lonza, Walkersville, MD, USA, n.d.). The standard curve was subsequently used to determine the endotoxin concentration in each sample in Microsoft EXCEL 2010. The same procedure was used for all experiments with exception of the experiment to determine the effect of dilution of raw serum samples and the experiment to determine the effect of combinations of dilution and heating of raw serum samples on the temporal profile of the reaction (see Sections 2.3.2.2 and 2.3.2.3 below). In these cases OD values were determined over a shorter period and data processed in a different software package. Hence, OD values were read every 30 s for a period of 70 min and 90 min, respectively, and data subsequently uploaded into the KC4 software (BioTek Instruments, Inc., USA). The KC4 software allowed the maximum velocity of the reaction (V_{MAX}) to be calculated from the linear part of the reaction curves of each standard and that of each sample [personal communication with

Douwes J, see (Hollander et al., 1993) for more details]. Standard curves based on the V_{MAX} values were generated for each of the two experiments for standards of known endotoxin concentration in the range between 0.003 EU/ml and 0.2 EU/ml and the curves subsequently used to determine the endotoxin concentration in each sample.

In addition to the quantification based on V_{MAX}, we also generated endotoxin standard curves using the same reaction time method that was used for the other LAL assays. The levels of endotoxin in serum samples were subsequently also determined based on the reaction time. The endotoxin values obtained using the reaction time and V_{MAX} methods were compared and both were also compared with those obtained using a novel method that compared the time for OD to increase from 0.5 OD above the baseline OD to 1.0 OD above the baseline OD (described in details in Section 3.2.4).

Kinetic-QCL™ LAL kits from two different batches were used in the study. The kit from one batch (lot 0000369831) was used for the two experiments that investigated the effect of dilution and the combination of dilution and heating of raw serum samples on assay performance. The remaining experiments used four kits all from batch 0000373909.

2.3.2. Experimental work

2.3.2.1. Assessment of interference by vacutainer additives. Four types of vacutainer, all sourced from Becton, Dickinson and Company (BD) were trialled: a plastic 'serum' vacutainer (6.0 ml, Ref. 367837; BD, UK), a glass 'serum' vacutainer (10 ml, Ref. 366430; BD, Franklin Lakes, NJ, USA), a plastic 'EDTA-plasma' vacutainer (K2E 7.2 mg, 4 ml, Ref. 367839; BD, UK) and a plastic 'heparin-plasma' vacutainer (LH 102 I.U., 6 ml, Ref. 367885; BD, UK). LAL reagent water (LRW) (Lonza, Walkersville, MD, USA, n.d.) and endotoxin free water (ETF water) (Sigma-Aldrich, Sigma® Life Science, Switzerland), respectively, were pipetted directly into appropriate vacutainers after their rubber stoppers had been removed. This procedure averted potential contamination by syringes or their lubricants. The assays were performed after those diluents had been kept overnight in the vacutainers at room temperature.

2.3.2.2. Effect of dilution of raw serum samples on reaction kinetics. The first experiment used raw serum samples from blood that had been collected in plastic vacutainers and stored at −80 °C. The temporal profiles of the LAL reactions obtained using undiluted raw serum were then compared with those using raw serum samples that had been diluted 1/5, 1/10 and 1/20 with LRW or similarly diluted with DA.

2.3.2.3. Effect of a combination of dilution and heating of raw serum samples on reaction kinetics and recovery of spiked endotoxin. The second experiment determined whether the denaturation of serum proteins by heating improved the reaction kinetics of the assay as evidenced by the temporal profile. We used aliquots of the serum samples from the previous experiment that had been diluted 1/5 with LRW and DA, respectively. All diluted serum samples were first transferred into endotoxin free glass tubes (pyrogen free, USP type 1 flint glass, 13 × 100mm, Lonza, Walkersville, MD, USA) and then heated in a water bath for 10 min at either 70 °C, 75 °C or 80 °C. The heated samples were allowed to cool before being decanted into micro-centrifuge tubes for quantification by LAL assay.

Aliquots of heated serum samples were also spiked with 0.5 EU/ml at a ratio of 1/10 (v/v) immediately prior to the LAL assay. The recoveries of the spiked endotoxin were used as an indicator of whether the heated serum inhibited or enhanced detection. An endotoxin recovery in the range between 50% and 200% of the spiked dose was considered acceptable according to the instructions provided by the manufacturer.

The pH values of heated samples that had been diluted with DA were between 7.5 and 8.0 whereas the pH values of heated samples that had been diluted with LRW were between 8.0 and 8.5. Hence, the pH values of the samples that had been diluted with DA lay in the range recommended by the manufactured (between 6.0 and 8.0).

2.3.2.4. Effect of the type of vacutainer on endogenous endotoxin levels in serum samples. Sera from blood of three healthy females that had been collected in plastic and glass 'serum' vacutainers, respectively, were used to determine whether the type of vacutainer influenced the concentration of endogenous endotoxin detected in serum. Serum samples prepared from blood taken into plastic and glass vacutainers were each decanted into endotoxin free micro-centrifuge tubes and stored at −80 °C prior to LAL assay. Raw serum samples were in each case diluted 1/10 and 1/30 with LRW and DA, respectively. The diluted samples were each then transferred into endotoxin free glass tubes and heated in a water bath for 10 min at 70 °C. The heated samples were allowed to cool before being decanted into endotoxin free micro-centrifuge tubes. An aliquot of each heated sample was then centrifuged at 1100 *g* for 15 min (Biofuge pico, Heraeus, Kendro Laboratory Products GmbH, Hanau, Germany). The supernatant of the centrifuged heated sample was again decanted into an endotoxin free micro-centrifuge tube. Aliquots of centrifuged and non-centrifuged heated serum samples were each spiked with 0.5 EU/ml at a ratio of 1/10 (v/v) immediately before LAL assay to determine the recovery of endotoxin.

2.3.2.5. Effect of storage temperature and time of spiking on the recoveries of spiked endotoxin from serum samples. In this experiment serum from the blood of one healthy female volunteer that had been collected into glass vacutainers was used to determine the effect of storage temperature at +4 °C and at −80 °C on the recovery of endotoxin spiked into raw serum samples. Aliquots of raw serum were each spiked with 5 EU/ml at a ratio of 1/10 (v/v) prior to overnight storage at either +4 °C or −80 °C. All samples were subsequently diluted at 1/10 and at 1/20 with DA. The diluted samples were then transferred into endotoxin free glass tubes and heated in a water bath for 10 min at either 70 °C, 75 °C or 80 °C. The heated samples were allowed to cool before they were again decanted into endotoxin free micro-centrifuge tubes.

We determined whether the recovery of endotoxin spiked into raw serum samples prior to storage differed from the recovery of endotoxin spiked into heated serum samples immediately prior to LAL assay by comparing results of this procedure with those spiked immediately prior to assay. Hence, aliquots of the heated serum samples that had not been spiked

with endotoxin prior to storage, were spiked with 0.5 EU/ml at a ratio of 1/10 (v/v) immediately prior to assay.

2.3.2.6. Effect of heating temperature and duration of heating period on the recovery of spiked endotoxin in raw serum samples. We determined whether an increase in the duration of the heating period at either 75 °C or 80 °C, improved the recoveries of endotoxin spiked prior to storage into raw serum samples from blood collected in glass vacutainers. Aliquots of raw serum were each spiked with 5 EU/ml at a ratio of 1/10 (v/v) prior to storage at −80 °C. The raw and spiked serum samples were then diluted 1/10 with DA. The diluted samples were then transferred into endotoxin free glass tubes and subsequently heated in a water bath at either 75 °C or 80 °C for either 10 min, 30 min, 60 min, 120 min or 240 min. The heated samples were allowed to cool before being decanted into endotoxin free micro-centrifuge tubes. As before, the results from the above test were compared with those from aliquots of heated serum samples that had not been spiked with endotoxin prior to storage and then spiked with 0.5 EU/ml at a ratio of 1/10 (v/v) immediately prior to assay.

2.3.2.7. Reliability of the micro-plate LAL assay. Two sets of standard curves were generated the first based on the time it took for the initial OD to increase by 0.2 units and the second on the time taken for the OD to increase from 0.5 OD above the initial OD to 1.0 OD above the initial OD. Standard curves generated by the first method were used to determine whether the assay was valid i.e. had a correlation coefficient from −1.00 to −0.980, slope from −0.400 to −0.100 and y-intercept from 2.500 to 3.500 according to the manufacturers instructions. All parameters of each of the standard curves generated on a basis of reaction time were within the recommendations defined by the manufacturer.

2.4. Statistical analysis

Statistical analyses were performed in the SYSTAT version 13 (Systat Software Inc., Chicago, IL, USA). Variations between the rates of the reactions of the two batches of the LAL kit were determined from the standard curves. Hence, the slopes of standard curves obtained by plotting the log of the endotoxin concentration against time from 0.5 to 1.0 OD above the initial OD, were compared by a two-sample Student's two-sample t-test and overall differences in the y-intercept of these standard curves were assessed by ANCOVA.

3. Results

3.1. Endosafe® PTS method

The recoveries of spiked endotoxin ranged between 85% to 114% in pooled raw serum samples that had been diluted either 1/30, 1/40 or 1/100 with DA. In contrast, the recoveries of endotoxin spiked into raw serum samples that had been similarly diluted with LRW were below 55% and below 25% when ESB was used as a sample diluent. Similarly, only pooled serum samples that had been diluted either 1/30, 1/40 or 1/100 with DA prior to heating step, showed recoveries of spiked endotoxin between 90% and 122%, whereas samples that had been diluted with either LRW or ESB prior to heating step had recoveries below 55% (Table 1). Therefore, only DA was used as diluent for serum samples in all subsequent analysis.

Table 1
Recoveries of spiked endotoxin from diluted serum samples and from diluted and heated serum samples.

Dilution factor	Recovery (%) of endotoxin spiked after dilution			Recovery (%) of endotoxin spiked after dilution and heating		
	LRW	ESB	DA	LRW	ESB	DA
5	19	<11	22	37	26	20
10	55	<11	33	24	21	33
20	26	18	49	30	23	60
30	NA	NA	89	NA	NA	90
40	35	22	114	46	28	122
100	34	22	85	54	27	108

Comparison of endogenous endotoxin levels from heated serum that had been stored at −80 °C with those obtained from serum that had been heated straight after clotting and centrifugation of the blood sample, indicated that fresh serum contained more endogenous endotoxin than did serum samples that had been stored at −80 °C (data not shown). Hence, fresh serum samples were used in subsequent analysis.

The levels of endogenous endotoxin detected in heated fasted serum from 10 healthy subjects ranged between 0.21 EU/ml and 0.96 EU/ml. The recoveries of endotoxin spiked into heated serum ranged between 37.4% and 172.7% (median 107.7%). However, 12 out of 20 unspiked samples had CVs above 20% (range from 0.0% to 55.6%). Similarly, 12 out of 20 spiked samples had CVs above 20% (range from 1.7 to 79%). The analysis was repeated in six out of the 12 unspiked samples. Of these, only two contained similar quantities of endogenous endotoxin to those in the first measurement.

3.2. Micro-plate LAL assay method

3.2.1. Reliability of the assay and inter-batch variation

The intra- and inter-assay levels of precision for spiked and unspiked heated serum samples were below 10%. There were no significant differences between the two batches on the two-sample Student's t-test in the rates of reaction as assessed by the slopes of the standard curves (plots of log endotoxin concentration against time difference). There were however significant overall differences in the y-intercepts of the reaction curves from the two batches as assessed by ANCOVA ($df = 3.180$, $F = 208.1$, $p = 0.002$).

3.2.2. Levels of endotoxin detected in different vacutainers

No endotoxin was detected in either LRW or ETF water after overnight storage in glass 'serum' type vacutainers at room temperature. In contrast, endotoxin was detected in quantities ranging between 0.3 EU/ml and 0.8 EU/ml both in LRW and ETF water that had been kept overnight in plastic 'serum' type vacutainers. Conversely, no endotoxin was detected in either LRW or ETF water had been kept overnight in plastic 'EDTA-plasma' type vacutainers. Endotoxin levels between 0.16 EU/ml and 0.20 EU/ml were detected in both LRW and ETF water when they had been stored overnight in plastic 'heparin-plasma' type vacutainers.

3.2.3. Effect of dilution, and of a combination of dilution and heating of unspiked serum samples on reaction kinetics

The yellow colour of undiluted raw serum caused the initial OD readings of undiluted serum samples to be higher than those obtained from the endotoxin standards, which were dissolved in LRW. However, this effect was reduced by diluting the samples. The shapes of the reaction curves obtained with raw and diluted sera differed from those of the endotoxin standards (Fig. 1A) indicating that dilution alone was not sufficient to reduce interference with the reaction kinetics. However, the shapes of reaction curves obtained with denatured sera were more similar to those of the standard curves indicating that a combination of dilution and heating was sufficient to reduce this interference (Fig. 1B).

3.2.4. Comparison of various methods for calculating endotoxin levels in heated spiked and unspiked serum samples

The levels of endotoxin in unspiked heated serum samples calculated using the method based on V_{MAX} were 10 times greater than those using the method based on the reaction time that was recommended by the manufacturer (Table 2). The recoveries of endotoxin spiked into heated serum samples calculated on the basis of reaction time, ranged between 20% with LRW diluent and 30% with DA diluent. Correspondently the range of recoveries calculated on the basis of V_{MAX} lay between 77% to 88% with LRW diluent and between 88% to 103% with DA diluent (Table 2).

The reaction curves obtained using the endotoxin standards differed from those of endotoxin spiked, heated, serum samples in that the initial phase of the reaction was more prolonged in the latter and the time to the development of a linear colorimetric response was correspondingly longer (Fig. 2A + B). Hence the delay in the commencement of the reaction caused the times taken for the OD to increase by 0.2 units above the initial value, to be longer when samples contained serum than with they contained LRW (endotoxin standards). However, since the recoveries of endotoxin spiked into heated serum were between 77 to 103% of the expected endotoxin concentration when the V_{MAX} method was used (Table 2) it was evident that the prolongation of the initial phase did not influence the slope of the subsequent linear portion of the reaction curve. Correspondingly, concentrations of endogenous endotoxin in heated serum samples and recoveries of endotoxin spiked into heated serum that were calculated using the reaction time method, were lower than those calculated using the V_{MAX} method. It is noteworthy that the reaction curves obtained with standards of lower concentrations of endotoxin did not plateau during the period of the assay. Hence, V_{MAX} could not be accurately determined in those standards.

Comparisons of the reaction curves obtained from standards diluted with LRW with those obtained from spiked and unspiked heated serum samples showed that most became linear between the point at which the OD was 0.5 units to 1.0 units above the initial value (Fig. 3A). The logarithms of concentrations of endotoxin in standards were linearly related to the slopes of the reaction curves when the endotoxin concentrations were between 0.0125 EU/ml and 0.2 EU/ml (Fig. 3B). Thus, we were able to calculate the concentration of endotoxin based on the time difference (in seconds) between the point at which the OD increased by 0.5 from the initial value and the point at which the OD increased by 1.0 from the initial OD value (Fig. 4A) which is a surrogate variable of the rate of the reaction (Fig. 4B).

