Endometriosis diagnostics: Meta-analysis implementation to develop a conceivable protocol for early detection.

A Thesis presented in partial fulfilment of the requirements for the degree of

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Abstract:

Background: Endometriosis is a common gynaecological condition affecting at least 10% of women globally. It is a common cause for infertility in women and can be asymptomatic. This condition can also cause chronic pain and include symptoms such as dysuria, dysmenorrhea, fatigue, and nausea. Currently, invasive laparoscopy is the gold standard for diagnosis. Endometriosis occurs when endometrial stem cells (EnSCs) invade the body, implant and grow into ectopic lesions. These lesions can be found in many areas of the body including the peritoneal cavity, lungs, and the brain. There are currently four theories surrounding the process of endometriosis: retrograde menstruation, lymphovascular metastasis, embryonic rest, and coelomic metaplasia.

Objectives: The main objective of this analysis was to discover a novel method to diagnose endometriosis at the earliest possible stage that could be used as a new gold standard procedure.

Methods: Primarily, reviewing the growth, structure, and function of the endometrium was necessary to investigate how EnSCs can migrate to various areas of the body, attach, and grow into painful ectopic lesions. Following this, a PRISMA compliant random-effects meta-analysis was performed on 21 studies of peritoneal fluid (PF) of TNF-α, a cytokine that changes concentrations in the menstrual cycle, and could therefore possess potential differences in endometriosis patients as well. Finally, a study protocol was developed that could validate an early stage endometriosis diagnostics procedure for future implementation.

Results: The meta-analysis of PF TNF-α yielded a significant difference ($p<0.05$). Although this study could not account for menstrual cycle stages, it has displayed support that protein concentration differences exist between endometriotic and non-endometriotic patients. Therefore, further analyses could be performed on different proteins in PF or serum to discover diagnostic candidates for endometriosis. This further supports the ideas described in the Study Protocol that possess the potential for implementation into diagnostic procedures.

Conclusions: There appear to be enough studies within the literature to perform similar PRISMA compliant random-effects meta-analyses of various endometriosis associated proteins. When these analyses are performed, the proteins with significant differences will be revealed and their potential explored further for diagnostic implementation.

Statement of Contribution

James Hansen – Study conception, design, sample collection, manuscript composition, statistical analysis, and interpretation.

Statement of originality

“I hereby declare that this thesis is my own word and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which a substantial extent has been accepted for the qualification of any other degree or diploma of a university or other institution of higher learning, except where due acknowledgement is made.”

Signed…….

Date……

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Keywords

Endometriosis, endometrium, retrograde menstruation, meta-analysis, study protocol, validation.

Chapter 1: Introduction

1.1 Study Background

Endometriosis is a gynaecological condition of ectopic lesion growth in areas distant from the uterus (Yang & Huang, 2014). This disease affects 10-15% of women (Jerman & Hey-Cunningham, 2015) and can present symptoms of infertility, dysmenorrhea, and abdominal pain (Nap, Groothuis, Demir, Evers, & Dunselman, 2004), which can become chronic. In some instances, endometriosis can also be asymptomatic and cause infertility (Hickey, Ballard, & Farquhar, 2014). There are four prevailing theories regarding endometriosis: retrograde menstruation, lymphovascular metastasis, coelomic metaplasia, and embryonic rest (Figueira, Abrão, Krikun, & Taylor, 2011). Retrograde menstruation states that some EnSCs detach from the basalis during menstruation (Lebovic, Mueller, & Taylor, 2011) and enter blood vessels throughout the body. These cells attach to a region distant from the uterus and develop into an ectopic lesion (Nap et al., 2004). Lymphovascular metastasis is similar to retrograde menstruation except that the EnSCs migrate through the lymphatic system and produce a lesion distant from the uterus (Sasson & Taylor, 2008). Coelomic metaplasia occurs when tissues are modified into endometriotic lesions (Burney & Giudice, 2012); however, this theory is the least supported. Embryonic rest occurs when specific embryonic cells remain dormant; but will develop into endometriotic lesions that tend to be of a uterine tube origin (Santamaria, Massasa, & Taylor, 2012). However, with the multiple case studies present regarding each theory, the evidence suggests that there is no single cause of endometriosis. It appears that, depending on the circumstance, one of these theories is the cause for endometriosis in an individual, for example, a case study in a male regarding the embryonic rest theory (Gonzalez, Vnencak-Jones, Shi, & Fadare, 2014). In this instance the diagnosis does not match the other endometriosis theories unless the lesion type described was incorrect.

As endometriosis is prevalent, it is worth discovering new methods for the earliest possible diagnosis. Presently, genome-wide associations are showing promising results that can predict or offer an alternative to some diagnostic practices (Nyholt et al., 2012; Painter et al., 2011). For example, one meta-analysis has shown that seven changes in single nucleotide polymorphisms (SNPs) have a strong genetic link associated with endometriosis that can be passed down to following generations (Nyholt et al., 2012). Alternatively, other analyses have shown that some genes such as Human Leukocyte Antigen (HLA), and Tumor Growth Factor beta Induced (TGFBI) are upregulated in endometriosis, while some genes associated with apoptosis such as Growth Arrest and DNA Damage 45A, 45B, and 34 (GADD) were down-regulated (Arimoto et al., 2003). Further support of these findings was evident in endometriotic lesions where HLA-G is expressed; whereas it is not expressed in normal endometrium (Barrier, Kendall, Ryan, & Sharpe-Timms, 2006). This may also explain some of the immune system evasion seen in endometriosis as when expressed HLA-G may reduce the sensitivity of endometriotic cells to NK and T-cell exposure (Barrier et al., 2006; Sundqvist et al., 2011).

Currently, the gold standard is an invasive laparoscopy (Nezhat et al., 2010). However, the issue with this is that a patient must be in pain and request for a diagnosis by a medical practitioner; or as in some asymptomatic cases (Hickey et al., 2014), will only be discovered upon patient request for an examination. Therefore, with this marked limitation, a different method of diagnosis is required to investigate if an early stage diagnostic procedure can be performed that is less invasive, cost effective, and allows for earlier patient treatment.

1.2 Purpose of this study

The purpose of this study is to evaluate if a new method for endometriosis diagnostics is plausible and less invasive than gold standard laparoscopy. To investigate the diagnostic plausibility; a review of the current literature is required regarding endometrial development and endometriosis. This can be used to discover if there are any specific protein concentration changes that could be targeted as an endometriosis diagnostic measure. Furthermore, this review can address what is currently known about endometrial development and how

endometriosis uses this development procedure to cause ectopic lesion growth. This review can also evaluate what areas are well-known as well as the unclear biochemical mechanisms required for endometrial and endometriosis development.

Subsequently, by investigating the mechanisms of endometriosis, a range of proteins can be targeted for diagnostics procedures. As endometriosis evades the immune system, various changes in multiple cytokines occur. Therefore, one of these proteins can be selected to further clarify its potential for endometriosis diagnostics. As diagnostic procedures can use a variety of sample types; two appear to be the most common for protein diagnostics: peritoneal fluid (PF) and serum. However, most cytokines in both sample types possess data set conflicts, therefore a meta-analysis will be performed to account for these conflicts and detect if a significant effect is present and consistent between studies. Following this, a study protocol is to be developed that can use this information to determine which proteins could be used from serum and PF for early-stage endometriosis diagnostics.

1.3 Study Objectives

For this meta-analysis, PF TNF-α was selected after searching the literature for proteins that commonly appeared in potential endometriosis diagnostic methods. In addition, no meta-analyses on PF TNF-α have been performed; furthermore, conflicts existed between the studies. Therefore, this random-effects meta-analysis has been performed to account for the conflicts between various studies. In addition, by using this same methodology, a study protocol has been proposed for further analysis of various proteins by using meta-analyses, reference range development, and a validation procedure to provide evidence for an early-stage endometriosis diagnostic procedure.

1.4 Study hypotheses

The main prediction of this study was that PF TNF-α possesses a significant difference between endometriotic and control patients when compared by meta-analysis. The endometriotic patients were expected to have higher concentrations of PF TNF-α (pg/ml) compared to controls. PF TNF-α was expected to possess significance ($p<0.05$) at the heterogeneity level for performing a meta-analysis, as well as the final result when comparing multiple studies. It was also expected that by utilising meta-analyses, a procedure could be developed to diagnose endometriosis using PF and serum samples as a less invasive alternative to laparoscopy.

1.5 Thesis layout

For this thesis, the format for each chapter has been presented in the style of a manuscript prepared for journal publication. Chapter two is a literature review of endometrial development and the four current theories of endometriosis. Chapter three is a meta-analysis and study protocol. The first half of chapter three covers the PF TNF-α meta-analysis and its diagnostic potential. Subsequently, a study protocol is described in the second half of chapter three. This proposes using multiple meta-analyses of various proteins within PF and serum for use in a diagnostic procedure. Following this, a reference range and validation criteria has been defined that can clarify which protein candidates at specific menstrual cycle stages can be selected for a standard diagnostic procedure.

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Chapter 2: Literature review of endometrial development from hormonal and environmental stimuli, and the theories of endometriosis

Abstract – Endometriosis is a painful chronic condition that commonly causes infertility in women. This review has been developed to analyse endometrial physiological development from environmental and hormonal stimuli as well as the current aetiology of endometriosis. Primarily endometriosis is most likely to be caused by the endometrial stem cells (EnSCs) detachment and dispersal through the body during the menstrual cycle (retrograde menstruation); followed by reattachment and growth at a new, non-uterine site, resulting in an endometrial lesion. In addition, EnSCs may travel through the lymphatic system (lymphovascular metastasis) and cause endometriosis in areas further away from the uterus. EnSCs are located in the endometrial basalis layer and proliferate by various hormones such as estrogen and progesterone in complex pathways discussed in this review in preparation for blastocyst reception. Therefore, by encompassing the physiological associations of EnSCs, the mechanisms of endometrial structure and development, and the current theories of endometriosis, this can allow for the potential of novel opportunities in medical laboratory diagnostics or treatments.

1. The Endometrium

1.1. Structure

The endometrium is the inner lining of the uterus composed of two layers; the basalis and functionalis (figure 1.1) (Salamonsen & Lathbury, 2000). The functionalis region consists of the uppermost two thirds of the endometrium separated into two layers: stratum compactum (furthest from basalis) and stratum spongiosum (nearest the basalis) (Ayachit & Kulkarni, 2017) with a shallow folded layer of various glandular, luminal, and ciliated epithelial cells (Fay & Grudzinskas, 1991; Kirk et al., 1978). The basalis is the lowermost third of the endometrium and involves the deeper interspersed crypts with endometrial stem cells (EnSCs). EnSCs are situated in the most basal region of these crypts to produce cells for the temporary functionalis layer (Ayachit & Kulkarni, 2017; Salamonsen & Lathbury, 2000). The basalis is attached to the subendometrial myometrium and remains attached after menstruation (Diedrich, Fauser, Devroey, & Griesinger, 2007; Figueira, Abrão, Krikun, & Taylor, 2011).

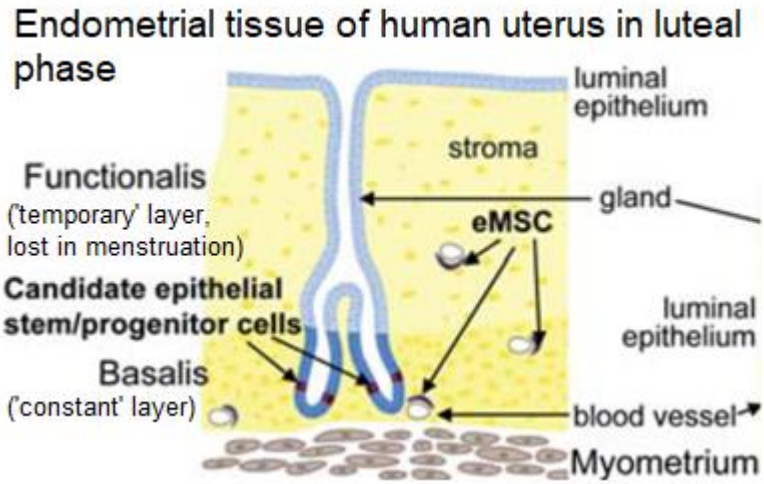


Figure 1.1: The basalis and functionalis regions of the endometrium as well as endometrial stem cells adapted from Gargett & Masuda (2010). eMSC: Endometrial mesenchymal stem cells.

Within basalis layer crypts, additional cell types exist that compose endometrial structure. These include: a dense stroma, large blood vessels, niche cells (Figueira et al., 2011), and a rich lymphatic network (Donoghue, Lederman, Susil, & Rogers, 2007). The dense stroma and large blood vessels provide a ready

supply of blood when the functionalis layer is constructed, and act as a stable foundation for growth, allowing endometrial regeneration after menstruation (Fay & Grudzinskas, 1991). The dense stroma provides a niche for the endometrial stem cells so that they are not lost during each menstrual cycle (Fayazi, Salehnia, & Ziaei, 2016). In regards to the lymphatic system, the growth and development of the basalis and functionalis layers are unclear; however, it does require vascular endothelial growth factors (VEGF) -C and -D, as well as VEGF receptor-3 (VEGF-R3) and that these are more prevalent in the basalis than the functionalis (Donoghue et al., 2007). Niche cells immediately adjacent to EnSCs prevent EnSC differentiation and transmit paracrine signals to induce or reduce cell proliferation (Figueira et al., 2011); thus, acting similarly to the Paneth cells of the small intestinal stem cell regions (Clevers & Bevins, 2013). Depending on the menstrual cycle stage, EnSCs can be stimulated to replace removed or dead epithelial cells after bleeding to assist in endometrial recovery (Diedrich et al., 2007) which rapidly develops a new cellular barrier, presumably to prevent potential infection. Alternatively, as seen in later stages of the menstrual cycle, EnSCs can be stimulated to proliferate to form layers of stroma (Deligdisch, 2000) with a loosely attached outer epithelial layer in the functionalis in preparation for blastocyst implantation (Sharkey & Smith, 2003).

Furthermore, depending on the type of division, EnSCs can form various cell types. If the division is symmetrical, either two transit amplifying progenitors (TA) or two daughter stem cells are formed (Figueira et al., 2011). TA cells will repeatedly divide and accumulate various molecular markers for differentiation purposes (Figueira et al., 2011), while regular stem cells likely retain the same function as their parent cell. If the divisions are asymmetric, one daughter cell will be moderately differentiated and the other will be identical to the parent (Figueira et al., 2011). In addition, separate EnSCs become incorporated into the functionalis layer (Darzi, Werkmeister, Deane, & Gargett, 2016). These will be altered further into decidual cells in preparation for the blastocyst, or to generate and support other associated glandular tissue (Darzi et al., 2016).

The functionalis layer consists of proliferated cells lost in each menstrual cycle if a blastocyst has not implanted (Salamonsen & Lathbury, 2000). The majority of this layer is a loose connective stroma surrounding dense regions of glandular tissue (Figueira et al., 2011) with a multitude of blood vessels

(Deligdisch, 2000) as a supply of nutrients. This glandular tissue is a combination of epithelial, vascular, and stromal cells (Darzi et al., 2016).

Prior to implantation, the functionalis undergoes a rapid preparation process involving a glandular and supportive loose connective stromal layer (Gargett & Masuda, 2010). The primary layer of glandular tissue is likely to be composed of a pseudo-stratified columnar epithelium (Gargett & Masuda, 2010). The formation of glands and various cell types in the functionalis (adenogenesis) occurs in conjunction with proliferating stroma as a response to gradually increasing levels of 17-β estradiol from the growing follicle (Messinis, Messini, & Dafopoulos, 2014) alongside other hormones such as prolactin, insulin-like growth factor 1 and 2 (IGF-1, -2), fibroblast growth factor -7 and -10 (FGF-7, -10), and hepatocyte growth factor (HGF) (Gray et al., 2001). In addition, other factors such as cell proliferation involving site-specific variations, as well as endocrine, paracrine, and extracellular matrix (ECM) interactions cause specific responses on various cell receptors within the developing gland (Gray et al., 2001). Upon completion, these glands secrete a mucosal layer filled with various substances atop of pinopodes such as: integrins, calcitonin, and leukaemia inhibitory factor (LIF) in a receptivity window that permits blastocyst implantation (Sharkey & Smith, 2003). Depending on the species this implantation window can be in the region of hours (e.g. rats: 24h) to days (humans, from day 5-10 after LH surge) (Sharkey & Smith, 2003). Outside of this window, the endometrium is no longer receptive of the blastocyst (Gray et al., 2001; Sharkey & Smith, 2003).

The growing stroma in the proliferative phase possesses a role in supporting blastocyst implantation (Sharkey & Smith, 2003). Upon implantation, these cells become highly secretory to facilitate growth and respond to progesterone to mediate further stromal cell growth and division (Sharkey & Smith, 2003). The stroma also possesses fibroblastic cells (Deligdisch, 2000; Diedrich et al., 2007) that establish a collagen framework for their own cells, glandular cells, and blood vessels to grow into (Fay & Grudzinskas, 1991). The stromal cells may also function in endometrial metabolism; as well as the production of: prolactin, epidermal growth factor (EGF), and pregnancy-associated plasma protein (Fay & Grudzinskas, 1991). When stimulated by 17β-estradiol and progesterone, stromal cells secrete heparin binding epidermal growth factor (HB-EGF) to support successful implantation (Cavagna & Mantese, 2003; Sharkey & Smith, 2003).

These roles of the stroma cooperating with glandular cells and the basalis layer of the endometrium are critical for preparation of the arriving blastocyst and if they are not co-ordinated, the endometrium will not undergo pregnancy resulting in unsuccessful reproduction.