The levels of endotoxin in unspiked heated serum samples that were calculated using this method were five times greater than those calculated with the reaction time method recommended by the manufacturer and were half those calculated on a basis of V_{MAX}. The values calculated for the recoveries of endotoxin spiked into heated serum using the new method were two to three times greater than those calculated by the reaction time but were generally lower than those calculated on a basis of V_{MAX} (Table 2). However when serum samples

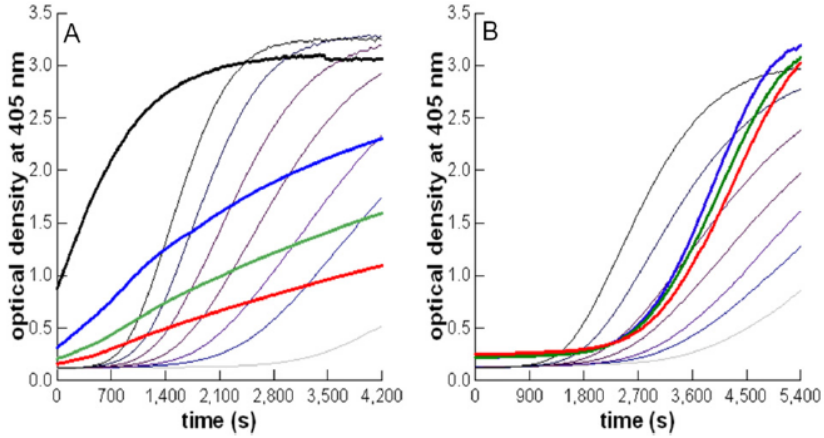

Fig. 1. Effect of dilution, and a combination of dilution and heating of unspiked raw serum samples on their reaction curves. A) Curve for raw (heavy black) and serum samples diluted with LRW (1/5 — heavy blue line, 1/10 — heavy green line and 1/20 — heavy red line) compared to curves for endotoxin standards and plain LRW (light lines). B) Curve for serum sample that have been diluted 1/5 with LRW prior to heating at either 70 °C (blue line), 75 °C (green line) or 80 °C (red line) in comparison to those of the endotoxin standards.

Table 2
Endotoxin levels in heated serum samples and percentage recoveries of spiked endotoxin in heated serum samples calculated on a basis of reaction time as recommended by the manufacturer, by V_{MAX} and by delta time.

Sample preparation		Calculation based on the reaction time		Calculation based on V_{MAX}		Calculation based on the delta time	
Sample diluent	Heating temperature	Endotoxin concentration in EU/ml	Recovery in %	Endotoxin concentration in EU/ml	Recovery in %	Endotoxin concentration in EU/ml	Recovery in %
LRW	70 °C	0.073	22.5	0.847	88.4	0.446	64.1
LRW	75 °C	0.069	19.9	0.610	77.1	0.366	59.4
LRW	80 °C	0.056	19.0	0.575	NA	0.300	54.6
DA	70 °C	0.092	29.7	0.921	102.8	0.446	78.1
DA	75 °C	0.076	26.8	0.778	87.6	0.366	88.3
DA	80 °C	0.045	31.1	0.556	91.4	0.446	52.6

Serum samples were diluted 1/5 either with LRW or DA and heated for 10 min at 70 °C, 75 °C and 80 °C, respectively.

were diluted with DA and heated for 10 min at 75 °C the recoveries calculated using the new method were similar to those obtained with the V_{MAX} method.

3.2.5. The influence of the type of vacutainer on endotoxin concentrations in unspiked serum samples

No endotoxin was detected when heated unspiked serum samples were diluted either with LRW or with DA when the serum had been prepared from blood that was collected into glass vacutainers. Conversely, endotoxin levels around 1 EU/ml were found in unspiked heated serum from blood from the same subject when the blood was collected into plastic vacutainers.

A white sediment was occasionally observed at the bottom of the micro-centrifuge tubes after heated serum samples had been centrifuged. In these cases the supernatant was again decanted into micro-centrifuge tubes before being assayed. However, the levels of endotoxin were found to be identical to those in the uncentrifuged samples. Further, the recoveries of endotoxin spiked into heated serum samples that had been centrifuged did not differ from those that had not been centrifuged (data not shown).

3.2.6. Effect of sample diluent, storage temperature, heating temperature and duration of heating on recovery of endotoxin spiked into heated serum samples

The recoveries of endotoxin spiked into heated serum samples from blood collected in glass vacutainers and diluted with DA, were around 100%. Conversely the recoveries of endotoxin spiked into heated serum from blood collected in glass vacutainers but diluted with LRW were below 50%.

The endotoxin concentrations in unspiked heated serum samples and the recoveries of endotoxin spiked into raw serum samples prior to storage were all significant higher when samples were stored at −80 °C than when they were stored at +4 °C (Table 3). The levels of endotoxin in most spiked serum samples that were subsequently diluted 1/10 and 1/20 with DA and heated for 10 min at either 70 °C, 75 °C or 80 °C, were close to the spiked endotoxin concentration of 0.5 EU/ml. However, in all cases these levels were below the calculated total concentration of endotoxin i.e. the sum of the spike plus the endogenous level detected in the unspiked serum. In contrast, the recoveries of endotoxin were between 88% and 118% of the total calculated concentration of endotoxin in serum samples that had been stored overnight at +4 °C or at

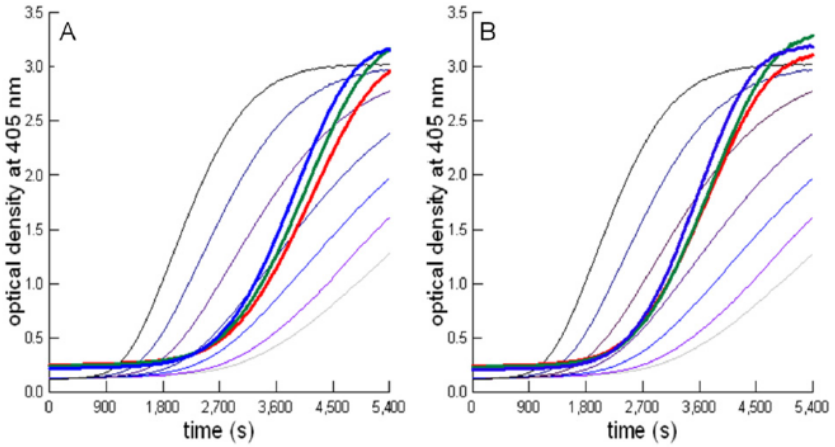

Fig. 2. Comparison of curves of endotoxin standards (light lines) and endotoxin-spiked serum samples diluted 1/5 with LRW (A) and DA (B), respectively, that were subsequently heated to 70 °C (heavy blue line), 75 °C (heavy green line) or to 80 °C (heavy red line) prior to spiking.

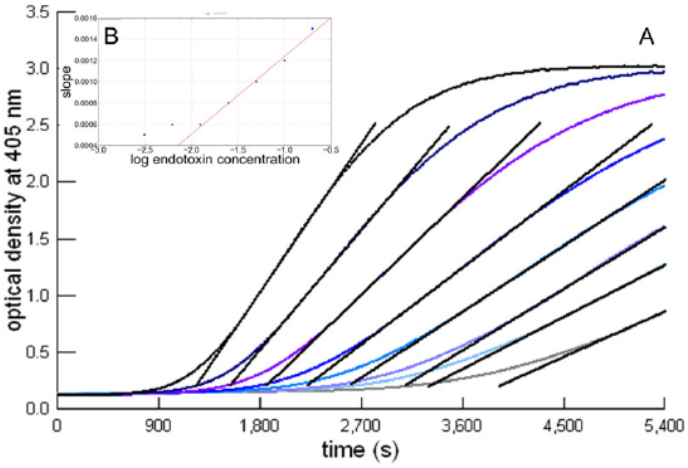

Fig. 3. Standardisation curves for various dose of endotoxin (0.2 EU/ml 0.0031) and plain LRW (0.0 EU/ml) showing the slopes of the linear portion of the curves (A) and the standard curve of log endotoxin concentration vs. calculated slope of the linear part of the reaction curve (B). B) The standard curve generated from the linear portion of the plot between 0.0125 EU/ml and 0.2 EU/ml ($y = 0.0007x + 0.0020$; $r^2 = 0.9918$).

−80 °C, followed by dilution and heating immediately prior to spiking with endotoxin (Table 3).

The recovery of endotoxin spiked into raw serum samples prior to their storage, subsequent dilution and heating, was influenced by the duration of the period of heating and the temperature to which it was heated. Hence the quantities of endotoxin detected in heated serum samples were significantly lower when the heating period was extended from 10 min to 4 h at either 75 °C or 80 °C (Fig. 5A + B). Again, the recoveries of spiked endotoxin were lower when diluted spiked serum samples were heated to 80 °C than that when they were heated to 75 °C.

4. Discussion

We have shown that when spiked heated serum samples were being assayed by the micro-plate LAL assay, the prolongation of the initial phase of the LAL reaction curve caused the time to the development of a linear colorimetric response to be longer than that in the reaction curves obtained when pure endotoxin was dissolved in LRW. Thus it seems likely that soluble components in heated serum influenced the initiation of the colorimetric reaction. Hence the calculation of endotoxin concentration based on the method described by the manufacturer as the time during which OD increases by 0.2 units above its initial value, is not appropriate for the determination of endotoxin even in heated serum samples. However, it appeared that presence of heated serum did not influence the rate of reaction once it had commenced i.e. the linear portion of the reaction curve. Since the reaction curves of standards with low concentrations of endotoxin failed to plateau during the assay period, V_{MAX} could not be determined accurately. Hence, the calculation method based on the V_{MAX} would also be unsuitable for any samples that contained low

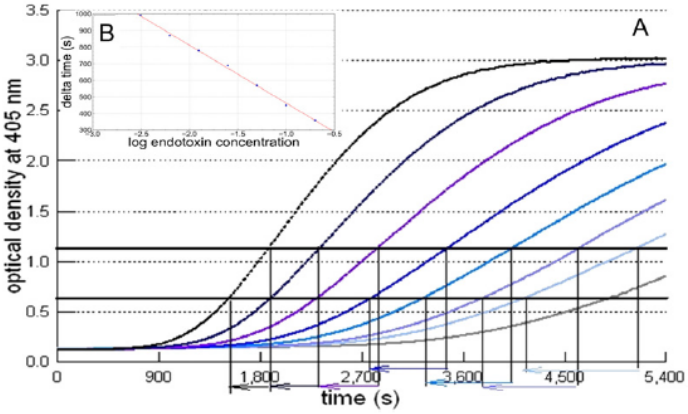

Fig. 4. Reaction curves of endotoxin standards showing the graphical principles underlying the determination of delta time between the time point at which OD reaches 0.5 unit above the initial value and that at which OD rises one unit above the initial value (A) and the corresponding standard curve of log endotoxin concentration vs. delta time (B). The standard curve of delta time vs. endotoxin dose rise between 0.0031 EU/ml to 0.2 EU/ml ($y = -348.80x + 114.05$; $r^2 = 0.9979$).

Table 3
Endotoxin concentrations in heated serum samples that were spiked with 5 EU/ml at a ratio 1/10 (v/v) prior storage overnight at either +4 °C or −80 °C with DA as diluent.

Sample preparation		Calculated endotoxin concentration in EU/ml		Calculated recovery (%) of endotoxin added to denatured serum samples	
Heating temperature	Dilution factor	−80 °C freezer	+4 °C fridge	−80 °C freezer	+4 °C fridge
70 °C	10	0.337	0.161	92.1	96.3
70 °C	20	0.460	0.352	115.5	117.9
75 °C	10	0.461	0.126	88.2	75.1
75 °C	20	0.555	0.352	118.8	107.8
80 °C	10	1.048	0.092	NA	NA
80 °C	20	0.504	0.252	NA	NA

concentrations of endotoxin. Plots of the log of endotoxin concentration vs. the slope of the linear portion of the reaction curve indicated that the relationship became non-linear between 0.0031 EU/ml and 0.0125 EU/ml. This departure from linearity was likely due to the difficulty in identifying the linear portion of the reaction slope when the concentration of endotoxin was low. Hence, the accuracy of quantification of endotoxin based on the calculated slope was no better than with the method based on V_{MAX}. Conversely, the method developed in the current work, that uses the time interval between two OD values, taken at a time where the reaction generally became linear, produces the best correlation between the quantities of endotoxin that were spiked into heated serum samples and the quantities that were detected in those spiked heated serum samples. Further, the standard curve generated by the rate curve of the reaction, i.e. plotting the log of the endotoxin concentration against the time difference between the two designated OD values on the linear portion of the reaction curve, were linear over the entire range 0.003 EU/ml to 0.2 EU/ml. This new method was independent of the time it took to initiate the colorimetric reaction and avoided any effect of serum proteins on its initiation. Further, there was no significant difference between batches in the rates of the reactions as assessed by the slopes of the standard curves when log endotoxin concentration was plotted against time difference. However, there was a significant overall difference between batches in the position of the curves on the y-intercept. This could be due to differences in shelf life between batches. The latter difference would not affect the comparisons conducted in this study as they were all based on differences in rates or surrogate variables of rates within batches. Hence, the new protocol is useful for the assessment of endotoxin levels in samples of heated human serum or plasma although it is difficult to see the circumstances in which this would be useful in studies of endogenous levels of endotoxin in blood, (see below).

The detection, by the micro-plate LAL assay, of significant levels of endotoxin in LRW and ETF water that had been stored overnight in plastic 'serum' vacutainers or in plastic 'lithium heparin-plasma' vacutainers, indicates that these type of vacutainers should not be used for collecting blood for LAL endotoxin assays. Conversely, the fact that no endotoxin was detected in either LRW or ETF water after similar storage in glass 'serum' vacutainers or in plastic 'EDTA-plasma' vacutainers, indicates these could be used for such collections. However, it is noteworthy that EDTA is known to sequester divalent cations in the blood fraction (Dawson, 2005) which can also affect the performance of the LAL assay (Sullivan and Watson, 1974).

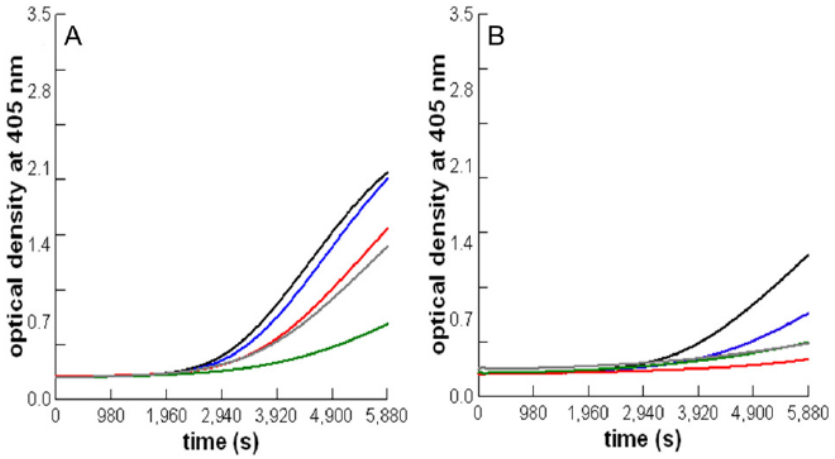

Fig. 5. Reaction curves of serum samples that were diluted with DA at a ratio of 1/10 and heated at 75 °C (A) and 80 °C (B), respectively for 10 min to 240 min. heating times: 10 min – black line, 30 min – blue line, 60 min – red line, 120 min – green line, and 240 min – grey line.

Whether the endotoxin that was detected in plastic 'serum' vacutainers or plastic 'lithium heparin-plasma' vacutainers is the result of contamination of heparin by endotoxin (which is of animal origin), or the result of direct or indirect interactions of vacutainer additives (such as the clot activator in the plastic 'serum' vacutainers and the heparin in the plastic 'heparin-plasma' vacutainers), with the agents that produces the LAL reaction is unclear. Other workers have reported that the quantity of endotoxin in plastic sodium heparin BD vacutainers varies between lots and that no endotoxin is detectable in glass sodium heparin BD vacutainers (Newhall et al., 2010). This suggests that the effect does not result from endotoxin contamination of heparin but perhaps from contamination by plasticizers either in the wall or the bung of the vacutainer. However, whilst endotoxin was detected by other workers in commercial heparin vacutainers, no endotoxin was detected when blood collection tubes that were prepared with endotoxin free heparin (Redl et al., 1992). Thus the LAL test conducted with commercial vacutainers of this type may have been detecting contamination by endotoxin in the added heparin. Regarding the differences in the levels of endotoxin detected in the plastic and the glass types of 'serum' vacutainers, it should be noted that no clot activators are added to glass 'serum' vacutainers but that they are added to plastic 'serum' vacutainers. Since this clot activator interacts directly with the clotting proteins in human blood it is conceivable that it also reacts with clotting proteins in the LAL reagent which are derived from the blood of the horseshoe crab. The likelihood of a false positive from interference with the test components of the LAL assay is supported by data from other work conducted in this laboratory using LPS-ELISA (data not published). This test detected no endotoxin when the sample diluent provided with the kit was kept overnight in plastic or glass 'serum' vacutainers at room temperature. Moreover, this test detected no differences between the amounts of LPS detected in serum from blood collected in glass 'serum' vacutainers and those in serum from blood collected from the same subject at the same time into plastic 'serum' vacutainers (data not published).

Since little or no endotoxin was detected by the LAL microplate assay when a known concentration of endotoxin was spiked into raw serum it seems likely that it was sequestered by serum proteins. The fact that heating serum before spiking with endotoxin leads to recoveries of around 100% indicates that sequestration of endotoxin is inhibited by denaturation. This finding fits in with the prompt clearance of endotoxin by components such as LPS-binding protein (LBP) and anti-endotoxin antibodies from the bloodstream to reduce its pro-inflammatory potential (Hailman et al., 1994; Harris et al., 1990; Wurfel et al., 1994; Munford, 2005). It is conceivable that variations in an individual's capacity to produce such agents and in their potency (Novitsky et al., 1985; Novitsky and Roslansky, 1985) will influence the quantities of 'free' endotoxin that remain in the circulation and are detected by the LAL kit. In order to properly describe any relationship between the quantities of endotoxin that enter the circulation and the quantity of fat in the diet it will be necessary to develop methods that can free any sequestered endotoxins into an environment that is favourable for quantification by LAL or other tests.

Since the Endosafe® PTS kit does not allow the reaction curve to be visualised and automatically quantifies endotoxin on the basis of the time it takes the OD to increase by 0.2 units above the initial value i.e. the initial function of the reaction curve, we could not apply our modified method determining the reaction rate at a later time. Hence the Endosafe® PTS kit is susceptible to substances e.g. serum, that delay the initial of the reaction. Further both the reliability as determined by intra-assay CVs and the reproducibility as determined by inter-assay CVs were much better with the kinetic-QCL™ LAL kit than with the Endosafe® PTS kit. Thus, the LAL reagents in the Endosafe® PTS seem to be more sensitive to the action of extraneous components in the reaction matrix than the LAL reagents in the kinetic-QCL™ LAL kit.

5. Conclusion

The quantification of endotoxin in blood fractions by LAL assay is confounded firstly by the type of blood vacutainer that is used, secondly by the sequestration of endotoxin by serum proteins and thirdly by the method that is used to calculate endotoxin content (Table 4). Further, our data indicate that combinations of dilution and heating are not sufficient to recover significant amounts of spiked endotoxin from raw serum samples. Hence, neither the Endosafe® PTS kit nor the kinetic-QCL™ LAL kit is an appropriate method by which to

Table 4
Overview of key problems with LAL assays.

Source of error	Remedy
Interference by serum with temporal dynamics of the assay	
Raw serum and diluted serum change the reaction kinetics of the LAL assay.	Use a combination of dilution and heating of samples prior to performing the assay.
Inhibitory effect of heated serum samples on the LAL reaction	
Slowing of the onset of the reaction by components in serum causes endotoxin concentration to be underestimated.	Increase the time over which reaction rate is determined.
Vacutainers contaminated with endotoxin	
Significant elevation of endotoxin levels in plastic 'serum' vacutainers and in plastic 'Li-heparin-plasma' vacutainers.	Use glass 'serum' vacutainers or plastic 'EDTA-plasma' vacutainers.
Sequestration of endotoxin by serum constituents	
Endotoxin spiked into raw serum or into diluted raw serum is not reliably detected by LAL assay even after a dilution and heating step. Hence sequestered endotoxin is not released during sample preparation.	Dilution and heating of serum prior to spiking with endotoxin prevents its sequestration. No current method available to dissociate sequestered endotoxin.

assess the total quantities of (sequestered and free) endotoxin in systemic blood. Further, if no suitable method for freeing endotoxin sequestered by components of serum or plasma is found, it may be necessary to use tests that indirectly determine whether there has been long term elevation of endotoxin or LPS. Hence, for example, the elevation of circulating levels of LBP may be more useful in determining the link between fat and circulating levels of endotoxin (Sun et al., 2010; Gonzalez-Quintela et al., 2013).

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APPENDIX VIII REVIEW PAPER – ENDOTOXIN MEASUREMENT

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REVIEW ARTICLE

Chasing a ghost? – Issues with the determination of circulating levels of endotoxin in human blood

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Abstract

Reliable quantification of bacterial products such as endotoxin is important for the diagnosis of Gram-negative infection and for the monitoring of its treatment. Further, it is important to identify patients with persistent subclinical level of bacterial products in their systemic circulation as data from animal studies also suggest this may be correlated with the onset of metabolic syndrome. In this review, we first aim to describe the principles of the Limulus amoebocyte lysate (LAL) test, an assay that is used to quantify endotoxin, and the various shortcomings that must be addressed before it can become a reliable means of quantifying endotoxin in samples derived from blood. We then review published data regarding endotoxin levels in healthy subjects and those with sepsis, inflammatory bowel disease, liver disorders and metabolic disorders such as obesity and diabetes. We also review the evidence regarding influence of macronutrients in augmenting the levels of systemic endotoxin. The results of this review show that reported mean levels of endotoxin in the systemic circulation of healthy humans and of those with various clinical disorders vary over a wide range. Further, this review shows that a significant proportion of this variation can be related to the method that was used to prepare plasma and serum samples prior to assay and its ability to reduce the effect of various blood borne factors that interfere with the LAL assay.

Abbreviations: **ALD**: Alcoholic liver disease; **AOAH**: Acyl-oxyacyl hydrolase; **BMI**: Body mass index; **BPI**: Bactericidal/permeability increasing protein; **CD**: Crohn's disease; **CSE**: Control standard endotoxin; **CV**: Coefficient of variation; **EAA**: Endotoxin activity assay; **EU**: Endotoxin unit; **FDA**: Federal Drug administration of the USA; **GALT**: Gut associated lymphoid tissue; **HDL**: High density lipoprotein; **IBD**: Inflammatory bowel disease; **IgG**: Immunoglobulin G; **IQR**: Inter-quartile range; **LAL**: Limulus amoebocyte lysate; **LDL**: Low density lipoprotein; **LBP**: lipopolysaccharide binding protein; **LPS**: Lipopolysaccharide; **LRW**: Reagent water for use with the LAL assay; **NAFLD**: Non-alcoholic liver disease; **OD**: optical density; **PPC**: Product positive control; **RSE**: Reference standard endotoxin; **TLR**: Toll-like receptor; **UC**: Ulcerative colitis; **WHO**: World health Organisation

Keywords

Blood, endotoxin, Limulus amebocyte lysate assay, LPS

History

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Introduction

The human gut contains around 10^{14} commensal bacteria, a number that is 10 times greater than the total number of human somatic cells. Under normal physiological conditions, these microbiota live in a symbiotic relationship with their human hosts[1]. The majority of gut-derived bacteria and their products that are able to penetrate the intestinal mucosa are promptly detoxified in the liver[2–6]. On occasion, for example after traumatic injury, significant quantities of these bacteria or their products may transit from the hepatic portal to the systemic circulation where they can induce sepsis and septic shock[7] (Figure 1). Further, work with mouse models indicates that other ''non-traumatic'' phenomena such as a fat-rich diet may chronically increase such transit and allow on-going entry of low concentrations of bacterial products in the systemic circulation. In this latter case, it remains unclear whether such elevation results from an increase in paracellular permeability of the gut mucosa[8,9] or from transcellular passage concurrent with the absorption of fatty acids (Figure 1). However, the latter route is favoured as it is thought to culminate in incorporation of the bacterial products into chylomicrons[10,11] with subsequent transport via the

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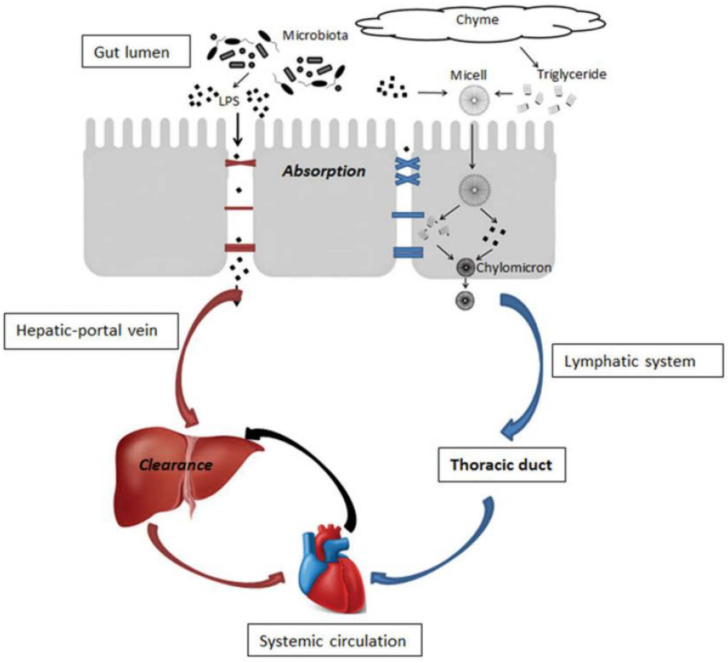

Figure 1. Schematic drawing of the transportation route of bacterial products such as lipopolysaccharide (LPS) from the gut lumen into the systemic circulation. Paracellular (red:left) and transcellular (blue:right) routes of absorption of LPS. The former undergoes immediate detoxification and removal by the liver via the portal circulation, the latter transports LPS via fat directly into the systemic circulation (figure after Figure 1 from Kelly et al.[174] with modifications).

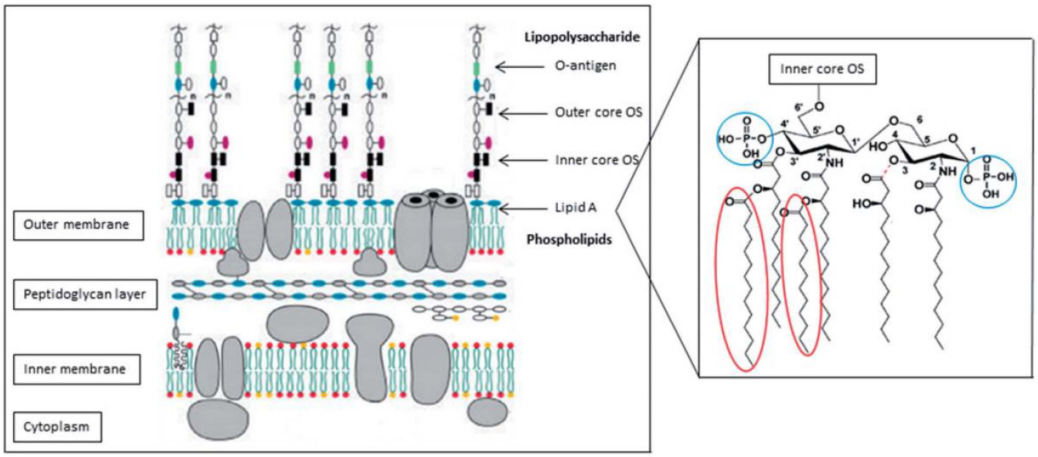

Figure 2. Diagram showing the inner and outer membranes of Gram-negative bacteria with inset enlargement of the lipid A moiety of LPS. The components of the outer membrane comprise an inner phospholipid layer and an outer lipopolysaccharide (LPS) layer. The components of the LPS layer comprise lipid A (inner and outer) core oligosaccharide (OS) and O-antigen (based on Figure 1 of Raetz and Whitfield[21]). The number of secondary acyl chains (red ellipse) on the lipid A moiety (inset) determines the endotoxic potential of LPS. The number and the position of phosphate groups (blue circle) have relative minor effects on endotoxicity (figure after Figure 4 from Raetz and Whitfield[21] with modifications).

lymphatic system and thoracic duct into the systemic circulation, effectively circumventing hepatic detoxification[12–15] (Figure 1).

The continued presence of small numbers of bacteria or their products in the systemic circulation may lead to on-going (local) low grade inflammation, i.e. the chronic production of subclinical levels of pro-inflammatory cytokines by circulating innate immune cells, epithelial cells or adipocytes, and may promote the development of disorders associated with the metabolic syndrome such as insulin resistance, type 2 diabetes, atherosclerosis and cardiovascular disease[16–20]. Lipopolysaccharide (LPS), the major component of the outer cell membrane of Gram-negative bacteria (Figure 2), is of particular interest in this matter as the

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contained lipid A fraction can act as ligand for the immune receptor toll-like receptor 4 (TLR4) that promotes a pro-inflammatory response in immune and other somatic cells[21,22]. However, the intensity of the TLR4-mediated immune response to LPS, i.e. the relative proportions of pro- and anti-inflammatory cytokines that are produced, and hence its endotoxicity, depends upon the chemical configurations of the lipid A moieties that stimulate the immune response, such as the number of secondary acyl chains and the number and the position of phosphate groups (Figure 2) (see recent review[23] for more details). The proportion of these chemical components varies with the organism that produces them[22,24–27]. Hence, the magnitude of the pro-inflammatory response to endotoxic factors in circulating enteric LPS varies according to the relative proportions of the various species of enteral microbiota that produce it and the particular doses of these species. Other factors that determine the type of immune response to LPS include the total dose of LPS[28] and the site at which it interacts with elements of the immune system, notably within the gut associated lymphoid tissue (GALT)[1,29,30] and the systemic immune system[7] (see recent review[23] for more details). The dose of endotoxin within a sample of LPS is quantified either by weight (ng) or in endotoxin units (EU), the use of the latter being currently predominant. The endotoxin unit (EU) reflects the endotoxic activity of the mixture of LPS molecules within a given sample relative to that of the control standard endotoxin. The WHO International Standard for endotoxin currently defines one EU as the biological activity induced in the endotoxin test by around 120 pg of LPS from *Escherichia coli* O113H10:K[31].