1.2. Function

The primary function of the endometrium is to prepare for an arriving blastocyst and allow implantation so that the offspring can grow *in-utero.* In order to accomplish this, the endometrium must proliferate in regular cycles for the highest probability of successful reproduction. This process varies across species and is known as an estrous cycles or menstrual cycle depending on whether the endometrium is resorbed following unsuccessful blastocyst implantation (Baerwald, Adams, & Pierson, 2012; Driancourt, Gibson, & Cahill, 1985; Larson & Ball, 1992; Sato, Nasu, & Tsuchitani, 2016) For example, in humans, the menstrual cycle (endometrium discarded with unsuccessful implantation) occurs every 26-35 days (Baerwald et al., 2012); however, in some mammals such as sheep, these cycles are controlled by the number of daylight hours and will occur every 17 days but only in the late autumn-early winter periods (Evans, Duffy, Hynes, & Boland, 2000). This is termed photoperiodism where certain mammals have reproductive cycles based around the number of daylight hours. It allows the individual to produce offspring at a period where nutrients will be the most plentiful and allow the greatest chance for offspring survival.

1.2.1. The Menstrual cycle

Menstrual cycles are induced by the hypothalamo-pituitary-gonadal (HPG) axis. Despite differences in time periods for the cycles, the pattern is relatively uniform across mammals with the exception of endometrial resorption (estrous cycle) or loss (menstrual cycle) at the end of the cycle (Sato et al., 2016). For humans, the primary cycle initiates at the onset of puberty, currently thought to be due to increasing kisspeptin levels (Liu & Herbison, 2016; Uenoyama et al., 2016) as well as elevated levels of glutamate, and decreased levels of γ-amino-butyric acid (GABA) in their respective neurons (Rojas et al., 2015). These changes in glutamate and GABAergic neuron activity are caused by naturally increasing levels of estrogen and progesterone (Messinis et al., 2014), which are thought to act on

their corresponding receptors of these two neuron types (Rojas et al., 2015) as the first cycle occurs. Interestingly, the mechanism for activation of estrogen and progesterone receptors in the glutamate and GABA neurons is unclear; however, the entire mechanism of puberty onset is being gradually revealed.

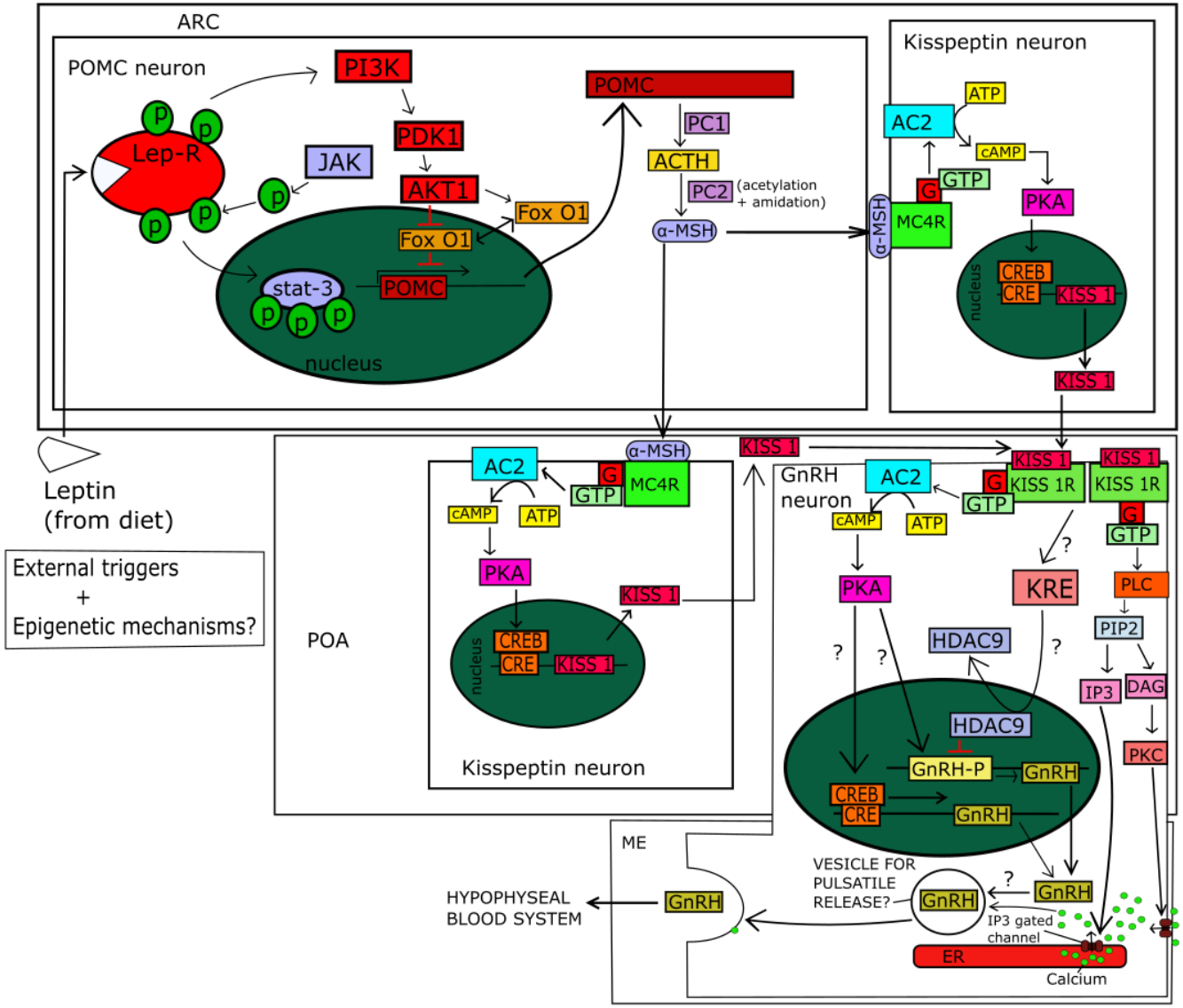
Gene expression of *Kiss1* in kisspeptin neurons of the hypothalamus are one of the central controls for GnRH secretion (Uenoyama et al., 2016). The protein product; kisspeptin activates kisspeptin receptors in GnRH neurons to begin GnRH secretion and puberty onset (Liu & Herbison, 2016). *Kiss1* is activated through epigenetic mechanisms regulated by estrogen (Uenoyama et al., 2016); however, the mechanism is unknown for the conversion from pubertal suppressor to activator. Current knowledge suggests it involves estrogen receptor-α (ER-α), as upon removal, it causes an immediate pubertal onset in mice; suggesting this receptor has an inhibitory function prior to puberty (Dubois, Wolfe, Radovick, Boehm, & Levine, 2016). One hypothesis suggests that ER-α has a bidirectional nature depending on the brain region in which it is located causing kisspeptin levels to increase or decrease; resulting in the primary GnRH surge for puberty onset (Uenoyama et al., 2016). Furthermore, depending on the brain region, kisspeptin either had an increase or decrease in expression around the time of puberty. For example, in the anteroventral periventricular nucleus (AVPV), kisspeptin levels increased while in the arcuate nucleus (ARC), levels decreased (Dubois et al., 2016; Uenoyama et al., 2016). Unfortunately, the exact mechanism for puberty onset is unclear prior to kisspeptin and ER-α expression and suppression. In addition, leptin also appears to act in a competitive manner as it also stimulates ER-α production in high fat diets, which suppresses puberty onset (Bless et al., 2016). Further investigation must be done in order to clarify the exact regulation of this mechanism. The current literature suggests that it involves a level of dietary requirement with leptin (Steinberg, Lowe Vandell, & Bornstein, 2009) activating a leptin - alpha-melanocyte stimulating hormone (α-MSH) pathway that stimulates kisspeptin (Manfredi-lozano et al., 2016; Watanobe, 2002).

The most probable pathway from leptin to kisspeptin is seen in figure 1.2 below. Leptin as a result of dietary intake, digestion, and absorption will act on a leptin receptor of pro-opiomelanocortin (POMC) neurons in the hypothalamic ARC (Watanobe, 2002). This will activate a signalling pathway involving Janus Kinase (JAK) which will phosphorylate the leptin receptor and activate signal transducer

and activator of transcription 3 (STAT3) (Varela & Horvath, 2012). Upon phosphorylation, STAT3 binds to the POMC promoter to stimulate its production (Varela & Horvath, 2012). Simultaneously the phosphatidylinositol-3-kinase (PI3K) pathway is also activated and causes inositol triphosphate (IP_3) to be developed from phosphatidylinositol 4,5-bisphosphate (PIP_2) leading to 3-phosphoinositide-dependent protein kinase 1 (PDK1) activation followed by Protein Kinase B (PKB) (Varela & Horvath, 2012). PKB will inhibit Forkhead box protein O1 (FoxO1) through phosphorylation, allowing further stimulation of POMC production as FoxO1 acts as a POMC inhibitor (Varela & Horvath, 2012). It should also be noted that insulin will act in the same manner as leptin on insulin receptors of the POMC neurons acting through the PI3K pathway (Varela & Horvath, 2012). Once POMC is made available, prohormone convertase 1 (PC1) will transform POMC into adrenocorticotropic hormone (ACTH) (Rousseau et al., 2008). Prohormone convertase 2 (PC2) will convert the ACTH into alpha-melanocyte stimulating hormone (α-MSH) with the assistance of acetylation and amidation (Rousseau et al., 2008). Following this, α-MSH will be secreted from the POMC neuron and received by melanocortin receptor 4 (MC4R) on Kisspeptin neurons in the preoptic area (POA) and the ARC (Manfredi-lozano et al., 2016). From this area of the pathway, the kisspeptin expression mechanism is not fully understood as in sheep α-MSH stimulates Kiss1 in the POA, yet decreases if bound to neurons in the ARC (Manfredi-lozano et al., 2016). In mice however, α-MSH in the ARC stimulates Kiss 1 (Manfredi-lozano et al., 2016) further showing that puberty regulation has minor differences among species.

Once α-MSH is bound to the neurons promoting kisspeptin production, the exact signal transduction pathway used to create kisspeptin is also unclear. However, it appears most likely to be via the adenylate cyclase pathway as well as the phospholipase C pathway to activate kisspeptin (Haskell-Luevano & Hadley, 1999), however this is yet to be confirmed in the literature. A similar process exists upon stimulating GnRH, where these same pathways promote GnRH secretion in the GnRH neurons of the respective areas of the mammalian hypothalamus such as the POA and AVPV in rodents, and the POA and infundibular nucleus of humans (Marques, Skorupskaite, Rozario, Anderson, & George, 2015). In addition, kisspeptin causes more frequent and longer durations of depolarization of the GnRH neurons (Krsmanovic, Hu, Leung, Feng, & Catt,

2010). This implies that GnRH may be kept in vesicles, and similar to other neurotransmitters, may be transferred to the blood stream through this pathway. Furthermore, kisspeptin may also have a role in GnRH expression by acting through a Kiss Responsive Element that transfers histone deactylase 9 (HDAC9) from the cytoplasm to the nucleus where it alters how various transcription factors function to promote GnRH expression (Salian-Mehta, Xu, & Mierman, 2016).


ARC
POMC neuron
PI3K
PDK1
AKT1
JAK
Lep-R
P
Fox O1
Fox O1
stat-3
POMC
nucleus
POMC
PC1
ACTH
PC2
(acetylation
+ amidation)
α-MSH
α-MSH
MC4R
Kisspeptin neuron
ATP
AC2
cAMP
G
GTP
PKA
CREB
CRE
KISS 1
nucleus
KISS 1
Leptin
(from diet)
External triggers
+
Epigenetic mechanisms?
POA
α-MSH
AC2
G
GTP
MC4R
cAMP
ATP
PKA
KISS 1
CREB
CRE
KISS 1
nucleus
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PKC
HDAC9
HDAC9
GnRH-P
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CRE
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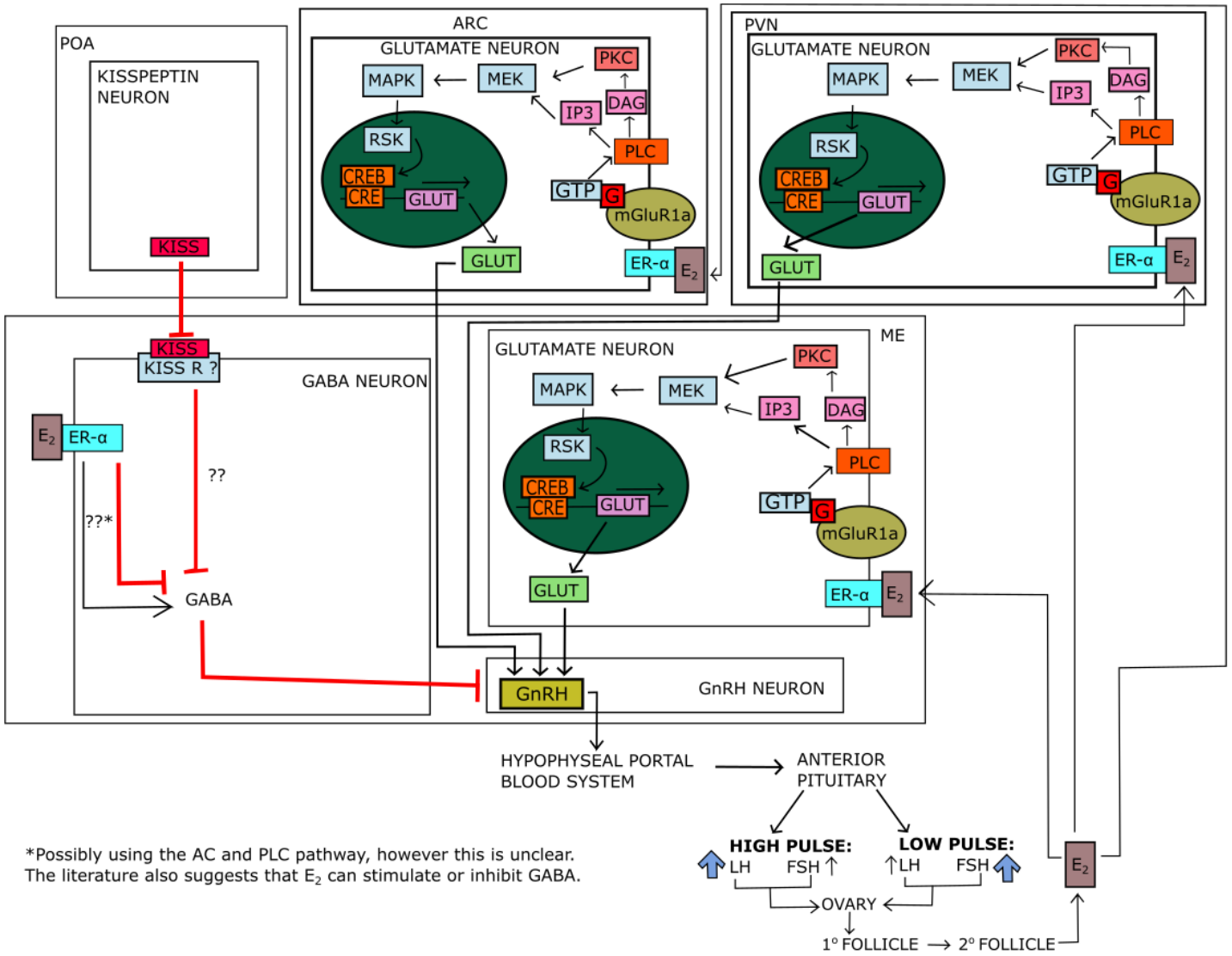
VESICLE FOR
PULSATILE
RELEASE?
GnRH
GnRH
GnRH
IP3 gated
channel
ER
Calcium
HYPOPHYSEAL
BLOOD SYSTEM
GnRH

Figure 1.2: Potential pathway used to initiate GnRH production in the primary menstrual cycle based on current literature (Altarejos & Montminy, 2011; Haskell-Luevano & Hadley, 1999; Messinis et al., 2014; Varela & Horvath, 2012). ARC: arcuate nucleus, Lep-R: Leptin receptor, p: phosphate, PI3K: phosphatidylinositol-3-kinase, PDK1: 3-phosphoinositide-dependent protein kinase 1, POMC: proopiomelanocortin, stat-3: signal transducer and activator of transcription 3, Fox O1: Forkhead box protein O1, AKT: protein kinase B, PC1: Prohormone convertase 1, PC2: Prohormone convertase 2, ACTH: Adrenocorticotropic hormone, α-MSH: alpha-melanocyte stimulating hormone, MC4R: melanocortin 4 receptor, G: G-protein, GTP: Guanosine triphosphate, AC2: adenylyl cyclase, ATP: Adenosine Triphosphate, cAMP: cyclic adenosine monophosphate, PKA: Protein Kinase A, CREB: cAMP response element binding protein, CRE: cAMP response element, Kiss 1 (inside nucleus): Kiss1 gene, Kiss 1 (outside nucleus): kisspeptin, kiss1R: Kiss1 receptor, POA: Preoptic area, KRE: Kiss response element, PIP2: phosphatidylinositol 4,5-bisphosphate, HDAC9: histone deacetylase 9, GnRHp: GnRH promoter, GnRH: Gonadotropin releasing hormone, PLC: Phospholipase C, IP3: inositol triphosphate, DAG: Diacylglycerol, PKC: Protein Kinase C, ER: endoplasmic reticulum, ME: Median eminence.