The concurrence of on-going low systemic levels of endotoxin with chronic inflammation that was reported in mice[16–20] has not been conclusively demonstrated in human subjects with metabolic disorders as the data varies with the particular medical condition. Hence, the levels of plasma endotoxin in subjects with the hepatic manifestation of the metabolic syndrome, i.e. non-alcoholic fatty liver disease (NAFLD), are reported to be elevated compared to those of healthy controls[32,33]. Conversely, Leber et al. [34] were unable to detect any circulating endotoxin (<0.05 EU/ml) either in obese subjects with type 2 diabetes or in healthy subjects[34], while five other studies reported that the levels of circulating endotoxin were elevated in subjects with type 2 diabetes [35–39]. However, the mean levels of circulating endotoxin reported in the latter five studies ranged widely from 5.19 to 70.73 EU/ml, as did the levels in healthy control subjects from the same five studies, which ranged from 3.89 to 61.06 EU/ml[35–39]. It is important to note that the range of means in the healthy subjects from these five studies was greater and the mean was higher than the means of healthy subjects reported by other workers (Figure 6)[8,9,32,33,40–50] and higher than those reported in patients with NAFLD, sepsis or septic shock (where the latter ranged between 1 and 4 EU/ml, with maximum levels of 12.5 EU/ml)[40,48,50–53]. Further, other studies report that between 89 and 11% of patients with sepsis or septic shock[40,48,51–56] and more than 50% of other ill and hospitalised subjects[57] had no detectable levels of endotoxin in their blood. These high degrees of variation may result from technical difficulties in adapting the currently available assays that are primarily designed for testing endotoxin in pharmaceuticals[58,59] for use with plasma or serum, or may be biological. Hence, a reliable assay of endotoxin in blood is much needed for prompt diagnosis and management of acute sepsis, and for detecting subclinical levels of circulating endotoxin in subjects with impaired gut barrier, liver dysfunction or metabolic syndrome.

In this review, we will discuss the principles underlying use of the current tests for endotoxin and issues that may arise when they are used to determine endotoxin levels in plasma or serum samples. We will then review, in the context of reliability of the results, the body of research that describes the levels of circulating endotoxin in healthy subjects and subjects with various conditions where these levels may be acutely and chronically elevated.

Selection of studies

A systematic literature search was conducted on Medline and Google Scholar for studies that reported circulating levels of endotoxin in healthy subjects and in subjects with various diseases or states that may promote uptake of endotoxin from enteral microbiota in the lumen of the gut. Hence, these included acute sepsis or septic shock, inflammatory bowel disease, i.e. Crohn's disease and Ulcerative colitis, hepatic disorders including acute and chronic hepatitis, alcoholic and non-alcoholic (fatty) liver disease, hepatic cirrhosis (alcoholic and non-alcoholic) and metabolic disorders such as obesity and diabetes (type 1 and type 2). The search also included studies that reported the effects of consumption of various macronutrients that may augment absorption of enteral endotoxins. Further, the reference lists of all identified reports were searched for additional publications that were not directly identified on electronic searches. Studies published in English and German were included in this review.

Only studies that determined endotoxin using either the chromogenic or the turbidimetric method for LAL assay were included. Where the methodology of the LAL assay was not described but references regarding the methodology were provided, the study was classified by methodology only if it could be reliably sourced from the original publication. Studies with either quantitative or qualitative data (either in pg/ml or in EU/ml) were used.

Formal statistical evaluation by meta-analysis could not be undertaken for a number of reasons. First, data from some reports were not normally distributed. Second, the bulking of results was confounded by differences in the methods used to prepare samples prior to the LAL assay. Third, reported levels of endotoxin appeared to vary to a greater degree with sample size and with the method of sample preparation than between a healthy and a diseased state. The results from healthy subjects were grouped on forest plots, with suitable classification and identification of methodologies and other relevant factors, in order to evaluate consistency of results in this respect.

The limulus amoebocyte lysate assay

In the 1960s, Frederik B. Bang and Jack Levin observed that the blood of the North American horseshoe crab,

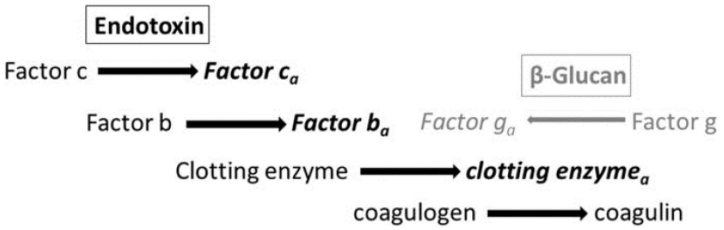

Figure 3. Clotting cascade generated in amoebocytes of the horseshoe crab showing point of activation by endotoxin and of β-glucan. Initiation by endotoxin (black box) is via Factor b and by β-glucan (grey box) via Factor g (based on Figure 1 of Novitsky[175]).

Limulus polyphemus, clots during infection with Gram-negative bacteria[60] presumably to prevent septicaemia. The clotting results from LPS/endotoxin initiating the coagulation of a protein in amoebocytes, (a cellular component of blood in *L. polyphemus*)[61] by a signalling cascade (Figure 3)[62]. Hence, an assay capable of detecting endotoxin was developed from a lysate of amoebocytes[61]. The original gel-clot LAL assay was based on clotting of these components in the presence of endotoxin to form a rigid gel[60,61]. Technological improvements in the isolation and purification of the enzymes and proteins involved in this reaction, led to the development of the turbidimetric and the chromogenic LAL assays in the 1980s. The latter two quantitative assays use the same clotting cascade in an assay based either on an endpoint or on reaction kinetics. In the turbidimetric LAL assay, endotoxin levels are quantified by the turbidity that develops in the presence of endotoxin and is measured by absorption at 360 nm[63]. The chromogenic LAL assay is based on the initial part of the amoebocyte clotting cascade but the coagulogen is replaced by a synthetic chromogenic substrate that is linked with *p*-nitroaniline (pNA)[64], such as Ac-Ile-Glu-Ala-Arg-pNA[65]. Activation of the cascade cleaves pNA from the colourless substrate, creating a yellow product[66]. The intensity of yellow colour is directly and quantitatively proportional to the concentration of endotoxin in the sample[65] and is quantified by absorption at 405 nm[66]. Both the chromogenic and the turbidimetric LAL assays were approved by the US Food and Drug Administration (FDA) in the late 1980s for the quantification of endotoxin in parenterally delivered drugs, for biological products and for medical devices[58]. It is noteworthy that the clotting cascade may also be activated by β-glucan components in the cell walls of fungi, leading to false-positive results (Figure 3). However, greater quantities of β-glucan than those of endotoxin are needed[64].

Problems with the LAL assay

Although the LAL assay was approved by the FDA for use with pharmaceuticals and other medical products, it is not approved for the determination of endotoxin in either human or animal blood plasma or sera or for monitoring other biological fluids, presumably as these samples contain a number of other materials that may compromise the accuracy of the test (Table 2).

Problems with the collection of blood samples

Since bacteria, and bacterial products such as LPS, are ubiquitous, suitable precautions must be taken to avoid any contamination during the sampling of blood, processing of subsamples and execution of the test. Further, any agents that are added to blood collection tubes, such as heparin to prevent coagulation, must be free of contamination with endotoxin. It is noteworthy that commercially available heparinised blood collection tubes have on occasion been reported to be contaminated with endotoxin[67,68]. Again the use of tubes containing agents such as the anti-coagulant EDTA[69,70] or a clot activator[71] that can directly interfere with the LAL assay should be avoided.

Problems with the kinetics of the LAL reaction

Certain components of the liquid phase of blood may also interfere with the LAL assay. The individual components that cause this interference have not been identified. However, this interference is manifest in the differences between the forms of the reaction curves obtained with the kinetic chromogenic LAL assay for endotoxin standards in endotoxin-free water and those assayed in raw or diluted serum[71] (Figure 4A). A range of treatments for serum samples, for example a combination of dilution and heating prior to assay, have been claimed to remove such components and to improve reaction dynamics[71] (Figure 4B). The problem is compounded by the differing sensitivities of the LAL tests, supplied by different manufactures, to interference from components of blood as assessed by ''abnormalities'' of the reaction curves[53].

Further, the forms of the reaction curves, either of endotoxin standards in aqueous solution or of plasma/serum samples, seem to vary between LAL kits from different manufacturers. Similar tardiness in the commencement of the LAL reaction curves of endotoxin standards in aqueous solution to those reported here, has been observed by others in both the gel-clot[61,72] and the chromogenic LAL assays[73,74]. Other work in which a modified chromogenic test from Kabi-Vitrum Diagnostica was used, showed no initial delay and produced reaction curves that were of linear configuration over the first 3 min of the assay, both with plasma spiked with endotoxin and with endotoxin-free water spiked with endotoxin[75]. To the best of our knowledge, this LAL test is no longer available.

It is noteworthy that in the endpoint LAL assay, the reaction is stopped after a given time and the turbidity or the intensity of the colour that develops during that time is used as a basis for quantification. Hence, the operator is not privy to any 'abnormalities' in the kinetics of the LAL reaction. Under such conditions, any effect of untreated plasma or

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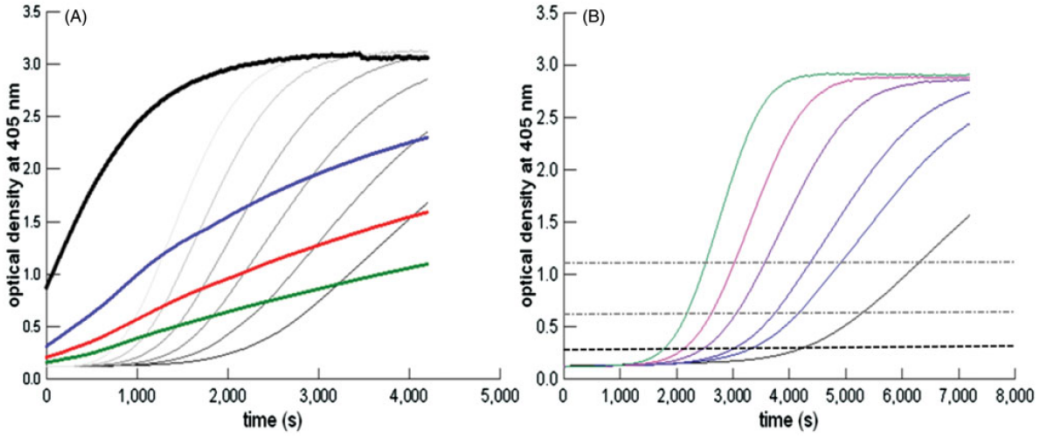

Figure 4. The effect of simple dilution of raw serum samples and of a combination of dilution and heating of endotoxin-spiked serum samples on the reaction curve of the LAL assay. (A) Reaction curves for raw serum (heavy black line) and serum diluted with endotoxin-free water (1:5, heavy blue line; 1:10, heavy red line; 1:20, heavy green line) compared with those for endotoxin standards. (B) Reaction curves of unspiked serum (black line) and endotoxin-spiked serum (+1 EU/ml, green line; +0.5 EU/ml, pink line; +0.25 EU/ml, violet line; +0.1 EU/ml, light blue line; +0.05 EU/ml, dark blue line) that were diluted and heated prior to assay. The portions of the sigmoid reaction curve used in the various methods for quantification are indicated as follows: calculation based on the time taken for the initial OD to increase by 0.2 U, dashed black line; calculation based on the time difference between the points at which the OD is 0.5 U and 1.0 U above the initial OD value, dashed and dotted grey lines. (A) Heavy black line: top; heavy blue line: below; heavy red line: below; heavy green line: bottom; (B) black line: bottom; green line: top; pink line: below; violet line: below; light blue line: below; dark blue line: below.

serum in promoting the commencement of the reaction would go un-noticed.

Problems with the method of calculation

When using kinetic chromogenic LAL assays produced by either Lonza or by Charles River Laboratories, the manufacturers recommend that endotoxin is quantified based on the reaction time, i.e. the time taken for the optical density (OD) to increase by 0.2 units above the initial value (see dashed black line in Figure 4B)[65,76]. While components in raw or diluted serum cause the LAL reaction to commence prematurely and its form to change, the dilution and heating of serum prior to assay is reported to slightly delay the commencement of the LAL reaction compared to that in aqueous solution but not to influence its form, notably the slope of the subsequent linear portion of the reaction curve[71]. Under the latter circumstances, quantification based on reaction time would likely underestimate the levels of endotoxin in diluted and heated serum[71]. However, the effect of a delay in commencement of the reaction may be avoided by suitably adapting the calculation algorithm from the kinetic chromogenic version of the LAL assay. The reaction curves that are obtained with endotoxin standards and with unspiked or endotoxin-spiked, diluted and heated serum, generally become linear between the point at which the OD is 0.5 U and the point at which the OD is 1.0 U above the initial OD value[71] (seen as the portion of the curve that lies between the two dashed and dotted grey lines in Figure 4B). Hence, the time difference in seconds between these two points reflects the rate of the LAL reaction during the linear phase and can be used as a basis for quantification[71]. Alternatively, the maximum velocity of the reaction (V_{MAX}) in the linear part of the reaction curve can be used[73,77] provided that the reaction curves for all standards and samples reach a plateau. The latter condition is necessary as the software supplied with the micro-plate reader appears only to accurately determine V_{MAX} from a reaction curve that reaches a plateaux[71].

Components in plasma or serum may influence the slopes of the endotoxin standard curves obtained on the LAL assay. Hence, the slopes of the standard curves obtained by spiking endotoxin into water differ from those obtained by spiking into plasma. Further, the slopes of standard curves may vary between plasma samples[78]. Previous workers attempted to avoid this problem by constructing standard curves for individual plasma samples[120,121,126,128,130,131,133]. However, some of this work was carried out using an endpoint LAL assay[121,131]where the researcher was not privy to any variation in the time of commencement of the reaction or in the rate of the reaction, i.e. slope.

Problems with the sample diluent

Serum clots and forms a gel when heated at 70–80 °C for 5–10 min (unpublished observation) due to denaturation of serum proteins. Hence, serum samples must be diluted prior to heating to prevent this. However, there are difficulties with maintaining the pH of the diluted serum samples during heat treatment, as the pH of diluted serum generally rises when it is heated (unpublished observation). Typically, the pH rises from 8.0 to 8.5 when the serum is diluted with LAL reagent water (LRW) (Lonza Walkersville, MD) by a factor of 1–10 and then heated at 75 °C for 10 min, and the pH typically rises from 7.5 to 8.0 when serum is diluted 10-fold with Endosafe® Dispersing Agent (DA) (Charles River Laboratories, Charleston, SC) and then heated at 75 °C for 10 min. The optimum pH of the LAL reaction lies between 6.0 and 8.0[65].

Hence, an appropriately buffered sample diluent is required to maintain pH values of serum samples within the stated optimal pH range of the reaction. It is noteworthy that the LRW that is provided with the assay, is of neutral pH and does not maintain the pH of diluted and heated serum within the optimum range whereas that of the DA is slightly acidic (pH around 6) and successfully maintains the pH of diluted and heated serum within the optimum range when samples are diluted by factor 10 or more[71].