The brain regions containing glutamate and GABA neurons stimulate and inhibit neuron activity respectively. They exhibit roles in the onset of puberty independently of kisspeptin (Cheong, Czieselsky, Porteous, & Herbison, 2015) as well as promoting further follicle growth in the menstrual cycle. This is evident due to estrogen receptor -α (ER-α) expression on their neuronal surfaces (Cheong et al., 2015) that respond to low levels of estrogen prior to puberty, as well as the increasing levels during menstrual cycles. In the hypothalamus, glutamate neurons promote GnRH production (Clasadonte, Sharif, Baroncini, & Prevot, 2011) and their neurons can be found in the ARC, paraventricular nucleus (PVN) and median eminence (ME) of the hypothalamus (Durand, Pampillo, Caruso, & Lasaga, 2008). GABA neurons, possess the function of inhibition and upon activation, inhibit other neurons from undergoing excitation. These neurons are also found in the ME (Durand et al., 2008) as well as various other brain regions to inhibit neuron activity. It is worth noting that other factors such as insulin and leptin have a role in GnRH secretion where leptin promotes FSH and LH

production (later resulting in greater GnRH production), while insulin promotes GnRH secretion and inhibits the GnRH suppressors GABA and neuropeptide-Y (NPY) (Rojas et al., 2015). It has been suggested that kisspeptin may have a role in glutamate activation and GABA inhibition, however this has not yet been confirmed (Liu & Herbison, 2016). In the glutamate neurons, ER-α is involved in the estradiol positive and negative feedback mechanism via different brain regions; however, in GABA neurons, ER-α is exclusively required for the positive feedback loop exhibited in puberty (Cheong et al., 2015) (see figure 1.3).



*Possibly using the AC and PLC pathway, however this is unclear. The literature also suggests that E_2 can stimulate or inhibit GABA.

Figure 1.3: GnRH secretion caused by estrogen acting through glutamate and GABA neurons and the effects of high pulse vs low pulses of GnRH (Boulware et al., 2005; Cheong et al., 2015; Moura & Petersen, 2010; Thompson & Kaiser, 2014). E_2: Estradiol, ER-α: Estradiol receptor-alpha, ARC: arcuate nucleus, POA: Preoptic area, PVN: Paraventricular nucleus, ME: Median eminence, mGluR1a: metabotropic glutamate receptor, G: G-protein, GTP: Guanosine triphosphate, PLC: Phospholipase C, IP3: inositol triphosphate, DAG: Diacylglycerol, PKC: Protein Kinase C, MAPK: Mitogen activated protein kinase, MEK: MAPK/ extracellular signal-regulated kinase (ERK), RSK: Ribosomal s6 kinase, CREB: cAMP response element binding protein, CRE: cAMP response element, GLUT: glutamate, Kiss 1 (inside nucleus): Kiss1 gene, Kiss 1 (outside nucleus): kisspeptin, kiss R: Kisspeptin receptor, GnRH: Gonadotropin releasing hormone, GABA: gamma amino butyric acid, LH: luteinizing hormone, FSH: Follicle stimulating hormone.

GnRH neurons possess a peculiar anomaly, they terminate at the ME (Campbell, Gaidamaka, Han, & Herbison, 2009). This is interesting because other neurons mentioned above also end at the ME suggesting that some neurotransmitters from the GABA and glutamate neurons may influence the GnRH neuron terminus in order to promote or inhibit GnRH secretion. However, it is more likely that glutamate and GABA also influence GnRH secretion from other brain regions, rather than the ME exclusively and directly. Furthermore, GnRH neurons are long and begin at various areas of the brain, including the nasal regions with cell bodies in multiple regions of the hypothalamus and medial septum (Campbell et al., 2009). GnRH neurons also aggregate together to allow the pulsatile releasing patterns observed to synchronize GnRH release (Campbell et al., 2009). When GnRH is released from the ME, it will travel through the hypophyseal portal blood system to the anterior pituitary. This activates the production of follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Figure 1.3) (Watanabe, Fukuda, & Nabekura, 2014).

When GnRH enters the anterior pituitary gland, it will bind to GnRH receptors found on gonadotrophs (Thompson & Kaiser, 2014). Gonadotrophs are cells that specifically secrete FSH and LH (Smith, Osianlis, & Vollenhoven, 2014). These gonadotrophs respond to the frequency of GnRH pulses to determine FSH

and LH secretion; where less frequent pulses promote greater FSH production and more frequent pulses promote both FSH and LH production (Thompson & Kaiser, 2014). It is not entirely understood how this mechanism functions; however, the proposed mechanism is discussed in thorough detail in Thompson & Kaiser (2014). Currently it is known that GnRH pulse frequency promotes the common sub-unit that FSH and LH possess; common subunit-α (CGA) (Thompson & Kaiser, 2014). The difference between FSH and LH is the second subunit; FSH-β and LH-β; and these subunits are controlled by the different pulsing frequencies of GnRH (Thompson & Kaiser, 2014).

Upon secretion, FSH and LH will be transported to the ovary to stimulate follicle growth (Rama Raju et al., 2013). At this stage many primary follicles will be growing and become secondary follicles. These are characterized by having at least two layers of cuboidal granulosa cells with receptors for FSH to allow for further growth (Chaves et al., 2012; Dorrington & Armstrong, 2013; Rojas et al., 2015). In response to FSH, secondary follicles will grow in size, secrete estradiol, and possess numerous granulosa cell layers (Baerwald et al., 2012; Rojas et al., 2015). In addition, FSH will induce LH receptor expression in the secondary follicles and will cause each to become dependent on LH over FSH (Baerwald et al., 2012; Rojas et al., 2015).

At this stage, the follicle selection process commences. This selection is associated with unique differences between secondary follicles. The follicle that is selected contains the greatest amount of LH receptor expression, aromatase activity, and secretions of 17-β estradiol (Baerwald et al., 2012). In addition, bone morphogenetic protein-15 (BMF-15) and insulin-like growth factor binding protein (IGFBP) appear to have a role in follicle selection and maturation, but it is unclear how these mechanisms function (Gasperin et al., 2014; Mihm & Evans, 2008; Rossi et al., 2015). The maturing follicle that becomes the dominant follicle will possess an oocyte, multiple granulosa cell layers, and a fluid filled antrum. As the follicle matures and grows, it will produce more 17-β estradiol (Grachev & Goodman, 2016). This estradiol will promote further GnRH production in the hypothalamus and cause a surge in LH and FSH (Messinis et al., 2014; Mihm, Gangooly, & Muttukrishna, 2011; Rojas et al., 2015). The LH secreted allows the final follicle growth and maturation so ovulation (release of the ovum into the fallopian tubes) can occur (Grachev & Goodman, 2016). LH also stimulates the

granulosa cells to start releasing progesterone via a prostaglandin E2 pathway (Elvin, Yan, & Matzuk, 2000); and it is the combination of estrogen and progesterone that promote the greatest stages of endometrial lining growth. The menstrual cycle is summarized in figure 1.4 below.

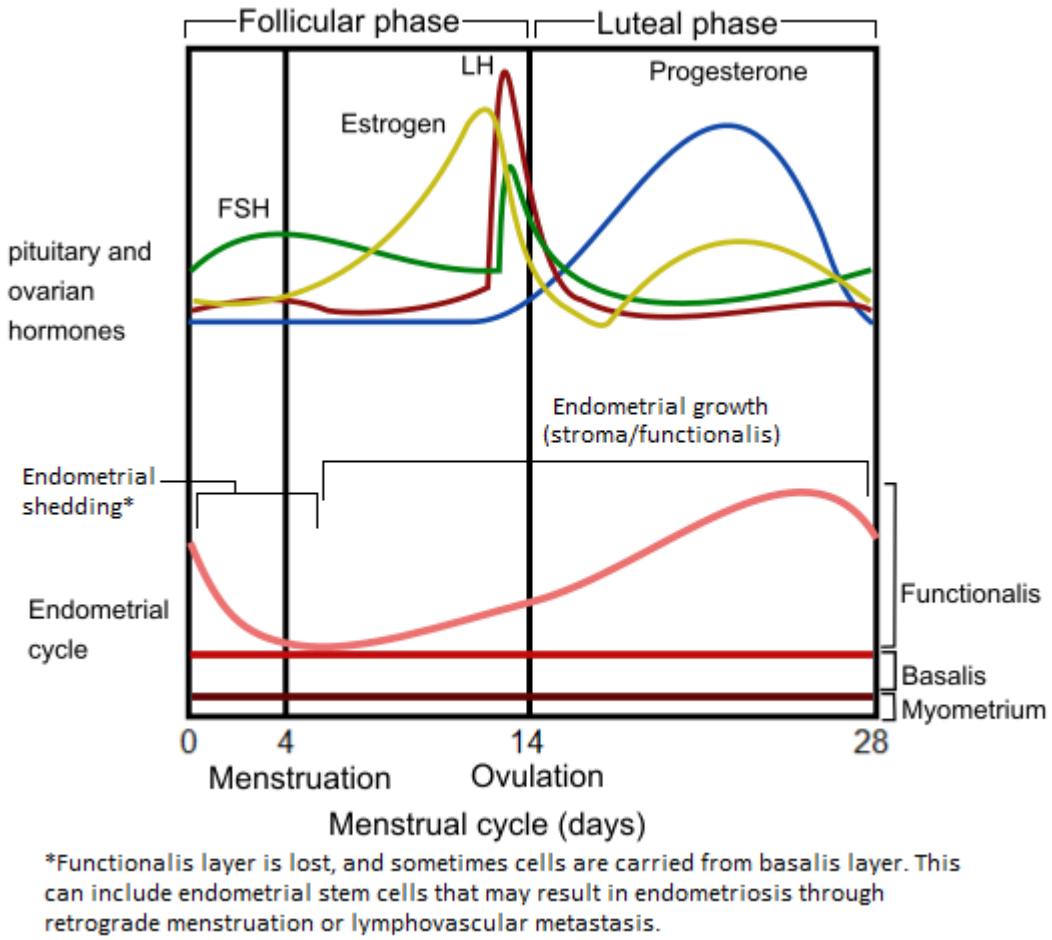


Figure 1.4: The menstrual cycle in human females adapted from Clayton (2019).

2. Mechanism of endometrial growth

2.1. The role of estrogen

As the follicle matures and secretes greater levels of estradiol and progesterone, the endometrium responds by proliferation of the stroma, endometrial vascular glands, and blood vessels in the functionalis (Rogers, Lederman, & Taylor, 1998; Salamonsen, Kovacs, & Findlay, 1999). For proliferation to occur, estrogen will bind to receptors on the nuclear membrane (Hewitt, Winuthayanon, & Korach, 2016) of the basalis epithelial layer in the first menstrual cycle and in each subsequent cycle after menstruation (Gargett, Chan, & Schwab, 2008). Stromal layer proliferation is determined by the estrogen receptor dependent pathway (Thompson & Kaiser, 2014) (see figure 1.3 and 2.1) acting on transcription factor CCAAT enhancer binding protein beta (C/EBPβ) (Hewitt et al., 2016). Furthermore, ER-α and ER-β are also found in this epithelial layer (Gargett et al., 2008; Lecce, Meduri, Ancelin, Bergeron, & Perrot-Applanat, 2001); however, these receptors are bound to chromatin sites prior to estrogen binding (Hewitt et al., 2016). Furthermore, progesterone may also have effects that inhibit estrogen receptor production (Koligan & Stormshak, 1977; Kurita et al., 1998) and prevent estrogen mediated stromal proliferation (Robertshaw, Bian, & Das, 2016).

This stromal proliferation inhibition by progesterone is interesting as in the menstrual cycle, there appears to be no requirement for progesterone to inhibit estrogen receptor production. It is unclear in the literature why this inhibition occurs; however, an adjustment is observed as estrogen levels decline at this stage of ovulation, while progesterone levels increase considerably (see figure 1.4) (Clayton, 2019). In addition, progesterone primes the endometrium for blastocyst reception at this stage and stimulates further stromal proliferation and glandular development (Robertshaw et al., 2016; Wetendorf & DeMayo, 2012). It is also important to note that the peak in progesterone levels indicate the transition from the proliferative phase into the secretory phase through preparing the endometrium for implantation by actions on the glandular functions within the functionalis (Slayden & Brenner, 2004).

Once menstruation has completed along with the loss of the progesterone source (i.e. the corpus luteum (CL)) and any residual progesterone; the follicles and endometrial tissue will commence their growth with the initial increase in estrogen from maturing follicles (Messinis et al., 2014). When estrogen binds to ER-α or -β within the endometrial epithelial or stromal cell nuclei, it will promote the production of: EGF, IGF-1, and transforming growth factor-α (TGF-α) (Gargett et al., 2008). These factors are all required for the proliferation of stromal and epithelial cells (Gargett et al., 2008). In order for this to occur, estrogen may use one of four pathways to promote the production of these growth factors: a direct ligand dependent pathway, a tethered pathway, a non-genomic pathway, or by growth factor signalling (Heldring et al., 2007). The ligand dependent pathway involves estrogen binding to its receptor inside the cell and migrating to a transcription site where it immediately begins synthesis of one of the afore mentioned growth factors (Heldring et al., 2007). The tethered pathway is similar, except that the estrogen-estrogen receptor complex will bind to a transcription factor, then stimulate growth factor production (Heldring et al., 2007). The non-genomic pathway involves the estrogen receptor using a second messenger to promote growth factor production (Heldring et al., 2007), and the growth factor signalling pathway involves a growth factor bound to a ligand acting on kinases to activate phosphorylated estrogen receptors to bind to the transcription site (Heldring et al., 2007). This has been presented in figure 2.1 below.

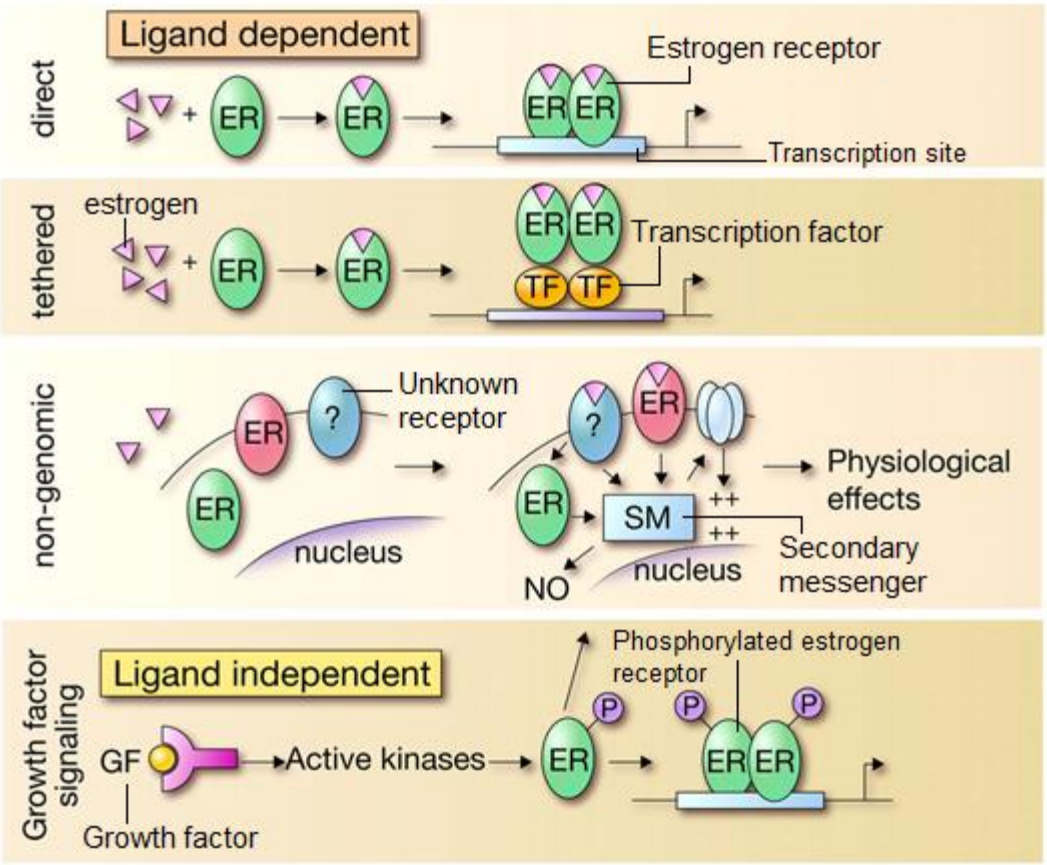


Figure 2.1: Various molecular pathways displaying how estrogen can stimulate other proteins to be produced for use in cellular proliferation (Heldring et al., 2007). ER: estrogen receptor, TF: transcription factor, SM: secondary messenger, NO: Nitric oxide, GF: Growth Factor, P: Phosphate molecule.

When estrogen causes greater expression of EGF, TGF-α and IGF-1, these will function in separate ways to promote mitosis. Specifically, these growth factors appear to have various functions in stromal and epithelial cells (Gargett et al., 2008). EGF co-ordinates mitotic spindle development for chromosome splitting of daughter cells after the nuclear membrane has disappeared in the parent cell (Mardin et al., 2013). When applied it will accelerate the mitotic process through binding to epidermal growth factor receptor (EGFR) and activating a more rapid form of centrosome construction and separation to opposite poles of the cell (Mardin et al., 2013). TGF may also have a similar role as it can bind to the EGFR and stimulate the same process (Heldring et al., 2007). Furthermore, TGF appears to have a second potential role in causing apoptosis of decidualized cells by

phosphorylating the PI3-K/Akt protein kinase enzyme (Caron, Frechette-Frigon, Shooner, Leblanc, & Asselin, 2009).

IGFs act in an autocrine/paracrine positive feedback loop to increase mitosis of endometrial stromal and epithelial cells (Gray et al., 2001). To cause proliferation, IGF-1 using the MAPK pathway acts to increase ER-α production (Gray et al., 2001). As ER-α is produced and bound with estrogen from follicles, it increases the expression of IGF-1 and IGFR (Gray et al., 2001). As IGF-1 and IGFR are expressed, upon binding, the IGFs stimulate further production of ER-α using serine phosphorylation via the MAPK pathway, resulting in further mitosis promotion (Gray et al., 2001). Moreover, when expressed, IGF-1 also assists in angiogenesis as well as endometrial growth and proliferation (Cavagna & Mantese, 2003). This occurs through binding to IGF receptors on cell surface membranes and various binding proteins such as IGFBP-1, -2, or -3 (Fay & Grudzinskas, 1991). It is likely that the presence of various IGFs, the pathways they activate (An, Cheng, Yuan, & Li, 2010; Lathi et al., 2005; Spicer & Aad, 2007), their receptors, and binding proteins cause the differentiation seen in the functionalis layer.

In addition, estrogen stimulates mitosis by increasing the expression of a cyclin-dependent kinase complex (CDK) (Groothuis, Dassen, Romano, & Punyadeera, 2007). CDK is an important part of the maturation promoting factor (MPF) protein complex (Casimiro, Crosariol, Loro, Li, & Pestell, 2012). This protein has many functions including: triggering the formation of a mitotic spindle, chromatin condensation, breakdown of the nuclear envelope, and fragmentation of the Golgi complex (Casimiro et al., 2012). Furthermore, estrogen also increases the expression of mitotic checkpoint component (MAD2) protein which interacts with the anaphase promoting complex used to promote the last mitosis stage (Groothuis et al., 2007).

2.2. The role of progesterone

While estrogen has the majority of its effects as a stromal and epithelial cell proliferator; progesterone has a role in stromal cell decidualization (Gargett et al., 2008). Decidualization is the process of the endometrium responding to progesterone causing less stromal cell growth and division, greater differentiation of stromal and epithelial cells, as well as an increase in endometrial glandular secretions (Gargett et al., 2008; Sharkey & Smith, 2003). These secretions assist in blastocyst implantation (Gellersen & Brosens, 2014) and this process possesses species-specific differences. For example, in most mammals decidualization occurs as a response to blastocyst implantation (Sharkey & Smith, 2003); however, in humans, this process occurs independently of implantation (Gellersen & Brosens, 2014; Wetendorf & DeMayo, 2012).

Progesterone is secreted near the time the mature follicle undergoes ovulation and continues for a time through the CL (refer to figure 1.4) (Clayton, 2019; Messinis et al., 2014). Upon secretion, it will bind to either the 'A' or the 'B' isoform of the nuclear progesterone receptor (nPR) (Cheon et al., 2002; Wetendorf & DeMayo, 2012). The nPR is bound to chaperone proteins within the cytoplasm (Wetendorf & DeMayo, 2012). As progesterone is lipophilic, it will pass through the cell membrane to bind to the nPR (Gellersen & Brosens, 2014). Upon binding, it will enter the nucleus and act with co-regulators to cause gene transcription of specific targets such as Indian hedgehog for further decidualization, as well as cause chromatin alterations (Cheon et al., 2002; Wetendorf & DeMayo, 2012). Also, it can activate an MAPK pathway similar to estrogen, suggesting it may have a role in mitosis (Wetendorf & DeMayo, 2012). However, a paradox is present as, although it can stimulate the mitotic pathway, it will also inhibit it (Kim, Kurita, & Bulun, 2013; Wetendorf & DeMayo, 2012). This is through nPRs acting on the Hand2 transcription factor (Kim et al., 2013). When acted upon, Hand2 will prevent the expression of fibroblast growth factor ligands (Kim et al., 2013). These ligands are used in the process of expressing ER-α, and therefore, by preventing their expression, mitosis is inhibited as well (Wetendorf & DeMayo, 2012) as estrogen cannot activate the MAPK pathway of mitosis (Gray et al., 2001).

In order for endometrial stromal cell differentiation to occur, progesterone will act through an Indian hedgehog (Ihh) signalling pathway leading to activation of cytoplasmic foxO1 (Kim et al., 2013; Li et al., 2012; Wetendorf & DeMayo,

2012). The progesterone-progesterone receptor complex will act as a transcription factor to target the Ihh protein (Large & DeMayo, 2012; Wetendorf & DeMayo, 2012). For the Ihh protein to be expressed, the gene must be transcribed and is only successfully produced when bound to the transmembrane receptor patched-1 (PTCH-1) (Varjosalo & Taipale, 2008; Wetendorf & DeMayo, 2012). This PTCH-1 will allow a transmembrane receptor smoothened (SMO) to activate, resulting in a downstream activation of the chicken ovalbumin upstream transcription factor-II (COUP-TFII) (Large & DeMayo, 2012; Wetendorf & DeMayo, 2012) (the Ihh protein after binding with progesterone-progesterone receptor complex will also promote COUP-TFII activity in a similar manner (Wetendorf & DeMayo, 2012)). COUP-TFII will consequently bind to bone morphogenetic protein 2 (BMP2) (Large & DeMayo, 2012; Li et al., 2012) to act on the wingless-related murine mammary tumour virus integration site 4 (WNT4) ligand (Large & DeMayo, 2012). This uses a β-catenin signalling pathway (Li et al., 2012) to act on the FOXO1 transcription factor (Large & DeMayo, 2012). This transcription factor will be used to promote genes such as: somatostatin (SST), decorin (DCN), left-right determination factor 2 (LEFTY2), and b-cell lymphoma 2-like 11 (BCL2L11) that allow for differentiation of endometrial stroma (Li et al., 2012). This process has been summarized in figures 2.2 and 2.3 below.

Interestingly, progesterone will only assist endometrial gland development if secreted at the correct time within the menstrual cycle (Wetendorf & DeMayo, 2012). If secretion occurs outside this window, it can be detrimental to the stage of preventing gland formation (Wetendorf & DeMayo, 2012). The main function of progesterone in gland development is leukaemia inhibitory factor (LIF) production (Wetendorf & DeMayo, 2012). LIF is used as a binding site for the implanting blastocyst and is produced in the expanding endometrial epithelium (Jeong et al., 2010). For LIF production, it is likely to be secreted using a similar pathway seen in figures 2.2 and 2.3 via FOXA2 (Jeong et al., 2010). This would occur through use of a similar transcription factor and activation (Jeong et al., 2010) to that of the genes described above such as SST and DCN.

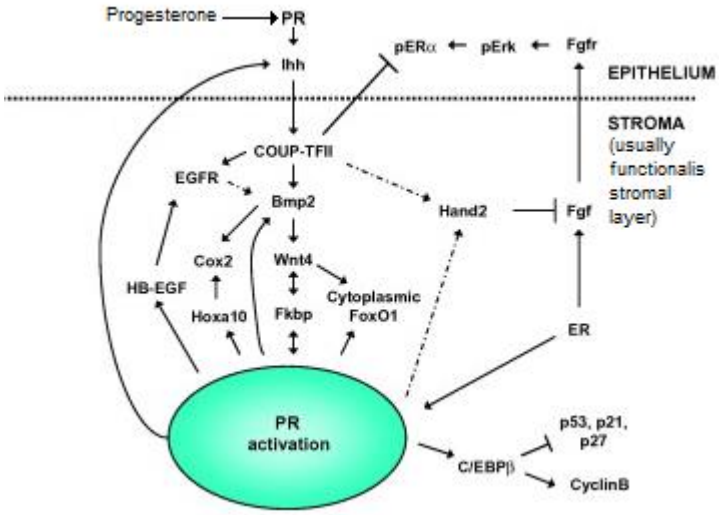


Figure 2.2: Complete pathway in endometrial stroma showing the effects of progesterone receptors on various signalling pathways adapted from Large & DeMayo, (2012). Solid arrows indicate induction, dashed arrows indicate potential pathways but with lacking evidence, barred solid lines indicate inhibition (Large & DeMayo, 2012). PR: progesterone receptor, Ihh: Indian hedgehog protein, pER-α: epithelial estrogen receptor α, pErk: eukaryotic translation initiation factor 2-alpha kinase 3, Fgfr: fibroblastic growth protein receptor, COUP-TFII: chicken ovalbumin upstream promoter-transcription factor 2, EGFR: epidermal growth factor receptor, Bmp2: bone morphogenetic protein 2, Hand2: Heart and neural crest derivatives-expressed protein 2, Fgf: fibroblastic growth factor, Cox2: cyclo-oxygenase-2, Wnt4: wingless-related murine mammary tumour virus integration site 4, HB-EGF: heparin binding-epidermal growth factor, Hoxa10: homeobox A10, Fkbp: FK506 binding protein, FoxO1: forkhead box protein O1, C/EBPβ: enhancer binding protein-β.

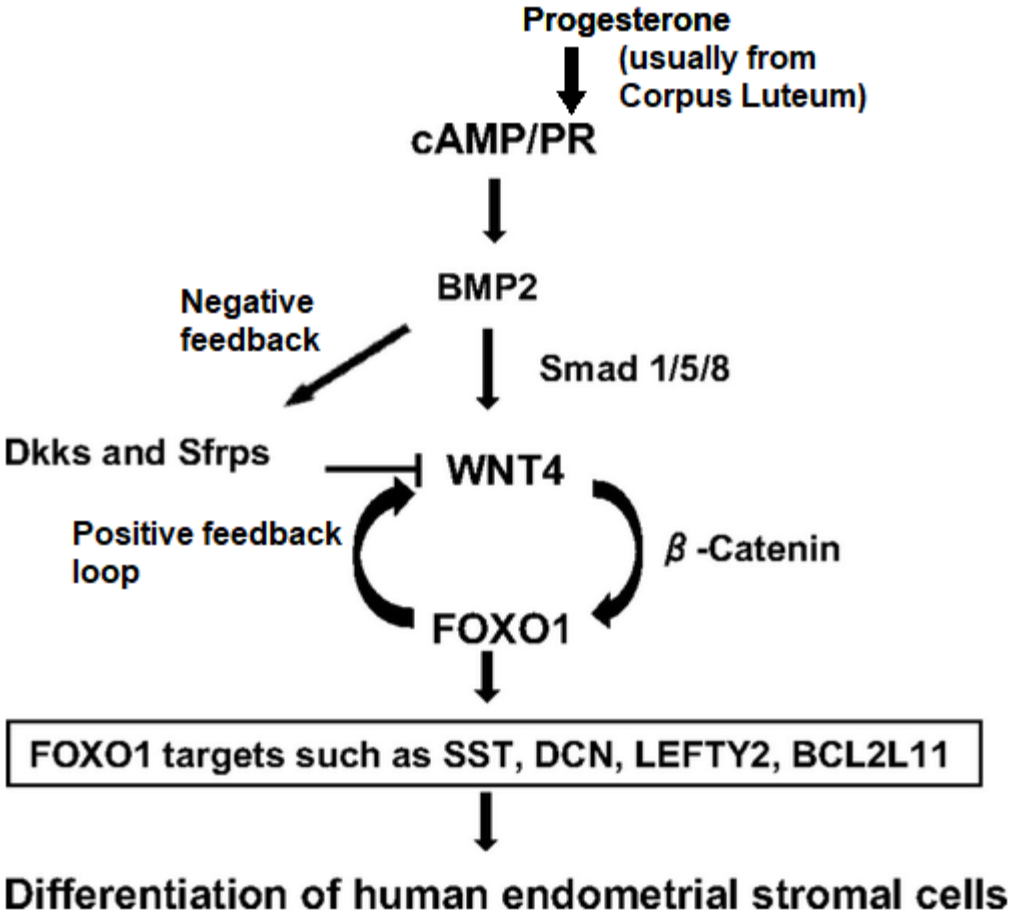


Figure 2.3: One of the pathways used by the nPR to activate FOXO1 to induce differentiation of endometrial stem cells in humans, adapted from Li et al., (2012). Abbreviations: cAMP: cyclic adenosine monophosphate, PR: Progesterone receptor, BMP2: bone morphogenetic protein 2, WNT4: wingless-related murine mammary tumour virus integration site 4, FOXO1: forkhead box protein O1, SST: somatostatin, DCN: decorin, LEFTY2: left-right determination factor 2, BCL2L11: b-cell lymphoma 2-like 11.

3. Endometriosis

3.1. Definition, and hypotheses

Endometriosis is the presence of functional endometrial stroma and glands outside of the uterine cavity (Nap, Groothuis, Demir, Evers, & Dunselman, 2004). It is a common chronic condition involving an endometrial tissue implant into various regions of the body, causing mild to severe pain (Yang & Huang, 2014). These implants are usually found in the fallopian tubes, uterus, ovaries, lungs, peritoneum, and renal tract (Bendon & Becker, 2012; Yang & Huang, 2014). Endometriosis is believed to be the cause of reproductive failure in women and can result in crippling psychological suffering and anxiety (Bendon & Becker, 2012; Yang & Huang, 2014). The cause of endometriosis is unclear; however, several theories have arisen such as: Retrograde menstruation, coelomic metaplasia, embryonic rest, and lymphovascular metastasis (Figueira et al., 2011). It is also plausible that endometriosis has multiple causes instead of a singular cause, however this is also unconfirmed (Sasson & Taylor, 2008). It should also be noted that the uterus is also an 'immunologically privileged site' (Jerzak & Bischof, 2002). This means an inflammatory response such as T-cell or Natural Killer cell responses do not occur while a lesion is present in the uterus (Jerzak & Bischof, 2002). This is likely due to the gene expression of HLA-G, which is known to prevent the NK and T-cells from destroying endometriotic lesions (Barrier et al., 2006; Sundqvist et al., 2011). The result of this makes the uterus vulnerable to diseases or conditions such as endometriosis or other infections in this area.

3.1.1. Retrograde menstruation

This is also known as the Sampson theory (Lebovic, Mueller, & Taylor, 2011) and describes shed cells from the menstrual cycle adhering to a non-uterine surface, causing an ectopic growth (Nap et al., 2004). This can occur through the spillage of viable endometrial cells from the peritoneum (Lebovic et al., 2011) entering the blood vessels. Initially when endometrial cells (resistant to apoptosis and phagocytosis) bind to the walls of any area to cause growth outside of the uterus (Lebovic et al., 2011); some of the immune system may not be activated as the cells are still considered to be 'self' and therefore an immune response is not

performed. This cellular adhering occurs through Intercellular adhesion molecule-1 (ICAM-1), focal adhesion kinase (FAK), and matrix metalloproteinases (MMP) (Kyama, Debrock, Mwenda, & D'Hooghe, 2003).

ICAM-1 is a cell adhesion molecule usually involved in leukocyte trans-endothelial migration, allowing endothelial binding prior to transmigration through the endothelial basement membrane into various tissues (Lawson & Wolf, 2009). However, when endometrial cells secrete and utilize ICAM-1, there are two effects: competition for space with leukocytes, and immunosurveillance evasion (Lebovic et al., 2011). This unique combination allows endometrial stem cells to grow into functional stroma and glands on non-uterine surfaces without immune system interference. Furthermore, interleukin-6 (IL-6) secreted by endometriotic cells may also play a role through the γ-interferon pathway to produce greater levels of soluble ICAM-1; resulting in further adhesion of other endometriotic cells (Kyama et al., 2003). In Sampson (1927), endometrial tissue fragments were discovered in uterine veins after menstruation suggesting that endometriosis could occur in any region of the body if the cells travel through the blood stream (Sampson, 1927). This observation was further supported by the detection of endometrial cells circulating in the blood stream (Chen et al., 2017). These observations greatly validate the idea of retrograde menstruation as this can explain the locations of endometriotic lesions in the various regions of the body described above (Bendon & Becker, 2012; Yang & Huang, 2014).

Focal adhesion kinase (FAK) is found in most cells of the body and is an important part of cell-cell adhesion (Flamini, Sanchez, Genazzani, & Simoncini, 2011; Mu et al., 2008). However, in endometriosis, this enzyme is expressed at greater levels in endometriotic tissue specifically, thus indicating its potential role (Mu et al., 2008). FAK acts as a signalling protein and is located on cell attachment sites that bind to the cytoskeleton using integrin receptors (Mu et al., 2008). In order to function, this tyrosine kinase enzyme must be phosphorylated to allow cell adhesion to occur (Flamini et al., 2011). When estrogen binds to ER-α or -β, it can cause this phosphorylation (Flamini et al., 2011). Therefore, if the cells survive through the use of ICAM-1 to avoid immunosurveillance (Lebovic et al., 2011), estrogen will be able to activate FAK to allow further binding and attachment (Flamini et al., 2011) as well as proliferation and growth of endometrial stroma and

glands (Rogers et al., 1998; Salamonsen et al., 1999) outside of the uterus resulting in endometriosis.

FAK and ICAM-1 act malevolently outside of the uterus through allowing painful ectopic endometrial growths as a result of being able to bind to the ECM as well as avoid and compete for space with the immune system. However; MMPs also possess an endometriotic role (Kyama et al., 2003). MMPs are zinc-containing, Ca^{2+}-dependent endopeptidases involved in cell adhesion (Verma & Hansch, 2007). These proteins break down collagen and the ECM (Nap et al., 2004) permitting an invasion site for endometrial cell binding. MMPs are usually expressed at their highest levels in the late secretory phase of the menstrual cycle. Due to this property, it can also lead to endometrial cells being able to implant into *ex-utero* areas (Nap et al., 2004). Also, MMPs are upregulated in the presence of tumour necrosis factor (TNF-α) and interleukin-1 (IL-1) (Kyama et al., 2003), which also increase in concentration at the secretory phase, and during the early and late luteal phase respectively (Philippeaux & Piguet, 1993; Simon et al., 1993), which may promote endometriotic growths. TNF-α also inhibits the tissue inhibiting metalloproteinases (TIMP), required to inhibit MMP overexpression (Amălinei, Căruntu, Giuşcă, & Bălan, 2010). When TIMP is inhibited, MMPs can effortlessly cause greater ECM degradation and increase the number of sites for potential endometrial cells to implant and cause endometriosis.