Problems with sequestration of endotoxin spiked into serum or plasma

Endotoxin may be sequestered or neutralised by components of the plasma or serum, making it undetectable by the LAL assay. Such sequestration may be mediated by LPS-binding protein (LBP), which acts by forming electrostatic linkages between positively charged amino groups at its reactive site and negatively charged phosphate groups on the lipid A component of LPS[79,80]. LPS can subsequently be transferred from LBP either to CD14 or to HDL or other systemic lipoproteins such as LDL and chylomicrons[81,82]. LPS may also be sequestered by specific antibodies within the immunoglobulin fraction, as well as other antibacterial peptides such as bacterial permeability increasing protein (BPI)[83,84] via similar cationic sites that interact with lipid A. Again, other innate immune cells such as macrophages may phagocytose and deacylate LPS, e.g. via lipase acyloxyacyl hydrolase (AOAH), to detoxify it[85]. Hence, the ''neutralisation capacity'' of a particular serum or plasma depends upon the magnitude of these various binding and detoxifying agents in relation to LPS content[81,84,86–90]. The endotoxin ''neutralisation capacity'' can be quantified by determining the proportion of a known concentration of endotoxin that is recovered following spiking into samples of raw plasma or serum.

Hence, if serum is diluted and heated for 10 min at 75 °C before spiking with endotoxin, the recovery can reach 100% with a kinetic chromogenic assay and suitably adapted method of calculation[71]. Again 90% of endotoxin is recovered when spiked into plasma that has been diluted with endotoxin-free water before the spiked sample was heated at 75 °C for 5 min[75]. However, little or no endotoxin, estimated by either the endpoint or by the kinetic chromogenic LAL assay, is recovered when the endotoxin is spiked into untreated serum or plasma that has not been diluted and heated (the endotoxin presumably having been sequestered by bioactive components in either the serum or plasma)[72,95,96].

Hence, it would appear that a combination of dilution and heating at 75 °C for 5–10 min is sufficient to inactivate the components that sequester endotoxin spiked into plasma or serum. However, the same treatment performed after the spiking of endotoxin into raw serum does not promote its release once it has been sequestered.

Further, such sequestration is not easily reversed once established. It is possible that specific treatments to separate the antigen–antibody complex, notably manipulation of the ionic environment[91,92] or selective digestion of the immune protein may prove useful in this respect. In regard to the latter, work has shown that the digestion of solutions of endotoxin complexed with human IgG by proteinase K improved endotoxin recovery[93], but as far as we are aware this process has not been used with serum or plasma.

A number of other chemical treatments have been investigated. Treatment of spiked raw plasma samples, either with perchloric acid[94] or with an Endotoxin Sample Preparation (ESP™) kit[95], with either an endpoint chromogenic assay[96] or an endpoint fluorescence assay[95], were reported to improve endotoxin recovery rates up to 100%. However, the same treatments have not been reported to be successful either with serum or with the kinetic LAL assay.

Variation in the endotoxin test between manufacturers and between batches

Given that the LAL assay ultimately depends upon the function of a set of blood components obtained from a biological source (North American horseshoe crab, *Limulus polyphemus*)[60,61] it follows there may be variation in the relative proportions and function of these components with source and hence with batch. Manufacturers generally standardise the activity of each batch relative to that of a Control Standard Endotoxin (CSE), i.e. a standard curve based on USP Reference Standard Endotoxin (RSE)[96]. Nevertheless, published work indicates that there is considerable variation between kits produced by different manufacturers. Hence, one group reports significant differences in mean levels of plasma endotoxin in subjects with sepsis or septic shock on assay with kinetic chromogenic LAL kits from two different manufacturers (1.99 and 3.50 EU/ml)[53]. Further, that the reaction kinetics obtained with one kit were less consistent than with the other. Again, the variations in the reaction kinetics in the less consistent kit required ''correction'' by further dilution of the samples.

The differences in intra-assay CVs and in reproducibility determined by inter-assay CVs have been reported when serum samples from healthy subjects were assayed using kinetic chromogenic LAL kits from different manufacturers[71].

Problems with the endotoxin

Apart from the technical difficulties in quantifying levels of endotoxin in blood with the LAL assay, there are difficulties regarding the quantitative relationship between the levels of endotoxin and the levels of LPS in the sample. Ultimately, the term ''endotoxin'' refers to the pro-inflammatory potential of LPS molecules. As discussed previously, the endotoxicities of the LPS is dependent upon the chemical configurations of its lipid A moiety that are identified by, and interact with, elements of the immune system. The endotoxicity of LPS molecules vary considerably with the species of Gram-negative bacteria that produce them[22,24–27]. A meta-analysis of 45 studies published between 1970 and 2013 indicated that the sensitivity of the test for circulating endotoxin in patients with bacterial infections ($n = 3868$) depended upon the species of Gram-negative bacteria that caused the infection[57]. Further, it seems that the sensitivity of the test is higher for products of Gram-negative bacteria that are found in soil and water such as *Pseudomonas aeruginosa* than for those of normal human enteral Enterobacteriaceae such as

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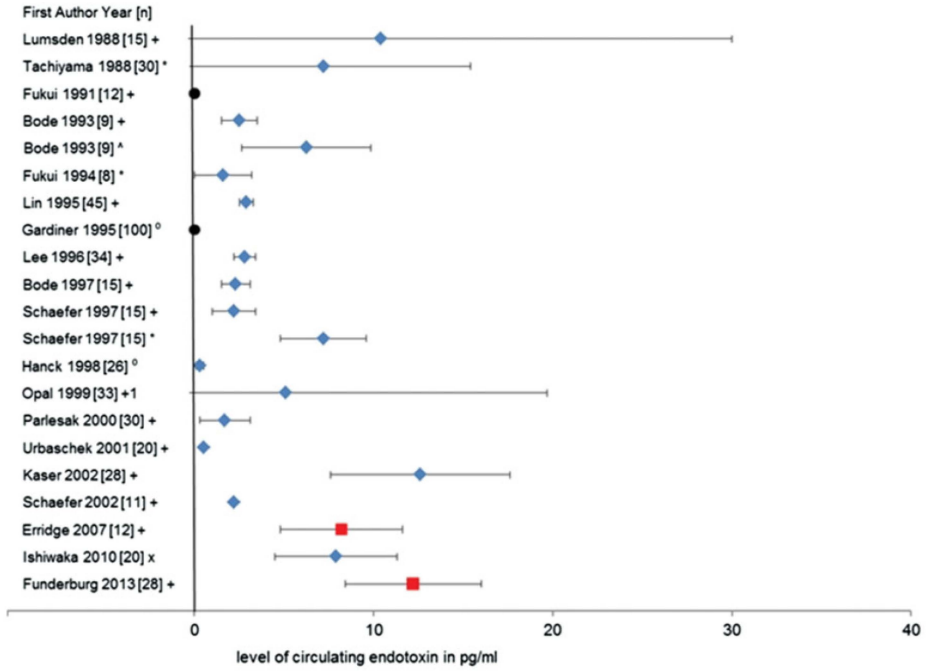

Figure 5. Mean levels of systemic endotoxin reported in pg/ml in healthy subjects. Black circles, no endotoxin detected; blue diamonds, display means and 95% confidence intervals; red rectangles, display medians and 25–75% interquartile ranges. Bracket number next to authors indicates the sample size. Symbols next to the sample size indicate methods of sample preparation: +, dilution and heating; *, perchloric acid method; ^, Tween 80; x, Trition X-100; °, no details provided. Number next to the methods of sample preparation indicates: 1, no endotoxin detectable in more than 50% of the samples. Note: The portions of the 95% confidence intervals that lie below zero have been removed.

Escherichia coli[97]. The fact that the proportion of endotoxin contained within a given dose of LPS varies according to the bacterial species that produces it[25,98,99] leads us to question the relative proportions of the endotoxic epitopes that can be detected by the LAL test and whether these also vary between enteral microbial species. A body of work suggests that in human subjects the level of pro-inflammatory immune response varies with the type of LPS produced by a particular bacteria specie[25,98,99]. However, a complicating factor is that the LAL assay seems to be more sensitive to LPS molecules that are less endotoxic to humans[25], i.e. the epitopes in LPS that have been already detoxified for example by the intestinal alkaline phosphatase that is secreted into the intestinal lumen[12].

The half-life of circulating LPS is short as it is rapidly sequestered by bioactive components such as LBP and BPI[12,83,100]. Indeed, the same mechanism that appears to sequester endotoxin *in vitro* may also operate *in vivo*. At all events, it is difficult to relate the levels of circulating endotoxin to the levels of circulating LPS. Hence, the clinical relevance of the levels of endotoxin in human plasma or serum that are determined by the LAL assay is questionable and hypotheses regarding the effects of on-going low-grade elevation of systemic endotoxins are correspondingly suspect.

The levels of systemic endotoxin in health and disease

In this section, we review and discuss published findings regarding the levels of systemic endotoxin in healthy human subjects and in subjects with conditions where the enteral microbial barrier is compromised and there is consequent acute elevation in circulating LPS. These conditions include sepsis, inflammatory bowel disease and compromised hepatic function. We also discuss work relating chronic low-grade increase in endotoxin levels to the genesis and maintenance of metabolic disorders such as obesity, type 1 and type 2 diabetes.

Our literature search identified a total of 63 publications during the period between 1984 and 2014, in which systemic levels of endotoxin were determined by chromogenic or turbidimetric LAL assay, either in healthy subjects or in subjects with the disorders mentioned above.

Systemic endotoxin levels in healthy humans

The literature search identified 47 studies in which the levels of systemic endotoxin were determined in healthy subjects. Forty-five mean/median plasma or serum endotoxin levels for healthy subjects were found in a total of 38 publications. Of these, 19 studies reported levels in pg/ml (reported

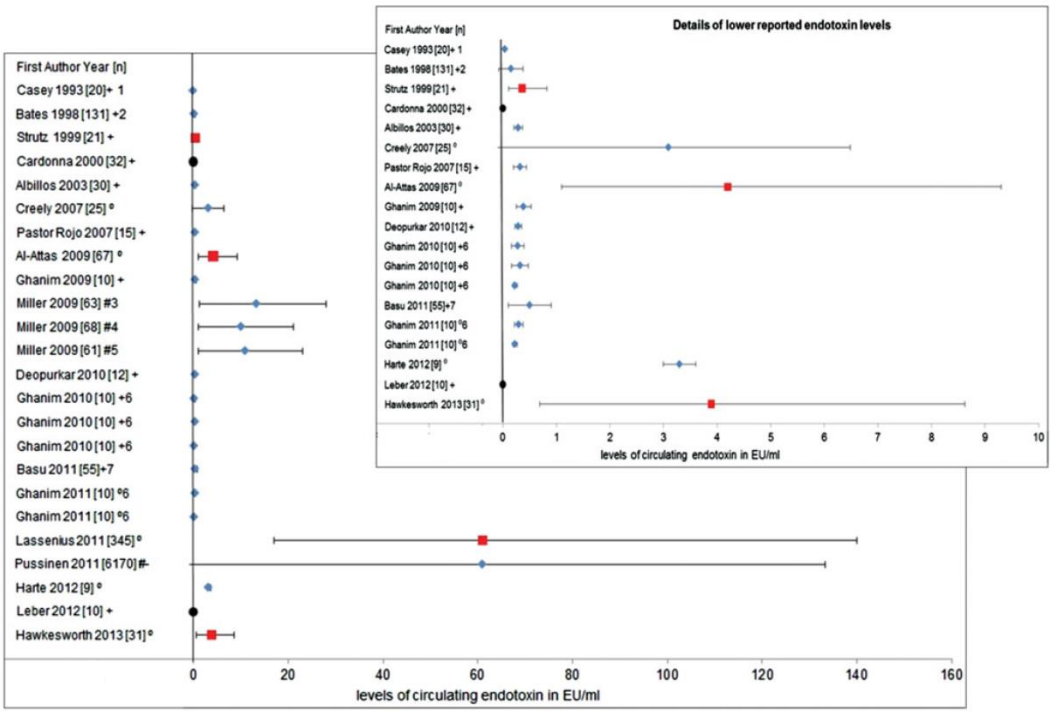

Figure 6. Mean levels of systemic endotoxin reported in EU/ml in healthy subjects. Inset shows reports with values below 10 EU/ml. Black circle, no endotoxin detected; blue diamond, display mean and 95% confidence interval; red rectangle, display median and 25–75% interquartile range. Bracket number next to authors indicates the sample size. Symbols next to the sample size indicate methods for the preparation of samples: + dilution and heating, # simple dilution, ° no details provided. Numbers next to the method of sample preparation indicate as follows: 1, no SD given; 2, levels determined in whole blood sample; 3, levels of British with South Asian ethnicity; 4, levels of British with African ethnicity; 5, levels of British with Caucasian ethnicity; 6, fasted levels of different treatment groups; 7, levels of pregnant women. Note: The portion of the 95% confidence intervals that lie below zero have been removed.

mean/median levels: $n = 21$; Figure 5) and 19 studies in EU/ml (reported mean/median levels: $n = 24$; Figure 6). The data of another study were not included in the forest plot as the data were from the same study population as reported in a separate paper[48]. Further, eight studies were identified in which data were only presented in figures[33,101] or in which either quantitative data, i.e. the percentage of samples with detectable level of endotoxin[32,55], or qualitative data, i.e. the range of detectable levels without any information about the mean or median[49,95,102,103], were given. The values covered a broad range that varied significantly according to method used to prepare the plasma or serum samples prior to assay (see below).

Three out of the 47 studies failed to detect any endotoxin (<0.005 EU/ml, < 0.015 EU/ml or < 10 pg/ml) in plasma or sera from healthy subjects[34,47,104]. In three other studies, the proportion of healthy subjects with detectable levels of systemic endotoxin ranged from 16% to less than 50%[32,48,105].

The overall mean levels of systemic endotoxin in healthy subjects ranged widely from 0.15 to 61 EU/ml [median 0.32 EU/ml, 25–75% interquartile range (IQR) 0.23–3.89 EU/ml; Figure 7A) or ranged from 0.5 to 65 pg/ml (median 5.1 pg/ml, IQR 2.2–7.9 pg/ml; Figure 7B).

In the studies that reported data in pg/ml, various methods were used to pre-treat the plasma or serum prior to LAL assay; 15 used a combination of dilution and heating, six a treatment with perchloric acid, one a treatment with Tween 80 and one a treatment with Triton X-100. Two of the studies provided no information regarding the method that was used for the pre-treatment of the samples[104,106] (Figure 5). The distributions of the mean levels of systemic endotoxin (in pg/ml) from healthy subjects varied according to the method that was used (Figure 7A). The median of the mean levels of plasma endotoxin from healthy subjects was slightly higher when samples were treated with the perchloric acid method (median 6.6 pg/ml, IQR 3.8–7.2 pg/ml) or with Tween 80 or Triton X-100 prior (mean 7.075 pg/ml) (Figure 7A) than when they were treated with dilution and heating (median 5.1 pg/ml, IQR 2.2–10.4 pg/ml) prior to the LAL assay. Hence, with all treatments, the mean levels of systemic endotoxin were generally below 10 pg/ml in healthy subjects (Figure 5).