FAK, ICAM-1 and MMPs are the major proteins involved in endometriotic implantations. These three factors also present support for the retrograde implantation theory as one method of how endometriosis can occur. By escaping through broken blood vessels in each menstrual cycle, it would not be difficult for a portion of the endometrial cells to enter the bloodstream and implant in other locations using ICAM-1, FAK, and MMPs. In addition, as ICAM-1 acts as a protein to assist in membrane binding as well as its immunological evasion properties; along with the adhesive nature of FAK; and the lysis of the ECM by the MMPs, invasion of endometrial cells would be very difficult to prevent. Furthermore, as the endometriotic cells respond to the same hormones as EnSCs in the menstrual cycle for growth, lesions of endometrial tissue appear to be very difficult to prevent once invasion into the bloodstream from a menstrual cycle has occurred. Furthermore, this theory has validity from more cases of endometriosis occurring on the intestinal left side than the right (Al-Fozan & Tulandi, 2003). This is due to

the left side colon being closer to the fallopian tube when compared to the right, resulting in a higher probability of cellular adherence from endometrial cells upon shedding in menstrual cycles (Story & Kennedy, 2005).

3.1.2. Coelomic metaplasia

This theory involves peritoneal tissue modification into ectopic endometrial tissue (Burney & Giudice, 2012). It is believed that hormonal or immunological influences can cause this alteration (Sourial, Tempest, & Hapangama, 2014); however, such growth factors have not been identified. This theory is not as well supported in the literature compared to retrograde menstruation. In addition, few reports exist on how this mechanism operates. It is believed that this form may play a role in deep endometriosis observed in pre-pubescent (Sourial et al., 2014), and adolescent girls (Brosens, Gordts, & Benagiano, 2013). Evidence for this comes from a study on female rabbits where metaplasia was induced (Vinatier, Orazi, Cosson, & Dufour, 2001). Also, an *in-vitro* study suggests that endometrial stromal and surface epithelia with a 10-fold increase of 17-β-estradiol cause metaplasia of the adjacent mesothelial cells to become ectopic endometrial cells (Vinatier et al., 2001). Moreover, evidence from case studies of patients with Mayer-Rokitansky-Küster-Hauser syndrome presents evidence from individuals with no functioning uterus, yet endometriosis has still occurred (Mok-Lin, Wolfberg, Hollinquist, & Laufer, 2010; Troncon et al., 2014) giving more plausibility to this theory.

3.1.3. Embryonic rest

Embryonic cell rest involves a dormant state of embryonic cells with a probable Müllerian (uterine tube) origin (Santamaria, Massasa, & Taylor, 2012). The theory proposes these cells exist in the peritoneal cavity or elsewhere that can be induced into endometriotic tissue (Figueira et al., 2011). Alternatively, this type of endometriosis could be the result of an embryogenesis defect by some embryonic cells persisting and developing into endometriotic lesions upon estrogen exposure (Sourial et al., 2014). There is little evidence for this theory (Sasson & Taylor, 2008); however, verification of this theory exists where males exhibit endometriosis (Gonzalez, Vnencak-Jones, Shi, & Fadare, 2014). This occurs through initial growth stages by the development of female-specific

structures usually inhibited in the presence of the male genome (Sasson & Taylor, 2008). Therefore, for this type of endometriosis to occur, a currently unknown method must exist where cells remain uninhibited until estrogen exposure occurs. This form of male endometriosis is rare and exists only in case reports (Gonzalez et al., 2014). Cases of male endometriosis are linked to estrogen therapy for prostate cancer (Gonzalez et al., 2014); further suggesting the plausibility of the embryonic rest or coelomic metaplasia theory for endometriosis onset in males. After exposure to estrogen, the now active cells will likely use ICAM-1, FAK, and MMPs to bind and allow growth of such endometriotic lesions.

3.1.4. Lymphovascular metastasis

This theory states that endometrial cells enter through the lymphatic system and as a result, will cause endometriosis in regions further away from the uterus, reaching as distant as the brain (Sasson & Taylor, 2008). It was thought that if endometrial cells could enter the blood stream and cause endometriosis, therefore it could do something similar using the lymphatic system (Sampson, 1927). Within the endometrial basalis layer, there are large and great numbers of lymph nodes (Jerman & Hey-Cunningham, 2015; Red-Horse, 2008). These draining lymph nodes allow effortless access for endometrial cells to invade far-reaching areas of the body in the menstrual cycle (Jerman & Hey-Cunningham, 2015). In addition, as described above, the endometrial cells evade white blood cell detection (Lebovic et al., 2011), and pass through tight endothelial layers (Lawson & Wolf, 2009) thus permitting them to travel great distances, implant, and grow into painful lesions through the same method described in the retrograde menstruation theory.

4. Conclusion

Endometriosis is the result of EnSCs escaping the uterus, adhering to various body regions, and causing painful lesion growths. Due to the prevalence of this condition; a review and analysis of endometrial development, the menstrual cycle with its underlying molecular mechanisms, and the resulting potential causes of endometriosis was performed. As the endometrium develops, it will go through regular cycles where the basalis will develop the temporary functionalis layer from EnSCs located in the basalis crypts. The first cycle is likely initiated from kisspeptin stimulation resulting from leptin production caused by natural dietary intake. Kisspeptin acts to cause the early surge of GnRH in pulsatile stages to act on the anterior pituitary gland where LH and FSH are developed and transported to the ovary for follicular development. As follicles mature, a single follicle is selected by various molecular mechanisms and causes further stimulation to allow endometrial functionalis growth in preparation for blastocyst arrival. However, should there be no blastocyst reception, menstrual bleeding will occur and the functionalis layer is discarded. As a result, EnSCs are occasionally lost through menstrual bleeding, enter the body, and use ICAM-1, MMPs, and FAK adhering molecules to permit endometriotic lesion growth in various body regions once an attachment site is formed. The EnSCs escape through the blood stream (retrograde menstruation) or the lymphatic system (lymphovascular metastasis) to generate endometriotic lesions. Alternatively, embryonic rest and coelomic metaplasia may occur where stem cells are induced to become endometriotic lesions throughout the body. The resulting endometriosis is chronic and painful where lesions develop in abnormal locations while avoiding immune system detection. Therefore, by understanding endometrial growth mechanisms, the menstrual cycle, and endometriosis development; novel diagnostic methods and medical procedures can be established to act as future therapeutic strategies for endometriosis treatment.

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Chapter 3a: Tumour necrosis factor-alpha meta-analysis: Early endometriosis diagnostics

Abstract - Endometriosis causes symptoms of pelvic pain, discomfort, fatigue, nausea, and infertility. Diagnostic methods include invasive laparoscopies, testing serum, peritoneal fluid (PF), or peritoneal lavages (PL). Tumour necrosis factor-alpha (TNF-α) is a potential endometriosis diagnostic candidate which differentiates between endometriotic and non-endometriotic patients. However, contradictions exist between studies; therefore, this PRISMA compliant meta-analysis was performed to evaluate the potential of TNF-α for an early stage diagnostic procedure. PF studies were identified to compare TNF-α levels (pg/ml) between fertile controls against endometriosis patients. Where means and standard deviations were not available, estimation methods[1, 2] were implemented. Our inclusion criteria identified 19 studies with 1075 patients. A Standard Mean Difference (SMD) random model compared PF TNF-α differences. Statistical significance ($p<0.05$), with an SMD of 2.1753 (Confidence Intervals: 0.2598 and 4.0908) suggests that TNF-α can be used as an early endometriosis indicator. Analysis limitations included: no TNF-α gold standard, studies not including menstrual stage at time of PF extraction, and incomplete data sets. As TNF-α is a general inflammatory cytokine; for diagnostic procedures, other cytokines and growth factors such as transforming growth factor-β (TGF-β), and Interleukins-1 and -10 (IL-1 and -10) should also be included from specific menstrual cycle stages. For diagnostic testing, a biochemical analyser or multiplex immunoassay using PF samples for early stage endometriosis is proposed as both tests can assay multiple factors in one procedure.

Key Words: Tumour necrosis factor-alpha, endometriosis, ascitic fluid, cytokines

Abbreviations: rAFS: revised American Fertility Society, TNF-α: tumour necrosis factor-alpha, PF: Peritoneal fluid, PL: Peritoneal lavage, ICAM-1: Intercellular adhesion molecule-1, FAK: focal adhesion kinase, NF-κB: Natural Factor-Kappa B, PP14: Placental protein 14, IL-1: Interleukin-1, IL-10: Interleukin-10, MEKK's: mitogen activating protein kinase kinases, CSF: cerebrospinal fluid, IRMA: immunoradiometric assay, ELISA: enzyme-linked immunosorbent assay, VEGF: vascular endothelial growth factor

1. Introduction:

Endometriosis is a painful condition causing infertility and affecting the quality of life in women[3] and is problematic to diagnose non-invasively in early stages. It involves the presence of lesions from endometrial stroma and glands outside of the uterus[4]. These lesions can be present in many areas of the body including the pelvic cavity, lungs, and the brain[5]. There are four theories surrounding the cause of endometriosis: retrograde menstruation, embryonic rest, lymphovascular metastasis, and coelomic metaplasia[6]. Usually diagnosis of endometriosis involves invasive laparoscopy to confirm the afflicting condition by visual inspection for endometrial lesions in abnormal locations[7]. Furthermore, the timing of diagnosis is also difficult as it is generally diagnosed after a patient has reported symptoms aligned with endometriosis, or, as in some infertility asymptomatic cases, an evaluation is performed for endometriosis[8]. Therefore, with such treatments being required, it is highly desirable to develop a simple, less invasive diagnostic test to confirm endometriosis in women[9, 10]. To investigate if such tests can be implemented, this meta-analysis has been performed for the potential of TNF-α as a candidate as one of the diagnostic factors for developing a less or non-invasive endometriosis test.

TNF-α, a 27kDa cytokine in its uncleaved form is one of the pro-inflammatory cytokines involved in endometriosis[11, 12] and was originally identified in studies of haemorrhagic tumour necrosis[13]. It is part of the primary non-selective (innate immune) defence mechanism against infection and is one of the factors involved in tumour destruction within the body[13, 14] as well as roles in cell survival, and

inflammation[15]. This cytokine can be found in and is secreted from various white blood cell types including activated macrophages, B-cells, T-cells, and neutrophils[16]. These blood cells are associated with the endometriotic lesion and produce TNF-α. In addition, TNF-α is also produced in response to other diseases, for example: *Mycobacterium tuberculosis*[17], and bacterial meningitis from CSF (cerebrospinal fluid)[18], displaying how common elevated levels are in a variety of conditions or disease states.

The appearance of TNF-α in the formation of endometriosis is clear in the retrograde menstruation theory where endometrial tissue enters the peritoneal cavity from fallopian tubes[6] and begins with an adhesion stage. Adhesion occurs through Intercellular adhesion molecule-1 (ICAM-1), metalloproteinases (MMP), and focal adhesion kinase (FAK)[19]. Furthermore, to promote the growth of adhesions, the immune response is either not activated and becomes resistant to apoptosis[9], or tumour formation is enhanced by TNF-α activating signal transduction pathways to produce Natural factor–kappa B (NF-κB)[12]. TNF-α is regulated by Placental Protein 14 (PP14), Interleukin-1 (IL-1), and progesterone[20], and these three may unintentionally promote TNF-α in this instance. When TNF-α is produced it will bind to TNF receptors 1 and 2, activating the MEKKs (mitogen activating protein kinase kinases)[12]. When this occurs, a series of downstream phosphorylation reactions occur with resulting increases in NF-κB[12]. When activated in this manner, apoptosis is suppressed, allowing enhanced tumorigenesis[12].

Currently PF TNF-α is not used for diagnostic procedures due to conflicting results between studies. Therefore, this meta-analysis has been performed with the objective to investigate if and how this cytokine can be utilised as one of the early diagnostic factors for endometriosis although such variations of statistical and non-statistical significance exist[9, 15]. Studies possessing PF or PL TNF-α in pg/mL were obtained through various databases for patients with and without confirmed diagnosis of endometriosis. Where possible, studies with data for revised American Fertility Society (rAFS) stages I-II early endometriosis stages[21] were used in preference for this analysis. Interventions for all studies analysed were approved by the relevant human ethics boards for the associated research and

involved laparoscopies to confirm endometriosis prior to obtaining PF TNF-α samples. Various diagnostic assays were performed and all were included in this meta-analysis; for example, the ELISA (enzyme immunosorbent assay), bioassay, IRMA (immunoradiometric assay), multiplex immunoassay, the Immulite™ biochemical analyser, and Cytometric bead array. All diagnostic medical laboratory procedures were included as no official gold standard has been developed for TNF-α. Although few studies indicate that the ELISA is the gold standard[22]; it is important to note that there is no reference as to the reasoning or validation procedures involved to support the ELISA to be the most accurate test. The outcome of this analysis possessed statistical significance ($p<0.05$), however, an imprecise, large effect was also present. This effect possibly comprised of a number of variables not accounted for with PF samples further explained in the discussion. However, the large effect was reflected in the raw data as evidenced by the range of values in TNF-α levels. Consequently, once a number of conditions are met as described in the discussion, this cytokine should be re-evaluated by a secondary meta-analysis to confirm any conflicts between previous studies of its potential use in endometriotic diagnostics.

2. Method:

2.1 Searches and study criteria

The databases used for this analysis included: NCBI (PubMed), Scopus, ResearchGate, Ovid, EBSCO Business Source Premier, Web of Science, and Google Scholar using the following terms: “meta-analysis, endometriosis, macrophages”, “TNF-α”, “tumour necrosis factor-α”, “endometriosis”, “PF”, and “peritoneal fluid” with many terms used simultaneously. From these search engines, studies were identified and screened for potential use in this analysis. Furthermore, references from the included studies were also identified for analysis as well. Some studies were obtained from Science Direct by use of the reference screened papers. Where research papers were not available, authors were contacted through ResearchGate. The meta-analysis criteria involved any study measuring TNF-α (pg/ml) in PF or PL and comparing these levels between normal

and endometriotic patients. Comparisons of other infertility associated diseases to standard endometriosis were not included. For this meta-analysis, ethical approval was not required as all studies were based on published articles. Furthermore, a risk of bias according to Higgins et al[23] was performed on all studies (Table 1).

2.2. Data extraction and synthesis

All data were collected and collated in Microsoft Excel. Selected studies had raw data values for TNF-α in pg/ml with conversions performed as necessary. From these, sample sizes, means, and standard deviations were used for the analysis. Sample sizes were provided in all studies, however, where means and standard deviations were not provided, statistical estimation formulas from Hozo et al[1] and Wan et al[2] were applied in Microsoft Excel.

Table 1: Meta-analysis risk of bias assessment for studies according to Higgins et al[23].

Studies	Random sequence generation (selection bias)	Allocation concealment (selection bias)	Blinding of participants and personnel (performance bias)	Blinding of outcome assessment (detection bias) (patient-reported outcomes)	Incomplete outcome data addressed (attrition bias) (Short-term outcomes (2-6 weeks))	Selective reporting (reporting bias)
(Kalu et al., 2007)	High	Low	Low	Low	Low	High
(Podgaec et al., 2007)	High	High	Low	Low	Unclear	Low
(Cheong et al., 2002)	High	Low	Low	Low	Low	Low
(Harada et al., 1997)	High	High	Low	Low	High	High
Bedaiwy et al., 2002)	High	Low	Low	Low	Low	Low
(Richter, Dorn, Rosing, Flaskamp, & Ulrich, 2005)	High	Low	Unclear	Low	Low	Low
(Mier-Cabrera, Jimenez-Zamudio, Garcia-Latorre, Cruz-Orozco, & Hernandez-Guerrero, 2011)	High	High	Low	Low	Low	Low
(Dziunycz et al., 2009)	High	High	Low	Low	Low	Low
(Barcz et al., 2012)	High	Unclear	Low	Low	Low	Low
(Drosdzol-Cop & Skrzypulec-Plinta, 2012)	High	Low	Low	Low	Low	Low
(Wickiewicz et al., 2013)	Low	Low	Low	High	Low	Unclear
(Pizzo et al., 2002)	High	Low	High	Low	Low	Unclear
(Rana et al. 1996)	Low	Low	Low	High	Low	Low
(Salmeri et al., 2015)	Low	Low	High	High	Low	Low
(Skrzypczak 1995)	Low	Low	Unclear	Low	Low	Unclear
(Wang et al. 2018)	High	High	High	High	Unclear	Unclear
(Liu et al. 2000)	Low	High	High	Low	Low	Unclear
(Oepomo, T. D. 2006)	Low	High	High	Low	Unclear	Unclear
(Bedaiwy et al. 2007)	Low	High	Low	Low	Low	Low
(Foda et al. 2012)	High	Low	High	Unclear	Low	Low
(Vercellini et al. 1993)	Low	Low	Low	Low	Unclear	Low

2.3 Data analysis

Due to the nature of this analysis, a random effects model was selected as all studies sampled different women under similar circumstances according to the rAFS classification system[24]. Studies were analysed using the Standard Mean Difference (SMD) in RStudio (Open source, version v1.2.1335, with R version 3.0.1) which provides a heterogeneity test, the effect size; as well as confidence intervals that displayed test precision. If this range did not overlap '0' a significant difference existed, and the further away the confidence interval range is from '0', the more of an effect that is present. The size of the range indicates the precision with smaller ranges having more consistently accurate results after analysing all studies. The following RStudio 'library' packages were used: 'effsize', 'lsr', 'meta', 'metafor', 'zoom', and 'matrix'. Table 2 displays the layout of the CSV (Comma delimited) file used in Microsoft Excel for interpretation by RStudio.

Table 2: Data presentation in Excel for RStudio analysis[25]. Cmean = Control mean, CSD = Control Standard Deviation, Cn = Control no. of patients, Emean = Experiment mean, ESD = Experiment Standard Deviation, En = Experiment no. of patients

Trial	Cmean [1]	CSD[2]	Cn[3]	Emean [¶]	ESD	En
Study 1	'x' value (in pg/ml)	Standard Deviation of study	No. of patients	'x' value (in pg/ml)	Standard Deviation of study	No. of patients
Study X	'x' value (in pg/ml)	Standard Deviation of study	No. of patients	'x' value (in pg/ml)	Standard Deviation of study	No. of patients

[1] Control patients
[2] Control Standard Deviation
[3] Control sample size of patients
[¶] Endometriosis

3. Results

For this analysis 43 studies were selected. From these, 22 were excluded due to the incorrect sample type, or because the data type was considered unreliable for accurate mean and standard deviation estimates, e.g. log data, and extrapolated data from genetics studies. A further 2 studies (73 patients) were excluded because they contained data for endometriosis patients, and possessed values of '0' or 'not detected' for control samples. Due to this property, they added no weight to the meta-analysis calculation. The remaining 19 studies were considered eligible and included 1075 patients. Figure 1 illustrates the PRISMA compliant[26] method for study selection. Table 1 shows the risk of bias assessment for these studies. It should be noted that there is selection bias in the majority of studies as clinicians intentionally selected those who did and did not have endometriosis or an infertility related condition.

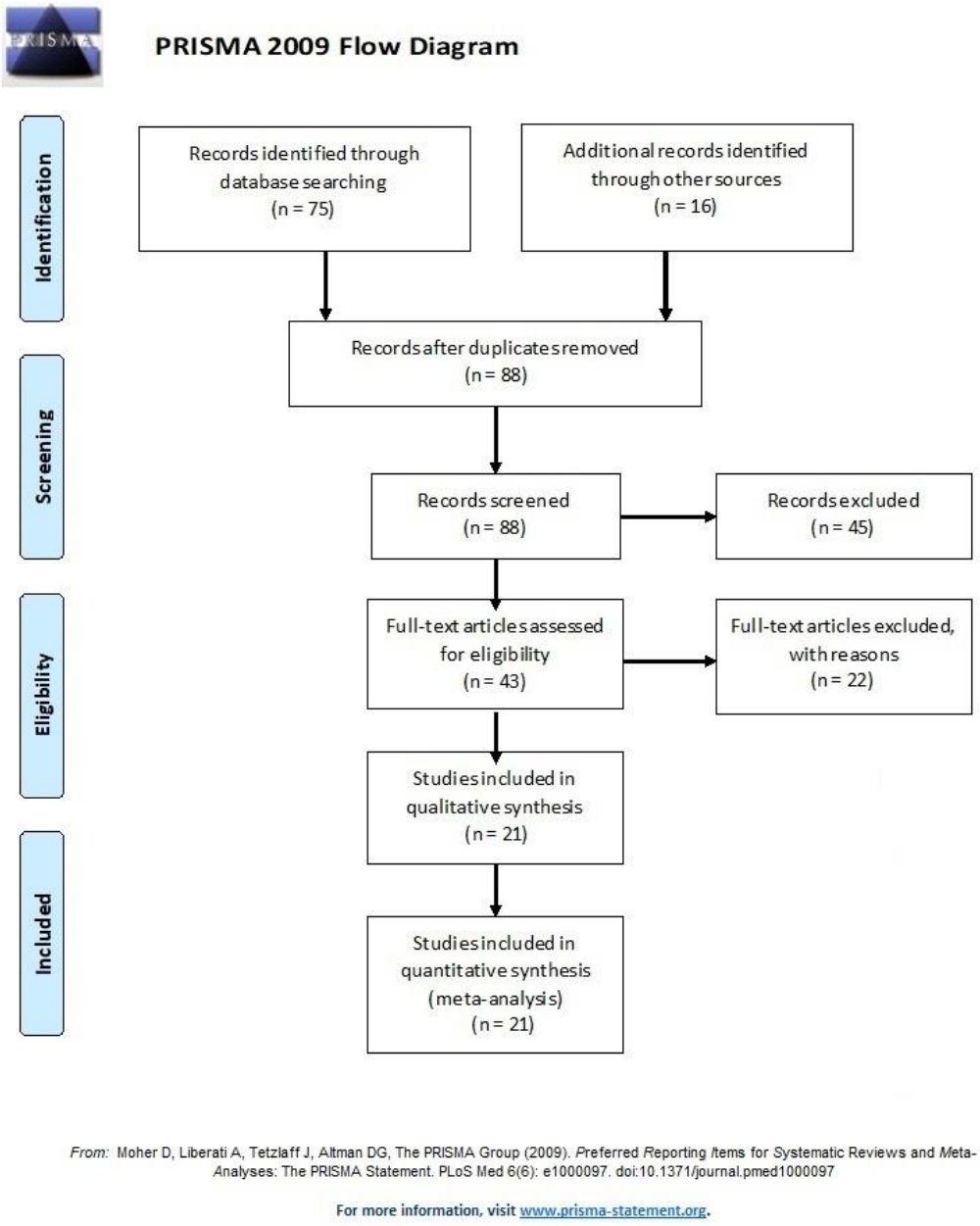

Figure 1: PRISMA flow diagram illustrating refinement of studies selected for this meta-analysis, adapted from template of Moher et al[26].

From this information, as the heterogeneity test possessed a low p-value ($p<0.05$) and from assessing the nature of the studies without statistical analysis, a random effects model was ideal while using the SMD calculation (Figure 2). Also, in Figure 2, according to the SMD calculation, statistical significance does exist ($p=0.0282$), and furthermore the effect is large (SMD: 2.1753, CI: 0.2598 – 4.0908). By consisting of a large confidence interval range close to '0' in the SMD calculation,

the result is also considered imprecise. This idea is explored further in the discussion and limitations of these studies. Furthermore, with refinement in future studies, the results suggest that TNF-α can be used as one of the early diagnostic markers for endometriosis. However, it cannot be the only predicting factor at this stage due to the inconsistency and data conflicts in previous studies and those seen in this meta-analysis. For further reference, Table 3 provides an overall summary of the 21 studies to show means, standard deviations, sample numbers, kits used, and whether or not there was a significant difference between the patients with and without endometriosis.

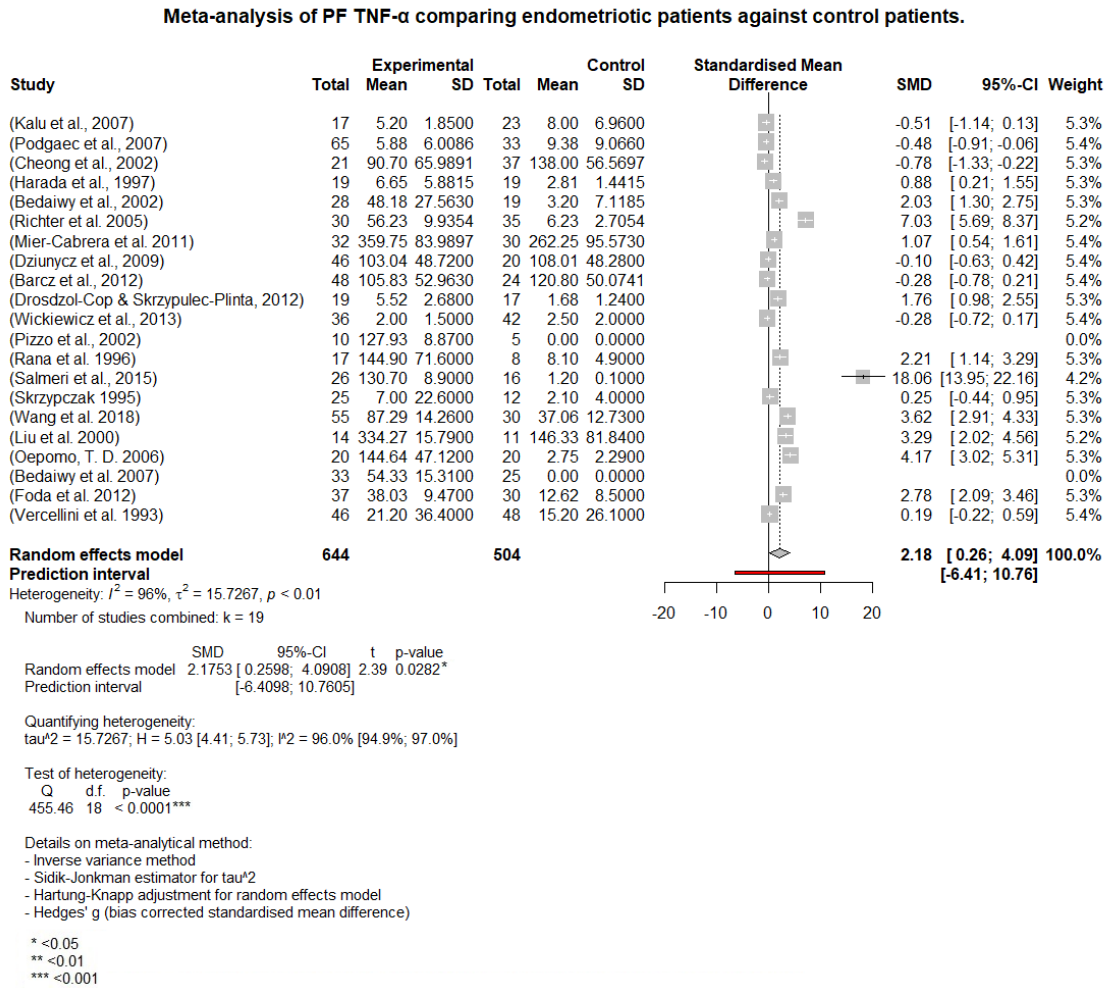

Figure 2: RStudio Meta-analysis and heterogeneity test of patient PF TNF-α levels (pg/mL) of women with and without endometriosis (SMD = Standardized Mean Difference (Hedge's g), SD = Standard Deviation, CI = Confidence Interval). Performed according to RStudio code from Harrer and Ebert[25].

Table 3: Summary of data for the 21 studies used in the meta-analysis. Any estimates performed were done according to formulas of Hozo et al[1] and Wan et al[2].

Study	Control			Endo[†]			Estimates required?	Kit used	p-values
	Mean	SD[‡]	N[§]	Mean	SD	N	Yes/No		
(Kalu et al., 2007)	8.00	6.96	23	5.20	1.85	17	Yes - sd estimated	Immulite™	0.053
(Podgaec et al., 2007)	9.38	9.06	33	5.88	6.01	65	Yes - mean and sd estimated	BD Cytometric Bead array using a flow cytometer	0.364
(Cheong et al., 2002)	138.00	56.57	37	90.70	65.99	21	Yes - sd estimated	Bioassay and staining via L929 cells	<0.05
(Harada et al., 1997)	2.81	1.44	19	6.65	5.88	19	Yes - mean and sd estimated	ELISA	<0.05
(Bedaiwy et al., 2002)	3.2	7.12	19	48.18	27.56	28	Yes - mean and sd estimated	ELISA	<0.001
(Richter, Dorn, Rosing, Flaskamp, & Ulrich, 2005)	6.23	2.71	35	56.23	9.93	30	Yes - Median = mean estimated	IRMA	<0.0001
(Mier-Cabrera, Jimenez-Zamudio, Garcia-Latorre, Cruz-Orozco, & Hernandez-Guerrero, 2011)	262.25	95.57	32	359.75	83.99	32	Yes - Median = mean, and SD estimated	Multiplex immuno-assay	<0.05
(Dziunycz et al., 2009)	108.01	48.28	20	103.04	48.72	46	No	ELISA	0.7743
(Barcz et al., 2012)	120.8	50.07	24	105.83	52.96	48	Yes - mean and sd estimated	ELISA	No signif. diff. (No value given)
(Drosdzol-Cop & Skrzypulec-Plinta, 2012)	1.68	1.24	17	5.52	2.68	19	No	ELISA	0.000001
(Wickiewicz et al., 2013)	2.50	2.00	42	2.00	1.50	36	No	Cytometric Bead array	0.1696
(Pizzo et al., 2002)	0	0	5	127.930	8.87	10	No	Diaclone France ELISA	<0.001

[†] Endometriosis
[‡] Standard Deviation
[§] Sample size

Study	Control			Endo[†]			Estimates required?	Kit used	p-values
	Mean	SD[‡]	N[§]	Mean	SD	N	Yes/No		
(Rana et al. 1996)	8.1	4.9	8	144.9	71.6	17	No	ELISA	<0.001
(Salmeri et al., 2015)	1.2	0.1	16	130.7	8.9	26	No	ELISA	<0.001
(Skrzypczak 1995)	2.1	4	12	7	22.6	25	No, values given in study assumed as the mean.	ELISA and RIA	No signif. diff.
(Wang et al. 2018)	37.06	12.73	30	87.29	14.26	55	No, values given in study assumed as the mean.	ELISA	<0.05
(Oepomo, T. D. 2006)	2.75	2.29	20	144.64	47.12	20	No	ELISA	0.00
(Bedaiwy et al. 2007)	0	0	25	54.33	15.31	33	Yes, mean and sd estimated	ELISA	<0.0001
(Foda et al. 2012)	12.62	8.5	30	38.03	9.47	37	No	ELISA	<0.001
(Vercellini et al. 1993)	15.2	26.1	48	21.2	36.4	46	No	ELISA	No signif. diff.

4. Discussion:

This meta-analysis was primarily designed to investigate if TNF-α can be a candidate for a future diagnostic test for early stage endometriosis. TNF-α is one of the generalized immunological cytokines[16]; a part of many pleural effusions[15], and is associated with many other diseases, for example tuberculosis[17]. Therefore, it must be noted that TNF-α can only be used for patients with no other illnesses or conditions as these may lead to confounding results. For further endometriosis diagnostic accuracy, a combination of TNF-α and other cytokines or hormones could be used. Results from this meta-analysis possess a large enough SMD value to support an existing effect and, once the limitations described are overcome to investigate a potentially narrower confidence interval range, TNF-α

[†] Endometriosis
[‡] Standard Deviation
[§] Sample size

should be considered as a candidate for an accurate diagnostic test. Furthermore, although sample size is small (n=19) the total sample size these studies represent (n=1075) provide sufficient support for TNF-α to become one of the endometriotic diagnostic markers.

In addition, of the 19 analysed, weighted studies, only one studied TNF-α exclusively[21]. The potential problem of exclusively researching one cytokine is that a greater pressure for a successful experiment exists as bias is present for only successful research to be published. Due to this successful experiment pressure, bias[27] can be introduced into meta-analyses as well, altering the confidence interval range and SMD. However, when multiple cytokines are measured, this suggests less pressure on successful experiments for TNF-α exclusively to be considered an accurate endometriosis indicator. This is due to other factors that may be significant. This provides studies measuring multiple factors a greater credibility as it allows for less publication bias based on successful results of a singular cytokine.

At this time, no other meta-analysis has investigated the value of PF TNF-α levels in endometriotic against non-endometriotic women. This makes it unfeasible to compare results of other papers to this analysis. However, these results share similar issues with other reports in the literature; there are agreements and conflicts of data and significance of TNF-α levels of PF samples. Some studies have found significant differences present[21, 28-30], and others possess no statistical difference[29, 31-33]. Once new data sets are developed with limitations overcome in multiple research publications; a new meta-analysis must be performed to investigate if the significance and confidence interval range is valid in a similar manner to Figure 2. Until this second meta-analysis is performed; PF TNF-α cannot be more accurately determined for clinical use in a less invasive endometriosis diagnosis.

4.1 Limitations:

A number of limitations were present in this meta-analysis. Primarily, there is no agreed gold standard for measuring endometriotic TNF-α in PF or serum[34]. This may explain some of the range in Figure 2 as different test types have the potential to yield different results, e.g. ELISA vs Cytometric bead array. This complicates matters when performing meta-analyses as comparisons between different studies using different methodology increase the probability of a wider TNF-α (pg/ml) range of varying data sets. Also, further variation exists via inter-company testing, i.e. ELISA kits developed by different companies. For instance, the same sample can be measured with ELISA kits from two different companies and yield different results[35]; although this should not occur with kits that assert similar specificity and sensitivity. This can also be seen in a commercial setting as validation methods will use ELISA kits from different companies to confirm the ideal diagnostic test for their laboratory. These details are recorded for evidence and as a reference for the selected test with the reasoning behind it.

As a Quality Assurance system, possessing a gold standard for a diagnostic test is essential. By implementing the gold standard and comparing the same measured factor on the selected test (e.g. ELISA for a specific protein) from kits of different companies; results can be compared to find the most appropriate diagnostic test as well as allow for validations or improvements to current methodologies. For example, if a sample of known concentration is performed using the current gold standard test and compared to a new test for accuracy; this allows for test validation or the potential for new gold standard development. Upon searching the literature, few comparisons between different test types for TNF-α have been performed, for example those done by Talvitie[36] and Leister et al[36, 37]. In addition, only Perdomo-Celis and Narváez[22] have suggested that the ELISA is the gold standard for TNF-α; however, beyond this, no other test has been proposed. This is understandable as TNF-α is currently under analysis to investigate if it can be used for endometriosis diagnostics. However, for the purposes of a meta-analysis and clinical use, a gold standard for TNF-α (pg/ml) would be ideal to clarify its true PF concentration range between studies.