The studies that reported results in EU/ml used either a combination of dilution and heating ($n = 11$) or dilution alone ($n = 2$) for sample preparation (Figure 6). Six of these studies provided no details regarding the method for preparation of the samples (Figure 6). The median of the

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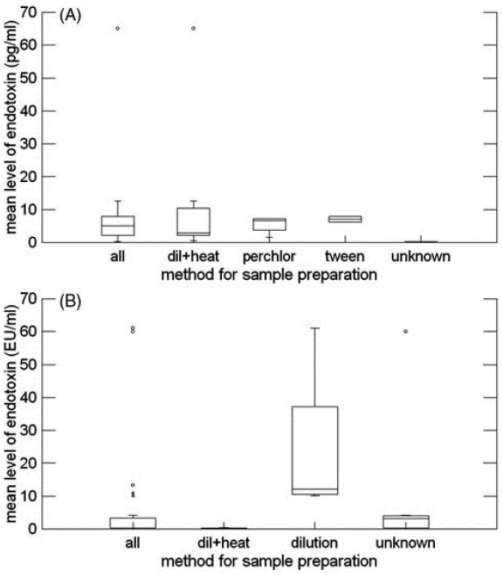

Figure 7. Reported mean levels of systemic endotoxin in healthy subjects classified by method of preparation of samples prior to assay. (A) All publications that reported endotoxin levels in pg/ml ($n = 21$). Dil + heat, diluted and heated samples ($n = 14$); perchlor, samples pre-treated with perchloric acid ($n = 4$); tween, samples pre-treated with either Tween 80 or Trition X-100 ($n = 2$); unknown, data from work that provided no details regarding method used for preparation of samples; $n = 1$). (B) All publications that reported endotoxin levels in EU/ml ($n = 24$). Dil + heat, diluted and heated samples ($n = 12$); dilution, samples diluted with endotoxin-free water ($n = 4$); unknown, no details provided method used for preparation of samples; $n = 8$). * – value above 1 SD. ° – value above 2 SD.

mean levels of systemic endotoxin in healthy subjects was higher, and the distribution wider, when samples were simply diluted (mean 12.10 EU/ml) than when samples were diluted and then heated (median 0.29 EU/ml, IQR 0.177–0.34 EU/ml) or in the group where methods of treatment were not reported (median 3.195 EU/ml, IQR 0.265–4.045 EU/ml; Figure 7B).

Taken together, these results indicate that the method that was used for the plasma or serum sample preparation prior to the LAL, had a marked effect on the levels of endotoxin detected and, in the case of dilution alone, markedly increased the range. It is therefore difficult to determine whether significant levels of endotoxin are normally present in the systemic circulation of healthy persons, and if so, what the levels are. Further, in studies of disorders that involve acutely and chronically elevated levels of systemic endotoxin, it is important to compare the levels obtained with those of healthy individuals that were determined using the same method of sample preparation, preferably concurrently.

Acute elevation of systemic endotoxin

Sepsis or septic shock is a condition characterised by an overwhelming pro-inflammatory immune response to an acute elevation of systemic endotoxin, which can be fatal[7]. We found a total of 12 studies that had reported some form of data regarding systemic endotoxin in patients admitted to hospital with sepsis or septic shock. Significant levels of systemic endotoxin were detected in the circulation of between 11 and 89% of patients in these studies[40,46,48,50–56,105,107]. Hence, circulating endotoxin was not detected in all patients. All these studies had endeavoured to eliminate any source of endotoxin contamination, i.e. had used endotoxin-free blood collection tubes and a strict protocol that avoided endotoxin contamination of any sources.

Some of this variation may result from variation in the levels of the factors that sequester endotoxin such as circulating levels of LBP, BPI and the relative proportion of certain lipoproteins[81,84,86–89]. Further, some patients may have developed temporary tolerance to LPS, a condition that is accompanied by an increase in the rate of bacterial clearance[108–110]. However, it is hard to find a biological explanation for the complete absence of endotoxin in some sera.

Four out of the 12 studies did not provide the mean or median levels of systemic endotoxin[46,54,55,107]. One other study reported a median value of 300 pg/ml, the choice of statistical parameter suggests that the values were not normally distributed[105]. The remaining seven studies provided mean levels in EU/ml. The median of the mean levels of systemic endotoxin reported in these seven studies was 1.99 EU/ml with an IQR from 0.84 to 3.47 EU/ml (Figure 8B)[40,48,50–53,56]. It is noteworthy that in one study, the levels of endotoxin detected in plasma of subjects with sepsis or septic shock were tested with two LAL kits from different manufactures, which gave mean values of 1.99 and 3.50 EU/ml, although the significance of this difference was not reported[53].

Five of the 12 studies compared the systemic levels of endotoxin detected in patients with sepsis or septic shock with those of healthy control subjects that were determined concurrently by the same method. The mean levels of systemic endotoxin in these healthy subjects ranged from 0.04 to 0.36 EU/ml in four studies[40,46,48,50] and was 5.1 pg/ml in the other study[105], while the mean levels detected in subjects with sepsis or septic shock in these four studies ranged from 0.96 to 3.45 EU/ml[40,46,48,50] and was 300 pg/ml in the other study[105].

Six of those seven studies that reported mean endotoxin values in EU/ml and the one study that reported median endotoxin level in pg/ml determined the levels of systemic endotoxin with reference to endotoxin standards prepared by spiking endotoxin into endotoxin-free pooled plasma from healthy donors[40,50–53,105]. Two of these six studies also repeated the analysis when the levels of recovery of product positive controls (PPCs) (endotoxin spiked into diluted and heated plasma/serum) were below 75% or above 125%[52,53]. Hence, the reported levels of endotoxin in subjects with sepsis or septic shock seem reliable. Taken together, it seems that higher levels of systemic endotoxin are detected in plasma from patients with sepsis or septic shock than in healthy controls. However, it is noteworthy that different proprietary kits were used in these studies and thus there may be superadded variation from differences between kits.

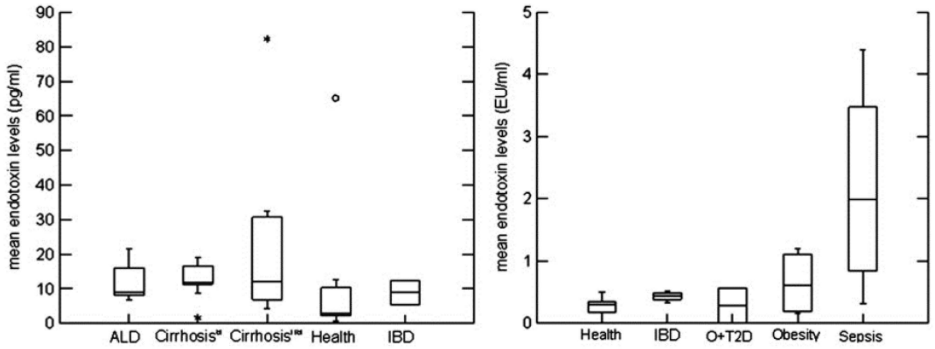

Figure 8. Comparison of reported mean levels of systemic endotoxin in healthy subjects and those in subjects with various diseases where samples were diluted and heated prior to LAL assay. (A) Mean levels of systemic endotoxin reported in pg/ml. Health – healthy non-obese ($n = 14$); IBD – inflammatory bowel disease ($n = 2$); ALD – alcoholic liver disease ($n = 10$); cirrhosis [a] – alcoholic ($n = 9$); and cirrhosis [na] – non-alcoholic ($n = 9$). (B) Mean levels of systemic endotoxin reported in EU/ml. Health – healthy non-obese ($n = 12$); IBD – inflammatory bowel disease ($n = 4$); O + T2D – obese subjects with Type 2 diabetes ($n = 2$); obesity – obese subjects ($n = 4$); sepsis – hospitalised subjects with sepsis or septic shock ($n = 7$). ° – value above 2 SD.

Chronic elevation of systemic endotoxin in intestinal and hepatic disorders

Inflammatory bowel disease

Inflammatory bowel diseases (IBD) such as Crohn's disease (CD) and ulcerative colitis (UC) are characterised by chronic and relapsing inflammation in sections of gastrointestinal mucosa in genetically susceptible subjects. This inflammation may be triggered or exacerbated by an unduly aggressive pro-inflammatory immune response to bacterial antigens that permeate from the gut lumen[111]. The ability of antigens to penetrate the gut wall is thought to be facilitated in patients with IBD[43,47] by a general increase in the permeability of their gut mucosa, which has been identified on oral dosage with inert sugar probes[112–116].

We found five studies published between 1986 and 2013 that assayed the systemic levels of endotoxin using a chromogenic type of LAL assay in patients who had been diagnosed with IBD[43,47,104,117,118]. The results of these studies are inconsistent. One study reported that 17 out of 18 patients (94%) with active CD had detectable levels of endotoxin in their plasma[118], while another reported only 31% of patients with CD (18% with active CD and 47% with non-active CD) and 17% of patients with UC (22% with active UC and 10% with non-active UC) had detectable amounts of endotoxin (>0.015 EU/ml) in their plasma[119]. Two other studies failed to mention whether all of the patients they tested had detectable levels of systemic endotoxin[43,117].

Four studies published values for the levels of systemic endotoxin and all four studies compared their results with those of healthy controls that were determined concurrently by the same method. However, only two of these studies provided details regarding the preparation of serum or plasma (dilution and heating) prior to the endpoint chromogenic LAL assay that was used. One of these studies reported the results in EU/ml and the other in pg/ml[43,117]. In the first of the latter two studies, the mean level of systemic endotoxin in subjects with CD or with UC was higher than that in healthy controls (mean CD = 0.46 EU/ml, mean UC 0.45 EU/ml and mean control = 0.32 EU/ml). Further, this study reported the levels of systemic endotoxin were greater than two standard deviations from the mean of the concurrently determined levels of systemic endotoxin in healthy controls. Hence, these levels were >0.44 EU/ml in 63% of subjects with active CD, in 36% of subjects with inactive CD, in 41% of subjects with active UC and in 5% of subjects with inactive UC[43]. However, in the second study, the mean endotoxin level from healthy controls did not differ from these with CD and UC (mean control = 12.2 pg/ml, mean CD = 12.4 pg/ml and mean UC = 5.5 pg/ml) [117]. Since one of these studies reported endotoxin levels in EU/ml and the other in pg/ml (without providing the requisite conversion factor to EU) the two cannot be directly compared. Pooled the means from the latter two studies of subjects with both active or inactive CD and UC gave a median level of systemic endotoxin in subjects with IBD of 0.44 EU/ml with an IRQ between 0.38 and 0.48 EU/ml, and of 8.90 pg/ml with an IQR between 5.5 and 12.4 pg/ml (Figure 8A and B). Hence, the overall median level of systemic endotoxin in the IBD group (0.44 EU/ml or 8.90 pg/ml) is slightly greater than the overall median of healthy subjects in all other studies (0.29 EU/ml or 2.85 pg/ml; Figure 8A and B).

When the data from the remaining two studies that provided no details on method used for sample preparation were combined with those from the former two studies that provided this information, according to unit used, the mean levels of endotoxin detected in subjects with CD ranged from 0.1 to 0.52 EU/ml[43,47] and from 12.4 to 29.1 pg/ml[104,117]. Similarly, the levels of systemic endotoxin in subjects with UC ranged from 0.136 to 0.45 EU/ml[43,119] and from 5.5 to 26.1 pg/ml[104,117]. The mean levels of endotoxin in the systemic circulation in concurrently determined healthy controls ranged from non-detectable (<0.015 EU/ml) to 0.32 EU/ml[43,119] and from non-detectable (<10 pg/ml) to 12.2 pg/ml[104,117]. The interpretation of bulked data from all four studies is complicated both by the lack of information regarding the methods of sample preparation and by the form of the chromogenic LAL assay that was used in three of the

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studies. Given the shortcomings and difficulties in comparing data it is unclear from these studies whether patients with CD or UC had either greater levels of systemic endotoxin or greater proportions of subjects with detectable levels of endotoxin, than did healthy subjects.

Liver disease

Since the liver has an important role in the clearance of endotoxin from the portal blood and systemic circulation (Figure 1)[2,6,12], it is likely that subjects with impaired liver function will be exposed to greater levels of systemic gut-derived LPS than healthy subjects[5,6,120–122]. We found 25 studies published between 1984 and 2014 that determined the levels of systemic endotoxin in the plasma of patients with acute or chronic hepatitis, with early and advanced stage of chronic degenerative disease of the liver, i.e. alcoholic liver disease (ALD), non-alcoholic fatty liver disease (NAFLD) and with alcoholic and non-alcoholic cirrhosis, using either the chromogenic ($n = 22$) or the turbidimetric ($n = 2$) LAL assay. Five out of 25 studies reported their results in EU/ml[2,32,33,123,124]. The remaining studies reported their results in pg/ml. The latter group used mainly LPS from either *E. coli* O111:B4[6,78,94,103,122,125–129] or LPS from *E. coli* O55:B5[120,130] or NP3 as reference endotoxins[131].

The mean levels of endotoxin and ranges of endotoxin concentrations obtained in plasma with the various methods of preparation and various conditions are summarised in Table 1. The data from three studies in the survey were not included in Table 1, either because they provided no information regarding the method of sample preparation[106,123], or because the reported levels of systemic endotoxin were significantly higher than those reported in the remaining 21 studies[2].

In sum, the findings suggest that the levels of endotoxin in the systemic circulation increase with severity of disease in both acute disorders of the liver, e.g. in acute hepatitis[94,103,120], and in chronic hepatic pathologies, i.e. from mild to moderate degeneration of the liver such as ALD (median 9.015 pg/ml, IQR 8.2–16.0 pg/ml) to more advanced chronic disorders such as hepatic alcoholic cirrhosis (median 11.7 pg/ml, IQR 11.1–16.75 pg/ml) and hepatic non-alcoholic cirrhosis (median 12.0 pg/ml, IQR 6.9–30.7 pg/ml) (all three sets of values being determined from diluted and heated plasma), and with the extent of complications such as bleeding from the gastro-intestinal tract[78,128]. Hence, the medians of the mean levels of systemic endotoxin were increased according to the level of hepatic compromise in comparison to that from healthy subjects reported in 14 other studies (median 2.85 pg/ml, IQR 2.2–10.4 pg/ml) that also used diluted and heated plasma (Figure 8A). However, it is noteworthy that those studies that used a kinetic chromogenic type of the LAL assay but did not employ individual endotoxin standards (i.e. addition of known concentrations of endotoxin to plasma from each subject), nor a modified method of calculation, reported higher levels of endotoxin in plasma from subjects with chronic hepatitis and liver cirrhosis[5] than did the studies that did use refinements[85,125,127,129,132].

It is less clear whether chronic active hepatitis causes the systemic levels of endotoxin to increase. A number of reports

Table 1. Range of reported mean levels of systemic endotoxin in subjects with various hepatic diseases with concurrently determined mean levels in healthy control subjects.

		Hepatitis		Fatty liver		Liver cirrhosis	
Disease group/sample preparation	Healthy	Chronic	Acute	Non-alcoholic	Alcoholic	Non-alcoholic	Alcoholic
Dilution and heating							
pg/ml	0.5–12.6[6,85,122,131,132,136,168–170]	4.6–57.1[136,168]	10.1[168]		6.9–52.3[a122,131,132,136,171]	5.7–82.3[6,131,132,136,168,170]	8.8–19[85,93,131,132,169,171]
EU/ml	ND–0.29[5,33,64,169,172]			0.28–1.52[b5,33,121]	0.1–0.2[a131,169]	0.14–0.36[136,172]	0.13–0.23[131,169]
Perchloric acid method							
pg/ml	ND–10[70,78,85,94,132,173]	ND–10[94,173]	<5–86[94,173]	ND–10[b94]	12.4[a132]	9.2–75[70,78,94,132]	18.1–40.9[85,93,132]
Tween 80 method							
pg/ml	6[a85]						25.4–40.7[a85,93]

ND = non-detectable.
[a]Data obtained with kinetic chromogenic or turbidimetric LAL assay.
[b]Data obtained with endpoint chromogenic LAL assay.

in which plasma samples were diluted and heated prior to assay, indicate that the levels of endotoxin are higher than those concurrently assayed in healthy subjects[5,120]. Other work in which plasma was treated with perchloric acid prior to assay found no such differences[94,103] (Table 1).