In addition to the lack of a gold standard, multiple diagnostic methodologies were performed across the analysed studies which could add to the imprecision in the results. The following test types were performed using PF TNF-α: ELISA, Immulite™, Cytometric bead array, Bioassay using L929 cells, immunoradiometric assay (IRMA), and the Multiplex immunoassay. With such inconsistencies, inaccuracies, and potential imprecision in the test types used, or a possible unknown background effect; any can produce variations in TNF-α. To investigate this, validation studies would need to be performed on a number of known TNF-α concentrations in samples with all tests compared. This will ascertain which test contains the greatest accuracy and precision for a gold standard procedure for TNF-α for future use in endometriosis diagnostics.

Stages of menstrual cycle were also not taken into account in the majority of the studies used in this meta-analysis except for one study[28]. This research examined TNF-α levels across different stages in serum and PF with variations being present in both sample types[28]. If menstrual cycle stage is accounted for when testing PF TNF-α in multiple studies and a narrower confidence interval range is present in the subsequent meta-analysis; this would further explain the imprecision within the SMD calculations regarding this meta-analysis, especially regarding early diagnosis. By understanding the differences that may exist on PF samples during the menstrual cycle, a more accurate range can be found for TNF-α as well as a comparison that shows the greatest difference between endometriotic and non-endometriotic patients.

Technical error, as well as calibration and maintenance of all equipment used for experiments can also decrease precision and accuracy within datasets. Although implied, no study mentioned current Quality assurance measures such as equipment calibration and maintenance. This introduces further potential error as false results can occur with unintentionally inaccurate measurements. Also, human error in sample preparation or test performance can influence results. Only with appropriate GLP or by separate technicians performing the same test on the same sample with currently calibrated equipment can this data be considered precise and accurate. However, in practice, using separate technicians on the same sample is usually unrealistic and limitations can arise that prevent such ideal testing such as cost, time, or the volume of sample obtained from a patient.

Furthermore, estimation of means and standard deviations introduce error as they are not true values. Although statisticians have developed methods for estimation[1, 2], original data are preferable. For any paper to be considered for a meta-analysis, the following factors should be included: number of samples, minimum, maximum, 1st and 3rd quartiles, standard deviation, 95% confidence intervals, mean, and median. This allows the most accurate calculations for SMD and confidence intervals to be processed in a meta-analysis as well as further use in accurate data synthesis methods should these be required.

5. Future research and conclusion:

The next stages of development for early stage endometriosis diagnostic research and testing is one that requires careful precision and accuracy. It also requires menstrual cycle stages to be recognized regarding such a test, as well as the less invasive aspects of sample collection. These procedures are less invasive as they involve using a syringe to draw PF from the patient; however, there is more inconvenience as the patient will be required to have samples collected on specific days of their individual menstrual cycle. In regards to protein analysis, in conjunction with TNF-α, other cytokines and hormonal candidates for endometriosis must be identified and evaluated for implementation to isolate endometriosis diagnosis without laparoscopy. These proteins would be identified by meta-analyses using either PF or serum. Furthermore, for the most accurate endometriosis diagnosis, secondary conditions that may interfere with results must be accounted for. To investigate this, a study on patients with a disease or condition causing elevated TNF-α in serum or PF must be performed and compared to endometriosis patients. This could be done by implementing a blind test comparing endometriotic patients with a secondary condition that also raises PF or serum TNF-α against three other groups: patients with endometriosis exclusively, patients with the secondary condition measured exclusively, and a control group. The resulting information and diagnostics could be used to show the accuracy or inaccuracy (e.g. when false-positives arise) of the test when a prevailing second condition exists that may interfere with the diagnosis.

The main contribution of this meta-analysis is to evaluate the potential of PF TNF-α as one of the specific diagnostic markers for early-stage endometriosis. Furthermore, once limitations are overcome, and a subsequent meta-analysis is performed on TNF-α from research using fixed menstrual cycle stages exclusively; a comparison of early endometriotic and non-endometriotic women can be performed to confirm if a narrower range does exist for TNF-α to validate its application in early stage endometriosis diagnostics. In addition, similar to the study of Cheong et al[28], analyses must be performed on various selected cytokines and hormonal candidates at different stages of the menstrual cycle in PF. These must compare menstrual cycle stages of control patients against those who have early stage (rAFS 1-2) endometriosis. Upon completion, the data must be used to develop reference ranges for the selected proteins of each group. This will reveal which candidate combination presents the greatest difference in concentrations within PF for development of a more reliable diagnostic test at a specific menstrual cycle stage. For this type of testing, a biochemical analyser is proposed as a foundation for gold-standard early endometriosis rAFS stage 1-2 testing, because of the ease of being able to measure multiple factors in one procedure and comparing them to reference ranges. Alternatively, using a multiplex immunoassay for cytokine and hormone levels could also be used as these are similar to an ELISA but with an added benefit of measuring multiple proteins in one procedure. Either of these would require significant validation and inter-laboratory comparison panels to confirm, irrespective of laboratory, that this test could accurately diagnose early stage endometriosis. This would only be the first proposition for future research into this area, and it is plausible that other diagnostic tests may be more accurate and precise than the multiplex immunoassay or biochemical analyser. However, it appears that a biochemical analyser using PF would be an ideal model for early onset endometriosis diagnostics until a more precise, accurate, and novel test can be developed.

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Chapter 3b: Study Protocol - Serum and Peritoneal fluid implementation for less invasive endometriosis diagnostics

Abstract - Many women are affected by endometriosis, a condition of endometrial lesion growth outside the uterus able to initiate chronic pain and infertility. Current gold standard diagnosis is via invasive laparoscopy. Attempted less-invasive methods use protein analyses including: Interleukins -1β, -6, -8, -10, -12, and -13 (IL-1β, IL-6, IL-8, IL-10, IL-12, IL-13), tumour necrosis factor-α (TNF-α), Cancer antigen-125 (CA-125), vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), and Interferon-γ (IFN-γ) in serum and peritoneal fluid (PF). However, literature conflicts prevent implementation of these proteins for diagnostic procedures. To account for this, this study protocol has been designed. Initially, random-effects model meta-analyses on various proteins of both sample types will determine significantly different factors ($p<0.05$). Protein measurement considerations will occur at this stage, e.g. biochemical analyser or multiplex immunoassay. Subsequently, the selected factors will be measured for ($n>=50$) known control and rAFS (revised American Fertility Society) I-II endometriotic patients from both sample types with human ethics approval. Samples will be taken at each menstrual cycle stage and confirmed with gold standard laparoscopy. Data analysis will determine proteins with significant differences with selections based on a common menstrual cycle stage if applicable. A reference range for each selected factor and sample type for control and rAFS I-II patients will be employed. Test validation will require new patients following a single-blind procedure using reference ranges to diagnose patients that have undergone gold standard laparoscopy. This protocol aims to develop a less invasive method for diagnosing early-stage endometriosis using laparoscopy for confirmation purposes exclusively.

1. Introduction

Endometriosis is a condition affecting 10-15% of women (Jerman & Hey-Cunningham, 2015) in which uterine cells from the endometrial lining invade the body and become painful, parasitic-like lesions that are not eliminated by the immune system (Lebovic, Mueller, & Taylor, 2011). There is no consensus about the cause of endometriosis; however, retrograde menstruation theory yields the most support (Jerman & Hey-Cunningham, 2015; Lebovic et al., 2011). This theory states that some cells lost during menstruation can enter blood vessels specifically and attach at various sites in the body, e.g. brain, lung tissue, and the pelvic cavity (Yang & Huang, 2014). To confirm the diagnosis, the current gold standard procedure is an invasive laparoscopy (Nezhat et al., 2010; Tanahatoe & Lambalk, 2003). In addition, when this procedure is performed, the patient may be in any of the revised American Fertility Society (rAFS) stages of endometriosis (Richter, Dorn, Rosing, Flaskamp, & Ulrich, 2005). Endometriotic patients typically experience dyspareunia, abdominal pain, as well as infertility resulting from lack of implantation (Lebovic et al., 2011; Nap, Groothuis, Demir, Evers, & Dunselman, 2004), and becomes a chronic cause of pain and distress in women.

Several studies possess conflicting data sets when using serum and peritoneal fluid (PF) for endometriosis diagnostics. For example, in some studies, PF TNF-α show significant differences (Richter et al., 2005; Salmeri et al., 2015), while others do not (Cheong et al., 2002; Wickiewicz et al., 2013). Similar results have been observed in other cytokines, e.g. transforming growth factor-β (TGF-β) (Pizzo et al., 2002), IL-6 (Barcz et al., 2012; Liu & Luo, 2000), and IL-8 (Barcz et al., 2012). However, it appears that these cytokines may have potential for use in diagnostic testing. To confirm this, meta-analyses must be performed to account for the conflicts seen in the data sets of multiple studies. Due to the current and significant variety of datasets present for serum and PF; it would be further recommended to develop a single test using serum and PF to confirm endometriosis as a novel, more economical alternative to laparoscopy.

2. Method

Endometriosis, once established is known to cause changes in concentration of various cytokines and hormones (Barcz et al., 2012; Keenan, Chen, Chadwell, Torry, & Caudle, 1995). As a result, it is likely that protein candidates exist that can be used for endometriosis diagnostics once conflicts in data sets are accounted for. The study protocol proposed is to perform multiple PRISMA compliant meta-analyses (Moher, Liberati, Tetzlaff, Altman, & Group, 2009) on various cytokines and hormones from at least 20 studies per protein analysed according to serum or PF. This will confirm the candidates for a novel diagnostic quantitative or semi quantitative procedure, such as a multiplex immunoassay, or other biochemical analyser that can measure protein reference ranges. Should there be less than 20 studies for some factors, these may still be used for analysis; however, the number of studies must be emphasized and accounted for within each meta-analysis. Multiple factors must be used for the proposed diagnostic test as this further accounts for conflicting studies, resulting in the most feasibly accurate test design. Upon completion, the following studies require confirmation of patients by gold standard laparoscopy and must account for menstrual cycle stage using serum and PF samples. The selected proteins will require measurement through the chosen test design on a number of known rAFS I-II stage endometriosis against controls to develop the reference ranges for each protein. For validation, another number of patients at rAFS 1-2 stage endometriosis against controls must be measured and performed in a single-blind test where the individuals performing the diagnostic test are unaware of the status of each patient; however, the status is known by an outside panel so results can be compared for accuracy of the novel procedure.

2.1. Meta-analysis to determine cytokine or hormone candidates.

The meta-analyses data must account for the cytokines and hormones in any similar standard unit from individual studies, e.g. pg/mL or be converted to this accordingly. Due to the nature of these meta-analyses, the random-effects model will be used (Borenstein, Hedges, & Rothstein, 2007). The statistics for these

analyses are a Standardized Mean Difference (SMD or Hedge's g) with the Sidik-Jonkman method for estimating between study variance as well as the Knapp-Hartung-Sidik-Jonkman method (Harrer & Ebert). The accumulated studies must also possess the average, standard deviation, and number of patients for controls compared to experimental groups. If the averages or standard deviations are not provided, formulas from (Hozo, Djulbegovic, & Hozo, 2005; Wan, Wang, Liu, & Tong, 2014) in Figure 1 can be used or a request sent to the first author of the paper to receive such information. An example of the meta-analysis has been provided in Figure 2 using RStudio.

$$\text{Mean} \approx \frac{\text{minimum} + 2*\text{median} + \text{maximum}}{4}$$

$$\text{SD} \approx \frac{\text{3rd quartile} - \text{1st quartile}}{1.35}$$

$$\text{Mean} \approx \frac{\text{1st quartile} + \text{median} + \text{3rd quartile}}{3}$$

Figure 1: Formulas used for data synthesis of averages and standard deviations (q1 = Lower quartile (25^{th}), q3 = upper quartile (75^{th})), adapted from Hozo et al., (2005); Wan et al., (2014).

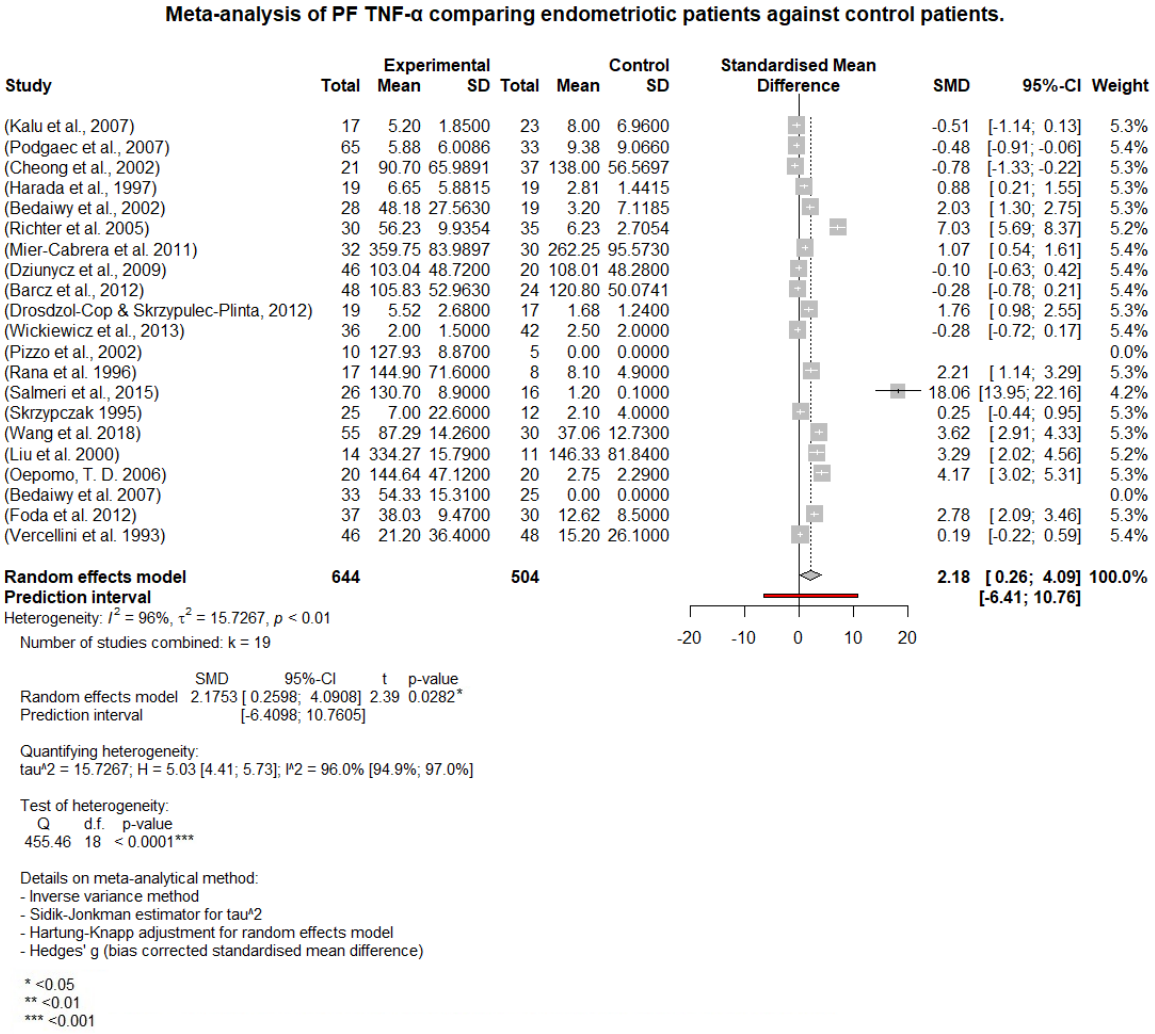

Figure 2: RStudio meta-analysis and heterogeneity test of PF TNF-α of women with and without endometriosis (SMD = Standardized mean difference (Hedge's g), SD = Standard Deviation, CI = Confidence Interval). A test of heterogeneity is provided as part of this test and as p<0.01, a random-effects model was applied. Meta-analysis performed according to RStudio code found in (Harrer & Ebert).

In addition, there may be meta-analyses performed prior to this hypothesis, therefore they can be used as reference guides for which cytokines to consider, e.g. cancer antigen-125 (CA-125) (Ben et al., 1998). For endometriosis diagnostics, each selected protein should have p<0.05 from the meta-analysis, with at least three cytokines per sample type being recommended for a diagnostic procedure. By using this method, the study further acknowledges data conflicts seen in the literature and will yield the proteins that can provide the most accurate diagnosis.

2.2. The study to confirm physiological candidates for early-onset endometriosis diagnosis.

For the following studies, every procedure must be accompanied by complete data sets. This includes the: minimum, maximum, range, 25th and 75th quartiles, interquartile range, median, mean, standard error, and standard deviation. With the information from the meta-analyses, a semi-quantitative test such as the multiplex immunoassay, or ELISA; or a quantitative diagnostic test such as a biochemical analyser can be developed to measure reference ranges of the three or more proposed proteins from each sample type to yield the greatest diagnostic precision. In addition, the same test type will preferably be used (i.e. biochemical analyser or multiplex immunoassay) for each protein analysed in the selected sample type.

This study requires at least 50 control patients and 50 experimental patients with rAFS stage I-II endometriosis including ethical and written consent with gold standard laparoscopy confirmation (Nezhat et al., 2010; Tanahatoe & Lambalk, 2003). The study must be performed according to menstrual cycle stage for serum and PF for analysing the proposed factors from the prior meta-analyses. In this instance, knowledge of the status of the patient is required. This will be to develop accurate reference ranges or other semi-quantitative conditions that display clear differences between rAFS stage I – II endometriosis and control patients in serum and PF. Although knowledge of the patient condition does increase the risk of bias for this study; it is necessary in order to develop a reference range for diagnostic procedures.