The consumption of alcohol may have a direct effect on hepatocytes as well as engendering chronic hepatic cell dysfunction. Both of these effects are associated with elevation of endotoxin[129,131]. The bulk of available data indicate that subjects with alcohol induced fatty degeneration of liver have elevated levels of endotoxin in their systemic circulation compared to those concurrently determined in healthy controls (mean 8.7–18.4 pg/ml versus mean <5 pg/ml)[119,127,129,132]. However, one study reported similar levels of plasma endotoxin in subjects with this condition (9.3 pg/ml) to those concurrently determined in healthy subjects (12.6 pg/ml)[5]. It is noteworthy that, in all these studies, the assays were carried out on diluted and heated plasma samples but that those which reported a relative increase in the systemic levels of endotoxin used individual endotoxin standard curves for each sample. This discrepancy highlights the influence of the diluent that is used for endotoxin standards on the quantification of endotoxin.

The plasma endotoxin levels, determined with dilution and heating of plasma samples prior to the LAL assay, were higher in subjects with alcohol induced hepatic cirrhosis (mean 11.7–19 pg/ml) than in those with non-alcoholic cirrhosis (mean 5.7–12 pg/ml)[129,132].

It is noteworthy that the range of mean levels of plasma endotoxin in healthy subjects in all studies overlapped with those reported in subjects with liver disorders. This makes it difficult to determine either a cut-off point for pathological elevation of plasma endotoxin, or (as was discussed previously) a valid ''normal'' range of plasma endotoxin levels that can be used in clinical practise. Bilirubin has been shown to interfere with the LAL assay[133]. Hence, the detection of endotoxin in the plasma of patients with biliary obstruction or acute hepatocellular disease may be complicated by elevated levels of bilirubin. Further, since the majority of studies on the effects of liver disease on endotoxin levels expressed their results in pg/ml and did not provide any information regarding a suitable conversion into endotoxin units, it is difficult to compare these values with those for other health conditions in which the results are reported in EU/ml.

Elevation of systemic endotoxin in chronic metabolic disorders

Obesity

We found six studies that determined systemic levels of endotoxin in obese adults (defined as BMI > 30)[39,45,101,134–136] and two studies in obese adolescents (defined as subjects with a BMI z-score ≥ 95th percentile for age and gender)[124,137]. Comparison of data showed that the mean levels of systemic endotoxin in obese subjects ranged widely between 0.16 and 5.16 EU/ml[39,45,134–137] and were around 80 pg/ml in another study[101]. It is noteworthy that none of the eight studies

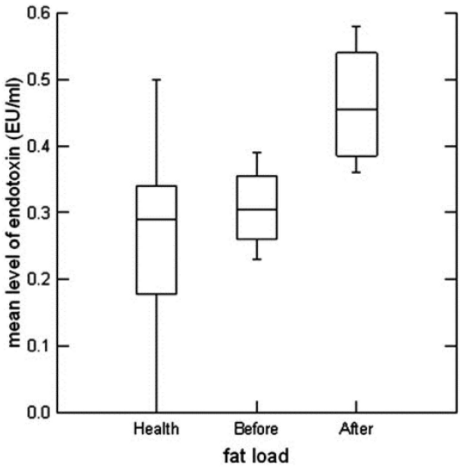

Figure 9. Comparison of reported mean levels of systemic endotoxin in healthy subjects before and after consuming a fatty meal where samples were diluted and heated prior to assay. Reported mean levels of systemic endotoxin in fasted and non-fasted healthy subjects. Health – healthy non-obese subjects from other studies ($n = 12$); before – level in healthy subjects prior to consuming fatty meal ($n = 4$); after – level in healthy subjects after consuming a fatty meal ($n = 4$).

reported the percentage of obese subjects in which endotoxin was not detectable. This implies that all obese subjects had detectable levels of endotoxin in their systemic circulation. When only the results from studies that used a combination of dilution and heating for the preparation of plasma or serum ($n = 4$) were included, the median of pooled mean levels of systemic endotoxin was 0.60 EU/ml with an IQR 0.181–1.10 EU/ml (Figure 8B). The studies that reported the highest levels of systemic endotoxin in obese subjects were those that did not describe the method by which the serum/plasma samples were prepared[39,135,137]. Hence, it is possible that the higher levels and greater variation of endotoxin in these studies resulted from undisclosed differences in the methods of preparation of serum/plasma.

Four of the eight studies that concurrently determined the levels of systemic endotoxin in obese and in lean healthy (defined as BMI ≤ 25) subjects using the same methodology, reported higher levels of systemic endotoxin in obese than in the lean healthy subjects[39,45,101,135]. However, with one exception, the method by which plasma or serum samples were prepared was not described. Moreover, the systemic levels of endotoxin in healthy subjects, which were determined concurrently in these three studies, were higher than those reported by other workers that diluted and heated their plasma or serum samples prior to assay (Figure 7B). The mean levels of plasma/serum endotoxin in obese subjects from the four studies that diluted and heated the plasma or serum samples before assay and reported their results in EU/ml, had a broad range which overlapped with that of the variously reported range in healthy subjects (Figure 8B).

Hence, it is unclear from this comparison whether obese subjects had significantly higher levels of systemic endotoxin than did healthy subjects.

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Table 2. Key problems with the LAL assay and possible remedies.

Technical issues with the LAL assay	Remedy
Blood collection tubes	
– Contamination of heparin plasma vacutainers. – Interference of the LAL reaction by additives such as serum clot activator.	– Use endotoxin-free certificated heparin plasma vacutainers. – Use plain glass ''serum'' vacutainers.
Interference of the LAL reaction by constituents of blood samples	
– Raw and diluted serum changes the kinetics of the LAL reaction. – Pre-treated serum samples inhibit the commencement of the LAL reaction. – Pre-treated serum/plasma inhibit or enhance the rate of the LAL reaction.	– Suitable sample preparation prior to assay possibly a combination of dilution and heating or digestion. – Use a kinetic chromogenic or turbidimetric LAL assay to check the temporal profiles of all samples for differences in the reaction kinetics. – Use a modified calculation algorithm that is based on the slope of the linear part of the reaction curve or use individual standard curves. – Perform Product Positive Controls (PPCs) for each sample by adding a known concentration of endotoxin to the pre-treated blood sample. The recovery of endotoxin added to pre-treated sample should lie between 50 and 200%.
Interference with optimal conditions for the LAL reaction	
– Cation concentration: EDTA-plasma may interfere with the LAL reaction. – pH range (6.0–8.0): heating of blood samples may increase the pH of the sample above the optimal range.	– Add divalent cation such as calcium and magnesium to EDTA-plasma if required. – Check the pH of heat-inactivated samples. – Use an appropriate sample diluent or adjust pH of the sample immediately after heating.
Sequestration of endotoxin by constituents of blood samples	
– Endotoxin may be sequestered by constituents of blood samples or physically sequestered by denatured proteins.	– Test the recoveries of endotoxin spiked into *raw* serum/plasma samples to assess the efficiency of the sample preparation method.

Diabetes

A review of the eight studies that were found showed that the mean levels of systemic endotoxin in subjects with type 1 diabetes ranged between 56 and 67 EU/ml[138], while those in subjects with type 2 diabetes ranged from non-detectable (<0.005 EU/ml) to 77 EU/ml[34,35,37–39,49,135]. However, five of these studies did not provide any information regarding the method by which plasma or serum samples were prepared prior to LAL assay, while one simply diluted them prior to testing[37]. Again, the study that simply diluted the samples, and those that did not provide information regarding sample preparation, were those that reported the highest levels of endotoxin. Hence, the mean was 77.03 EU/ml for sample preparation by simple dilution[138] and varied between 4.6 and 9.2 EU/ml[35,38,39,135] with the five studies that did not indicate the method of sample preparation. In contrast, in the remaining two studies in which plasma or serum samples were diluted and heated prior to LAL assay, one failed to detect any endotoxin in the systemic circulation of subjects with type 2 diabetes[34] and the other reported a much lower mean level of 0.567 EU/ml[49]. It is noteworthy that the study that failed to detect any endotoxin in the serum of diabetic subjects also found no endotoxin in the systemic circulation of healthy controls[34,139], whereas the other study found elevated levels of systemic endotoxin in the plasma of diabetic subjects (mean 0.567 EU/ml) compared with those in concurrently tested healthy subjects (mean 0.15 EU/ml)[49]. Given that diluted serum can accelerate the LAL reaction and that dilution and heating of serum can delay the commencement of the LAL reaction[71], and assuming that the recommended method for calculation (based on the time it takes to initiate the LAL reaction), is likely to have been used in these studies, it seems likely that the overall results are influenced by the differences in method and are correspondingly suspect.

The effect of macronutrients on the levels of systemic endotoxin

We found seven studies published between 2007 and 2012 that investigated the effects of consuming foods that contained differing proportions of macronutrients on the levels of endotoxin in plasma/serum of either healthy subjects ($n = 6$), obese subjects ($n = 2$), subjects with type 2 diabetes ($n = 1$) or subjects with impaired glucose tolerance ($n = 1$). None of the seven studies reported any subjects in whom there were no detectable levels of systemic endotoxin either before or after the meal. Five of the seven studies diluted and heated their plasma or serum samples prior to the LAL assay[8,9,41,134,140], while the two remaining studies provided no information regarding the method by which samples were pre-treated[42,135]. In these five studies, the ingestion of either, a 900 kcal meal containing around 50 g of fat (which comprised around 50% of the calories), or of a drink of plain cream (33 g), lead to a post-prandial rise in median plasma levels of endotoxin from 0.305 to 0.455 EU/ml[8,9,41,42,140] (Figure 9). Conversely, neither the ingestion of 300 ml of water, a 300 calorie solution containing 75 g of glucose (Glucola) nor an equicaloric quantity of pure orange juice, caused the levels of systemic endotoxin to rise[8]. Another study showed that the levels of serum endotoxin rose from 3.3 to 6.3 EU/ml when

healthy non-obese subjects consumed a single meal containing 75 g of fat, 5 g of carbohydrate and 6 g of protein, the fat comprising 94% of the calories[135]. Similarly, consumption of the same meal by obese subjects (defined as BMI > 30) caused serum endotoxin levels to rise from 5.1 to 7.7 EU/ml, while it rose from 5.7 to 7.5 EU/ml in subjects with impaired glucose tolerance, and from 5.3 to 14.2 EU/ml in subjects with type 2 diabetes[135]. However, this study provided no details regarding the method used for preparation of the samples. Moreover, the study used an endpoint chromogenic LAL assay, so that any variation in reaction kinetics from those of the standards would not have been detected.

Two studies examined the effect of co-consumption of other nutrients on these changes. Hence, after a co-consumption of a 900 kcal meal containing around 50 g fat (equal to around 50% of calories from fat) with either a drink of orange juice (300 kcal), or a muscadine grape extract containing a mixture of resveratrol (100 mg) and other polyphenols to a total of 75 mg, systemic endotoxin levels were assayed by endpoint chromogenic LAL kit following dilution and heating of the plasma samples. Neither of these two treatments caused post-prandial systemic endotoxin levels to rise[9,42]. Similarly, after the consumption of a 900 kcal meal in which the fat content had been reduced to around 27 g (equal to around 27% of calories from fat) there was no significant rise in levels of plasma endotoxin[41]. These findings suggest that either co-consumption of other dietary items or a reduction in the relative proportions of fat in the meal may ameliorate any post-prandial rise in systemic endotoxin although the problems outlined previously must be borne in mind.

Conclusion

Adaptation of the LAL assay to accurately quantify endotoxin in samples of plasma or serum is challenging as is the interpretation of the data obtained using currently available methodologies. Pooling and comparison of the results from various studies indicate that the methods used for the pre-treatment of the sample prior to assay significantly influence the levels of endotoxin that are detected and in many cases confound meaningful comparison. Hence, for example, meaningful comparison of systemic endotoxin levels in subjects with various medical disorders that compromise the barrier function of the intestine with those of healthy subjects is dependent upon selecting data that have been obtained using identical assay methodologies. Even then there may be difficulties. For example, in studies where plasma or serum were simply diluted, or when the method of sample preparation was not described, the levels of systemic endotoxin in diabetic subjects (range of means 4.6–77.0 EU/ml) were elevated compared with those from simultaneously tested healthy subjects (range of means 3.1–61.1 EU/ml)[35,37–39,135,138], but both these levels were higher than those reported in other studies in which plasma or serum were diluted and heated prior to the endotoxin test (Figure 8A and B).

While, a distinction between direct interference by constituents in serum or plasma with components of the LAL assay and interference by the sequestration of endotoxin by components of the blood would be useful in optimising methods for pre-treating samples, little work has been done in this regard. Some data suggest that a combination of dilution and heating is sufficient to reduce both direct interference with the assay by constituents of serum and direct interaction between (spiked) endotoxin and constituents of serum[71]. However, we can find no studies showing that such treatments are able to release sequestered endotoxins and render them detectable by current commercial LAL assay. In contrast, two publications indicate that the treatment of plasma by either perchloric acid or the ESP kit is able to release sequestered endotoxin and improve recoveries[94,95].

A particular methodological pitfall lies in the use of the endpoint LAL assay in which the reaction is stopped at a given time point and the intensity of the colour or turbidity that has developed at this time is used as a basis for quantification. As we have seen, the manner in which the sample is pre-treated prior to the test is likely to alter the speed at which the LAL reaction commences. Thus, for example, any slowing of this speed compared to that of endotoxin standards in aqueous solution will result in a decrease in the amount of endotoxin that is detected after a given interval. Hence, the data obtained from the endpoint LAL test can only be compared with results from studies that used different methods of sample preparation if it has been demonstrated that neither method of sample preparation engenders any differences in the times at which the reactions commence. This can be verified only by the use of kinetic versions of the chromogenic or turbidimetric LAL assays. Further, since reaction kinetics may vary between LAL kits and between manufacturers, it is important to perform such tests with kits from the same supplier and batch.

A particular problem in the reporting and interpretation of data lies in the reported absence of endotoxin in samples of plasma or serum from a significant proportion of both healthy[34,47,48,104,105] and diseased subjects[34,40,47,48,51–56]. This may again result from the method of sample preparation prior to assay. However, it may equally represent a real finding in the population that is being studied. At all events studies should report and compare both the proportions of negative tests and the levels of endotoxin in the various study groups. It should also be borne in mind that significant proportions of negative tests may preclude the use of parametric statistical methods as well as rendering the mean a poor descriptor of that data.

It is possible that comparisons of healthy and ill populations using the same standard method of sample preparation prior to the LAL assay will be sufficient to detect when higher levels of endotoxin are entering the systemic circulation in clinical conditions where the intestinal barrier or hepatic function are significantly compromised. Recommendations regarding the use of such tests are listed in Table 2. These comments apply both to the assessment of LPS levels in systemic and hepatic portal blood, although it is generally not possible to access the latter system in studies on human subjects. While the levels of LPS may be significantly higher in hepatic portal than in systemic blood[2–6] its components are equally likely to interfere with the LAL assay. Caution is nevertheless advised in interpreting the clinical relevance of elevated systemic endotoxin levels as the magnitude of LAL

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reaction may not directly correlate with the pro-inflammatory potential of LPS in human subjects. Further, it is important to keep in mind that the LAL test determines the endotoxicity of LPS molecules in a given sample but does not give any information about the quantity of LPS in a given sample. Hence, results obtained with the LAL assay should be reported in endotoxin units (EU) rather than in pg/ml. If results are reported in pg/ml the relevant conversion factor from pg/ml into EU/ml should be provided.

A considerable improvement on existing levels of accuracy of the LAL test on use with biological fluids would be required for adequate discrimination of more subtle effects such as the detection of chronic low levels of endotoxin. A number of manufacturers state that a variation in the recovery of endotoxin spiked into (diluted and heated) plasma or serum (Positive Product Control, PPC) of between 50 and 200% (i.e. between half and double the spiked dose) is acceptable[65,76]. However, calculations based on the levels of endotoxin reported in healthy and in obese subjects following ingestion of a fatty meal[8,9,41,42,134,135,140] indicate that an assay with a CV of less than 15% would be required to reliably distinguish a 50% difference in levels of circulating endotoxin in these two groups ($p < 0.05$)[141].

Given the shortcomings of the currently available assays, methods that indirectly assess LPS by its effects are being increasingly used, even though they cannot directly establish causation. One example is the endotoxin activity assay (EAA), which is based on the quantification of free radicals released from neutrophils in response to endotoxin[142]. The assay is approved by the FDA for assessing the risk of septic shock[143] and has been used in this respect[139,144–149], in healthy human subjects[150] and subjects with disorders such as obesity[151–153]. However, the assay must be completed within 3 h of collection [e-mail correspondence with the manufacturer] and may be awkward to use in surveys containing large numbers of subjects. Alternatively, the quantification of LPS-binding protein (LBP) may be useful in inferring a chronic low level increase of systemic LPS, as the production of LBP, an acute phase reactant produced by hepatocytes[154], is up-regulated following immune challenge with LPS[154]. Human specific LBP-ELISAs have been used to indirectly assess the systemic levels of LPS in various infections and in obesity[43,155–167].

Declaration of interest

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Publication:	INTERNATIONAL REVIEWS OF IMMUNOLOGY
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Author: Anne Gnauck, Roger G Lentle, Marlena C Kruger

Publication: Innate Immunity (formerly known as Journal of Endotoxin Research)

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REVIEW PAPER – ENDOTOXIN MEASUREMENT

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Title: Chasing a ghost? – Issues with the determination of circulating levels of endotoxin in human blood

Author: Anne Gnauck, Roger Graham Lentle, Marlena Cathorina Kruger

Publication: Critical Reviews in Clinical Laboratory Sciences

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2-1	N		Y	N		Figure 1 from Raetz CR, and Whitfield C. 2002. "Lipopolysaccharide endotoxins." Annu Rev Biochem. no. 71:635-700.
2-2	N		N	Y	Y	Figure 1 from Tran A.X., and Whitfield C. 2009. "Lipopolysaccharides (Endotoxins)." In Encyclopedia of Microbiology, 513-528.
2-3	N		Y	N		Figure 2 from Raetz CR, and Whitfield C. 2002. "Lipopolysaccharide endotoxins." Annu Rev Biochem. no. 71:635-700.
2-4	N		N	Y	Y	Figure 8 from Tran A.X., and Whitfield C. 2009. "Lipopolysaccharides (Endotoxins)." In Encyclopedia of Microbiology, 513-528.
2-5	N		N	Y	Y	Figure 1 from Takeuchi O, and Akira S. 2010. "Pattern recognition receptors and inflammation." Cell no. 140 (6):805-20.
2-6	N		N	Y	NA*	Figure 1 from Munford RS, and Varley AW. 2006. "Shield as signal: lipopolysaccharides and the evolution of immunity to gram-negative bacteria." PLoS Pathog. no. 2 (6):e67.
2-7	N		N	Y	Y	Figure 2 from Suzuki T. Regulation of intestinal epithelial permeability by tight junctions. Cell Mol Life Sci. 2013;70(4):631-59.doi:10.1007/s00018-012-1070-x
2-8	N		Y	N		Figure 1 from Kelly CJ, Colgan SP, and Frank DN. 2012. "Of microbes and meals: the health consequences of dietary endotoxemia." Nutr Clin Pract no. 27 (2):215-25. doi: 10.1177/0884533611434934.
3-1	N		Y	N		Figure 1 from Novitsky TJ. 1994. "Limulus amebocyte lysate (LAL) detection of endotoxin in human blood." J Endotoxin Res. no. 1:253-263.
3-2	Y	N	N	N		
3-3	N		N	Y	Y	Charles River Laboratories. Endosafe-PTS Assay Guide.
3-4	N		N	Y	Y	epitomics, http://www.epitomics.com/products/product_info/1257
3-5	Y	N	NA	N		
4-1	Y	N	NA	N		
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5-2	Y	Y	N	Y	Y	Figure 2 from Gnauck A, Lentle RG, and Kruger MC. 2015. "Aspirin-induced increase in intestinal paracellular permeability does not affect the levels of LPS in venous blood of healthy women." Innate Immun. no. 21 (5):537-45. doi: 10.1177/1753425914557101.
5-3	Y	Y	N	Y	Y	Figure 3 from Gnauck A, Lentle RG, and Kruger MC. 2015. "Aspirin-induced increase in intestinal paracellular permeability does not affect the levels of LPS in venous blood of healthy women." Innate Immun. no. 21 (5):537-45. doi: 10.1177/1753425914557101.
5-4	Y	Y	N	Y	Y	Figure 4 from Gnauck A, Lentle RG, and Kruger MC. 2015. "Aspirin-induced increase in intestinal paracellular permeability does not affect the levels of LPS in venous blood of healthy women." Innate Immun. no. 21 (5):537-45. doi: 10.1177/1753425914557101.
6-1	Y	Y	N	Y	Y	Figure 1 from Gnauck A, Lentle RG, & Kruger MC. (2015). The Limulus Amebocyte Lysate assay may be unsuitable for detecting endotoxin in blood of healthy female subjects. J Immunol Methods, 416, 146-156. doi: 10.1016/j.jim.2014.11.010
6-2	Y	Y	N	Y	Y	Figure 2 from Gnauck A, Lentle RG, & Kruger MC. (2015). The Limulus Amebocyte Lysate assay may be unsuitable for detecting endotoxin in blood of healthy female subjects. J Immunol Methods, 416, 146-156. doi: 10.1016/j.jim.2014.11.011
6-3	Y	Y	N	Y	Y	Figure 3 from Gnauck A, Lentle RG, & Kruger MC. (2015). The Limulus Amebocyte Lysate assay may be unsuitable for detecting endotoxin in blood of healthy female subjects. J Immunol Methods, 416, 146-156. doi: 10.1016/j.jim.2014.11.012
6-4	Y	Y	N	Y	Y	Figure 4 from Gnauck A, Lentle RG, & Kruger MC. (2015). The Limulus Amebocyte Lysate assay may be unsuitable for detecting endotoxin in blood of healthy female subjects. J Immunol Methods, 416, 146-156. doi: 10.1016/j.jim.2014.11.013
6-5	Y	Y	Y	N	Y	Figure 5 from Gnauck A, Lentle RG, & Kruger MC. (2015). The Limulus Amebocyte Lysate assay may be unsuitable for detecting endotoxin in blood of healthy female subjects. J Immunol Methods, 416, 146-156. doi: 10.1016/j.jim.2014.11.014
7-1	Y	N	NA	N		
7-2	Y	N	NA	N		
7-3	Y	N	NA	N		
7-4	Y	N	NA	N		
7-5	Y	N	NA	N		
7-6	Y	N	NA	N		

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9-2	Y	N	NA	N		
9-3	Y	N	NA	N		
9-4	Y	N	NA	N		
10-1	Y	N	NA	N		
10-2	Y	N	NA	N		
10-3	Y	N	NA	N		
10-4	Y	N	NA	N		
11-1	Y	N	NA	N		
11-2	Y	N	NA	N		
11-3	Y	N	NA	N		
12-1	N		Y	N		Figure 1 from Kelly CJ, Colgan SP, and Frank DN. 2012. "Of microbes and meals: the health consequences of dietary endotoxemia." Nutr Clin Pract no. 27 (2):215-25. doi: 10.1177/0884533611434934.
12-2	N		Y	N		Figure 1 and 4 from Raetz CR, and Whitfield C. 2002. "Lipopolysaccharide endotoxins." Annu Rev Biochem. no. 71:635-700.
12-3	N		Y	N		Figure 1 from Novitsky TJ. 1994. "Limulus amebocyte lysate (LAL) detection of endotoxin in human blood." J Endotoxin Res. no. 1:253-263.
12-4	Y	Y	N	Y	Y	Figure 4 from Gnauck A, Lentle RG, and Kruger MC. 2015. "Chasing a ghost? – Issues with the determination of circulating levels of endotoxin in human blood." Crit Rev Clin Lab Sci.
12-5	Y	Y	N	Y	Y	Figure 5 from Gnauck A, Lentle RG, and Kruger MC. 2015. "Chasing a ghost? – Issues with the determination of circulating levels of endotoxin in human blood." Crit Rev Clin Lab Sci.
12-6	Y	Y	N	Y	Y	Figure 6 from Gnauck A, Lentle RG, and Kruger MC. 2015. "Chasing a ghost? – Issues with the determination of circulating levels of endotoxin in human blood." Crit Rev Clin Lab Sci.
12-7	Y	Y	N	Y	Y	Figure 7 from Gnauck A, Lentle RG, and Kruger MC. 2015. "Chasing a ghost? – Issues with the determination of circulating levels of endotoxin in human blood." Crit Rev Clin Lab Sci.
12-8	Y	Y	N	Y	Y	Figure 8 from Gnauck A, Lentle RG, and Kruger MC. 2015. "Chasing a ghost? – Issues with the determination of circulating levels of endotoxin in human blood." Crit Rev Clin Lab Sci.
12-9	Y	Y	N	Y	Y	Figure 9 from Gnauck A, Lentle RG, and Kruger MC. 2015. "Chasing a ghost? – Issues with the determination of circulating levels of endotoxin in human blood." Crit Rev Clin Lab Sci.

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FIGURE 2-2 AND 2-4

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Chapter: Lipopolysaccharides (Endotoxins)
Book: Encyclopedia of Microbiology
Author: A.X. Tran,C. Whitfield
Publisher: Elsevier
Date: 2009
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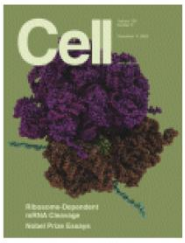

Title: Pattern Recognition Receptors and Inflammation
Author: Osamu Takeuchi,Shizuo Akira
Publication: Cell
Publisher: Elsevier
Date: 19 March 2010

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FIGURE 3-3

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Gnauck, Anne
Sent: Friday, 22 January 2016 11:07 a.m.
To: Lenich, Clare [Clare.Lenich@crl.com]
Cc: Patel, Mehul [Mehul.Patel@crl.com]
Attachments: chapter 03_overview method~1.pdf (324 KB)

Hi Clare,

Thank you for your immediate reply. Attached the relevant section of my thesis.

Kind regards
Anne Gnauck

From: Lenich, Clare [Clare.Lenich@crl.com]
Sent: Friday, 22 January 2016 7:43 a.m.
To: Gnauck, Anne
Cc: Patel, Mehul
Subject: RE: Asking for permission to use figures of Endosafe PTS cartridges for thesis

Hi Anne. Yes you can use the information, we just ask that we can see the section of your thesis that includes content about the PTS before it is used.
Thanks

Clare Lenich
Manager, Technical Service
Microbial Solutions | Charles River
P: 843.402.4900 | F: 843.766.7576
clare.lenich@crl.com | www.criver.com/microbialsolutions
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From: Gnauck, Anne [mailto:A.Gnauck@massey.ac.nz]
Sent: 21 January 2016 4:57 AM
To: Global-Endo Comments <endo-comments@crl.com>
Subject: Asking for permission to use figures of Endosafe PTS cartridges for thesis

To whom it may concern,

I'm Anne Gnauck, a PhD student at the School of Food and Nutrition at Massey University. I would like to use the two figures showing the details of the Endosafe PTS cartridges that are given in the manual of the cartridges for the methodology chapter of my thesis. Can I get your permission to use the two figures in my thesis?

Thank you for your help.

Kind regards
Anne Gnauck

FIGURE 3-4

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Abcam Scientific Support [nz.technical@abcam.com]
Sent: Wednesday, 27 January 2016 4:02 a.m.
To: Gnauck, Anne

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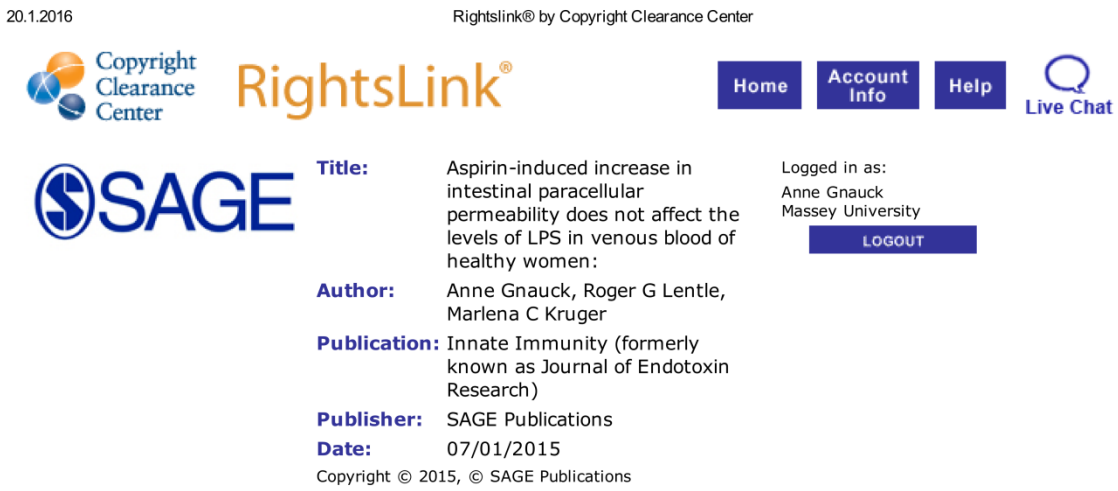
Please let me know if you have any further questions and have a great day.
Best regards,
Kevin

Kevin Hanson
Scientific Support Manager
Abcam Inc.
www.abcam.com

FIGURE 5-1 TO 5-4



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Title:	Aspirin-induced increase in intestinal paracellular permeability does not affect the levels of LPS in venous blood of healthy women:
Author:	Anne Gnauck, Roger G Lentle, Marlena C Kruger
Publication:	Innate Immunity (formerly known as Journal of Endotoxin Research)
Publisher:	SAGE Publications
Date:	07/01/2015

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Title: Chasing a ghost? – Issues with the determination of circulating levels of endotoxin in human blood

Author: Anne Gnauck, Roger Graham Lentle, Marlena Cathorina Kruger

Publication: Critical Reviews in Clinical Laboratory Sciences

Publisher: Taylor & Francis

Date: Jan 6, 2016

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