Furthermore, the menstrual cycle stage must be accounted for in this study as differences in cytokine levels have been found in the literature (Cheong et al., 2002) and few studies have accounted for this. Therefore, to develop the most accurate diagnostic test, this detail must be included. For example, TNF-α measured in stage I-II rAFS endometriosis from a serum sample and a PF sample against a control patient where both samples are taken in the proliferative phase (see Table 1). This is repeated for other proposed cytokines at each menstrual cycle stage. However, as ranges vary for cytokines in each menstrual cycle stage, e.g. across days 7-14 of proliferative phase; in diagnostic practice, targeting a specific day for a patient would be difficult to implement, and this must also be

taken into account. The potential for significant differences at each stage will be measured by an ANOVA comparing the factors at each menstrual cycle stage with $p<0.05$ as the criteria for each factor.

Table 1: Example of measurement of a selected protein in serum and PF at proliferative phase of menstrual cycle.

	Serum		PF	
Subject	Control (pg/ml)	rAFS I – II (pg/ml)	Control (pg/ml)	rAFS I – II (pg/ml)
1	X	X	x	X
N	X	X	x	X

Once completed, if all menstrual cycle stages are significantly different for a protein in serum and PF, samples can be used from any menstrual cycle stage; however, this appears unlikely. Therefore, of the proposed cytokines from this study, all those with significant differences at a common menstrual cycle stage should be employed for patient and medical practitioner convenience. If there are significant differences across multiple menstrual cycle stages, the stage with the lowest p-value should be utilized. However, if a protein exhibits a significance ($p<0.05$) across multiple menstrual cycle stages in common with others possessing a difference at a single common menstrual cycle stage; the protein with multiple significant differences across menstrual cycle stages must be used at the same stage as those with the single significant difference. The reasoning being that in medical practice, this will be a more feasible option. For example, if serum IL-6 and IL-8 are significant at the proliferative phase of the menstrual cycle, and serum IL-10 is significantly different across all stages ($p<0.01$) of the menstrual cycle, samples should be taken at the proliferative phase to measure the levels of IL-6, IL-8, and IL-10. The same protocol should be instituted for PF where applicable. Once the study is complete with significant differences and menstrual cycle stages accounted for; the collected data can be used to develop reference ranges if the biochemical analyser is selected. Alternatively, the conditions required for a semi-quantitative procedure can also be established for the diagnostic test by using the same datasets.

2.3. Diagnostic Validation Procedure

To validate the newly developed procedure, a single-blind test must be applied. For this validation, at least 50 new rAFS stage I – II endometriotic patients and 50 control patients will be analysed by the new procedure with the associated menstrual cycle stage accounted for. All patients must have some form of identification and their condition confirmed by gold standard laparoscopy (Nezhat et al., 2010; Tanahatoe & Lambalk, 2003). The laparoscopy results will be used as the reference to compare to the results of the proposed diagnostic test. The group performing the diagnostic test, on the other hand, must have no knowledge of the conditions of any patient, but will possess the identification of each patient. The procedure must be performed and results developed by the diagnostic group as to which patients were considered to be endometriotic or not according to the identification of each patient. To analyse this information compared to the gold standard, a simple percentage analysis can be performed. Correct diagnostics will be recorded and a total will be calculated to confirm diagnostic accuracy. For example, if 48 of 50 controls were diagnosed correctly and 49 of 50 endometriotic patients were diagnosed correctly, therefore 97% of the new diagnostic procedure was correct. A minimum of 95% accuracy is recommended for this procedure.

For a standardised diagnostic procedure, this screening test would need to be optional and recommended at 6-month, annual, or patient preference intervals. A confirmatory gold standard laparoscopy would only be required when results are positive from the new procedure and the patient or medical practitioner possess a preference of further evidence to support the medical laboratory diagnosis.

2.4. Alternatives and Troubleshooting

It is plausible that there will be no common menstrual cycle stages with two or more cytokines possessing a statistically significant difference in serum or PF ($p<0.05$). Should this occur, testing protocol would require measuring the significantly different factors from the two sample types at the closest intervals of the menstrual cycle for patient convenience. For example, if IL-6 is only significant at the proliferative phase, and IL-8 at the secretory phase, then a sample must be taken in between or at both of these phases. The same validation procedure (Method section 3) outlined above will be applied. If successful, when this method of two or more cytokines at separate menstrual cycle stages are used for regular diagnostic testing, a patient would have serum and PF samples taken at the two separate stages with the new diagnostic test performed.

To account for potential false-positives and false negatives, the patient must have their current health status taken into account, e.g. if a pre-existing or ongoing condition exists, the patient is taking any medication, or is ill at the time of testing. These three factors may cause elevation or depression of protein concentrations that can cause a false-result to occur. Furthermore, for the most accurate test, the specificity and sensitivity must be at least 95%. Additionally, the menstrual cycle stage as well as at least three proteins must be used from serum and PF for the diagnostic procedure in the initial validation stage. The reasoning for this being that due to the conflicts in the literature, using multiple proteins of different sample types will yield the most precise result and narrow the possibility of false-results. Furthermore, should fewer proteins be used, there is a greater potential for false-results to arise. Also, by taking these described factors into account, this will likely prevent unnecessary treatments should a false-positive occur. If there is still any uncertainty, a laparoscopy can still be performed if required.

If there are no significant differences in any cytokine measured irrespective of sample type at the meta-analysis or diagnostic testing level, or not enough studies are present regarding certain factors for testing, it is important for this information to be published with these critical evaluation points. Primarily; the proteins used may no longer be required for consideration in further studies for potential endometriosis diagnostic candidates. However, should the same or different proteins be considered, all research must include the original information: sample

size, mean, standard deviation, minimum, maximum, range, 25th and 75th quartiles, interquartile range, median, and standard error. This is so meta-analyses can possess the most accurate results regarding measurements of any potential protein for consideration in the diagnostic procedure. Secondarily, more research must be performed on factors with less studies or those not considered in the conducted meta-analyses along with examples. Subsequently, all following studies must be performed according to menstrual cycle stage for the most accurate meta-analyses to be performed to confirm significant or non-significant factors. Following this, the methodology described above should be re-evaluated to investigate new protein candidates.

3. Discussion

Primarily, no physiological based test has been designed for endometriosis, nor has any specific test been proposed for serum or PF with multiple factors. This is due to the numerous literature data conflicts, suggesting that such methods are unlikely to be accurately performed. In addition, due to these conflicts, a single factor from two sample types is not recommended for an accurate endometriosis diagnosis. At least three factors from two sample types are required for a successful, precise test design to justify the result with respect to conflicts in the literature with a significant difference for each protein found by meta-analysis. The design for a protein-based endometriosis test is ambitious due to: the amount of data required, the time required for analysis, the intensity of the studies to follow as described above to confirm candidates; and the design of the test for a single or multiple menstrual cycle stage analysis.

Furthermore, test validation would be time-consuming depending on the selected diagnostic test used to determine true endometriosis; as well as comparing this to known experiment and control patients. Other ailments, illnesses, or injuries could also be a potential confounding factor as cytokine and hormone level changes that are shared with endometriosis can occur. For example, elevated serum concentrations of TGF-β, and TNFα in endometrial cancer (Chopra, Ding, & Hannigan, 1997), which are also found in endometriosis (Bedaiwy et al., 2002; Pizzo et al., 2002). In addition, when analysed by serum,

some cytokine levels, e.g. IL-6 have differing levels of elevation between endometriosis and endometrial carcinoma (Slater, Cooper, & Murphy, 2006), and this must also be considered regarding interpretations when developing a diagnostic test. Therefore, a $p<0.01$ – $p<0.05$ between control and experiment patients for protein concentrations are recommended for the most accurate diagnosis with no adverse conditions that could affect results. Should such patients with pre-existing conditions appear, these factors must be taken into consideration when interpreting physiological results and may still require laparoscopy for confirmation.

By implementing multiple meta-analyses, the proposed study aims to alleviate the issue of invasive endometriosis diagnostic procedures and conflicts of data by promoting a comparison of multiple factors in serum and PF. These results can provide candidates for use in diagnostic procedures. Meta-analyses are recommended as they investigate for consistency between studies, being able to account for conflicts between datasets and varying sample sizes. As a result of this, they provide the most precise estimates of true effects (Haidich, 2010) and thus reveal the ideal proteins.

Furthermore, accuracy increases when menstrual cycle stage is recorded as this can potentially isolate candidates with the greatest observed differences in concentration. One study has shown that such differences exist at various cycle stages (Cheong et al., 2002), and therefore, for the most precise test design, these stages must be considered. By using a follow-up study to find and compare rAFS I – II patients to controls, reference ranges can be developed according to menstrual cycle phase that can be used to find which proteins possess the most significance as described above. However, if significant differences occur across all menstrual cycle phases ($p<0.05$), it facilitates diagnosis through only one test being performed at any phase, rather than targeting a specific day of the menstrual cycle in a patient.

If the reference ranges are developed, compared, and show significant concentration differences ($p<0.05$), the validation procedure can be performed as described above in the single-blind procedure. If successful, the designed test can proceed for diagnostic application. Table 2 provides an example using serum for test result interpretation. By possessing this earlier detection method for endometriosis, more conservative treatments can be used. The result being the

prevention of further chronic and increasing pain associated with progressive endometriosis. If testing was required at multiple menstrual cycle stages, there may be difficulties with a patient returning at the correct menstrual cycle stage for confirmatory diagnosis, however this method is still plausible.

Table 2: Testing protocol for early-onset endometriosis with two or more serum factors.

Serum factor 1 result	Serum factor 2 result	PF factor 1 result	PF factor 2 result	Final Result	Confirmatory result required
Positive	Positive	Positive	Positive	Positive	No, only required at doctor discretion or patient request.
Positive	Negative	Positive	Positive or negative	Positive	Yes, re-test, or confirm by laparoscopy at doctor discretion.
Negative	Negative	Positive	Positive or negative	Negative	Yes, re-test, or confirm by laparoscopy at doctor discretion.
Negative	Negative	Negative	Negative	Negative	No

This study protocol has been developed with the intention of designing a gold standard of testing at the earliest stage of endometriosis detection. By designing a simple, economic test as a routine procedure, patient request, or doctor recommendation for early diagnosis, it allows application of the earliest possible treatment. It is different from current methodologies as patients can request for a physical check using the rAFS system for endometriosis if desired, but this will be more invasive when compared to a physiological measure. Currently, unless a patient undergoes regular uterine physical exams, endometriosis can only be diagnosed after a patient is in pain and has visited a specialist medical practitioner. In addition, patients may report pain and discomfort

at any stage of endometriosis; however, this protein-based test can yield a more accurate early-stage detection method. Additionally, it must be noted, that in some instances, endometriosis may be asymptomatic (Hickey, Ballard, & Farquhar, 2014), further showing that a minimum of a number of a routine annual or patient preference check-up could be applied.

There is also potential for a business profit with the design of such a test, as no cytokine or hormonal test has been designed and used for endometriosis diagnosis at the earliest possible stage, on a routine basis, and in a protein-based manner. Furthermore, this design may address another area of concern regarding pricing compared to current methodologies. Depending on the type of test requested, e.g. physical exam or hormonal drug therapies (Kennedy et al., 2005) and where it is performed, prices will vary (Simoens et al., 2012; Simoens, Hummelshoj, & D'Hooghe, 2007). However, current methods appear to be similar or more expensive in cost than a hormonal or cytokine-based test.

For the proposed method to be put into practice, there is a high risk-benefit association; where the benefit has been described above. However, the risk of such a large study by meta-analyses and validation could result in not having any viable hormone or cytokine for endometriosis testing by physiological means. The scenario of no significantly different factors in two sample types by two menstrual cycle stages appears unlikely considering the number of cytokines and hormones undergoing analyses. If there is no difference in any cytokine or hormone, this information is important for publication. This is because it will assist other researchers to investigate different methods for less-invasive endometriosis diagnostics not yet considered, rather than repeating errors resulting in substantial loss of time and finances. The additional benefit, should this design succeed, would be a standard test for women globally that allows for earlier endometriosis treatment leading to less chronic-associated pains and symptoms.

4. Conclusions

The result of the proposed study protocol suggests that factors likely exist for a less-invasive physiological endometriosis diagnostic test. The observations from existing research by meta-analysis, for example serum cancer antigen 125 (CA-125) (Hirsch, Duffy, Davis, Plana, & Khan, 2016), also present potential for an early onset-endometriosis diagnostic procedure. Even though conflicts in the literature exist, multiple meta-analyses can account for these to provide the most accurate and precise final result for each analysed cytokine. Furthermore, to provide greater accuracy, the proposed meta analyses must be performed on serum and PF samples simultaneously. By using these two sample types, preferably with an identical diagnostic test such as the multiplex immunoassay or biochemical analyser comparing cytokines to standardized reference ranges; this should provide the most plausible method to confirm early-onset endometriosis. It is also intended to act as a new gold standard for future tests to be compared against.

Moreover, further research into each proposed diagnostic factor at each menstrual cycle stage is required to confirm if major differences between rAFS I – II stage endometriosis and control patients exist. This will also confirm if the proposed diagnostic test will be more accurate at certain menstrual cycle stages. In addition, when testing, the condition of the patient must be accounted for so that no confounding factors exist that could unintentionally produce false positives or negatives. The proposed method aims to discover which factors are the most significant for diagnostic analysis with the resulting medical laboratory test being less invasive than current methodologies. This diagnostic procedure is also designed to be an economical, routine, easy-to-perform screening test with a confirmatory laparoscopy only being required at the request of the patient or by recommendation of the medical practitioner. Should this method be successful, it will further allow for earlier endometriosis diagnoses and treatment; a result ultimately desired by patients and the medical community.

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Chapter 4: Conclusion

4.1 Overview

Due to the prevalence of endometriosis, investigating its mechanisms of action, as well as the ideal method for the earliest possible diagnosis and treatment is imperative. This thesis has covered endometrial development and the four current endometriosis theories. Following this, a PRISMA compliant meta-analysis was performed to research if PF TNF-α could become a candidate for an early stage (equivalent to rAFS I-II) endometriosis diagnostic procedure. As the results had $p<0.05$ for this analysis, it is reasonable to suggest that PF TNF-a can be used for future diagnostic procedures with some refinement. Consequently, a study protocol has been developed using the same meta-analysis methodology on serum and PF of multiple proteins. Should significant differences exist between endometriotic and control patients of the newly chosen proteins; they could be refined to develop reference ranges according to menstrual cycle stage between known endometriotic and control patients. These reference ranges could be developed using a semi-quantitative test, e.g. ELISA, or a quantitative test, e.g. biochemical analyser. Once data sets were compared, at least three proteins with significant differences ($p<0.05$) at a common menstrual cycle stage in serum and PF would be selected for the final validation procedure. This procedure would include a new set of patients with rAFS I-II stage endometriosis and controls; using a single-blind procedure with all patients being given unique identifications and diagnosed using gold standard laparoscopy. The validation process would use the newly developed reference ranges for PF and serum to diagnose the patients according to their identifications. The diagnoses between the gold standard and the new procedure would be compared to determine the accuracy, with $\geq$95% matching diagnoses required for a successful outcome.

4.2 Strengths and limitations of this study

4.2.1 Strengths

The strength in this thesis lies within the analytical process. By reviewing endometriosis and endometrial development, proteins could be utilized within diagnostics laboratories. Furthermore, by using a meta-analysis, literature conflicts are accounted for with weights given according to sample sizes in each study. This allows for greater precision and accuracy to determine if a specific protein has diagnostic potential. In addition, performing more meta-analyses on other proteins of different sample types is straightforward to repeat. This causes protein selection for diagnostics to become a much simpler process within a study protocol. Also, the study protocol is logical and could determine accurate reference ranges for women at each menstrual cycle phase for comparisons between endometriotic and control patients.

4.2.2 Limitations

Some limitations must also be accounted for in this thesis. Primarily, the full process of endometriosis is not understood as evidenced in the literature review. Not all biochemical pathways are known and there is no consensus on the development and process of ectopic lesion growth with the associated symptoms. Additionally, the meta-analysis possessed numerous limitations as discussed such as: no gold standard present for TNF-α (Freeman et al., 2016), the number of different tests used for measuring TNF-α, and the menstrual cycle stage not being accounted for in each study. More studies may also have been present that could have been included; however, many could not be used due to limited access to some research articles, as well as articles outside the scope of criteria for the meta-analysis. Also, in some instances, data estimation formulas were required (Hozo, Djulbegovic, & Hozo, 2005; Wan, Wang, Liu, & Tong, 2014) where original data could not be accessed, which could further increase the error and confidence interval range seen in the meta-analysis. The study protocol is limited in terms of the time and finance required for the reference range, validation procedure, and. the potential for no common menstrual cycle stage for proteins with significantly different concentrations between endometriotic and control patients.

4.3 Implications and final remarks

The implications of this thesis provide evidence that meta-analyses can be performed to study if the data from multiple studies is consistent, with PF TNF-α as a potential diagnostic candidate. If the data is consistent with proteins of significant differences, these candidates could proceed to the next phase of diagnostic validation. Once proteins are found, reference ranges can be developed and analysed to narrow which candidates from serum and PF possess the most support for the diagnostic procedure. Upon completion, a single-blind validation will be used to compare gold standard laparoscopy to the new diagnostic process for accuracy confirmation. If successful, this new test can be put into the next phase for human trials and be implemented on a patient preference; three-month, six-month, or annual basis as a routine screening procedure for the earliest plausible endometriosis diagnosis. This will result in earlier treatments and less chronic pain experienced by women who suffer from this condition.

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