The evaluation and use of the pig (*Sus domesticus*) as a model for determining the effects of dietary lipids on lipid metabolism and thrombosis in human beings.

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Dedicated to my children,
Sophie, Fergus and Phoebe

Abstract

The domestic pig (*Sus domesticus*) has been used extensively as a model in atherothrombosis research because this species has many physiological similarities to human beings. In the series of studies presented here, the domestic pig was used and evaluated as a model for determining the effects of dietary lipids on lipid metabolism and thrombosis in human beings.

In the first study, serum specimens were collected from five 8-week-old Large White pigs in the fasting and postprandial states to determine whether cholesterol fractions can be estimated without use of the ultracentrifuge. Sequential ultracentrifugation was used to determine cholesterol in VLDL (VLDL^{fuge}), LDL (LDL^{fuge}) and HDL (HDL^{fuge}) fractions. VLDL^{fuge} was compared with VLDL cholesterol (VLDL-C) concentration estimated as serum triglyceride concentration divided by four (VLDL^{trig}). HDL^{fuge} was compared with cholesterol remaining in the supernatant after precipitation of apolipoprotein B-containing lipoproteins with Mn^{2+} and heparin (HDL^{ppt}). LDL^{fuge} was compared with the concentration determined from the Friedewald formula (total cholesterol less HDL^{ppt} less VLDL^{trig}). After correcting the centrifuged fractions for recovery of total cholesterol, the mean difference between the LDL^{friede} and LDL^{fuge} of fasting samples was $<5\%$ and the mean difference between fasting HDL^{ppt} and HDL^{fuge} was $<8\%$. Fasting VLDL^{trig} was more than twice VLDL^{fuge}, after correction for cholesterol recovery, possibly because of very low recoveries in the ultracentrifuge or because of an incorrect divisor of total serum triglyceride. This study showed that whereas HDL cholesterol (HDL-C) and LDL cholesterol (LDL-C) can be reliably estimated in these specimens by simple methods, VLDL-C estimation requires further investigation. A secondary finding was that feeding significantly reduced the serum concentrations of total cholesterol and LDL-C but raised HDL-C.

Using the simple methods for determining serum HDL-C and LDL-C concentrations established in the first study, fasting serum cholesterol concentrations were then determined in eight-week-old pigs (6 pigs per group) fed diets containing either fish oil, milkfat, coconut oil, olive oil or cornstarch at an inclusion rate of 4% (w/w) for 3 weeks in the second study. Serum total cholesterol concentration was significantly higher in pigs fed coconut oil than in pigs fed cornstarch or fish oil ($p < 0.05$). Pigs fed coconut oil, olive oil and milkfat had a significantly higher serum HDL-C concentration than those fed cornstarch or fish oil. There were no significant differences in serum LDL-C concentrations between groups. The serum triglyceride concentrations were higher in pigs receiving coconut oil and milkfat. This study showed that dietary fats that have a hypercholesterolaemic effect in humans tended to raise serum HDL-C rather than LDL-C concentrations in pigs. These findings suggest differences in lipoprotein metabolism between human beings and growing pigs and caution is warranted when making inferences about human lipoprotein metabolism from porcine studies.

A balloon angioplasty model of arterial injury in pigs for the assessment of the thrombogenicity of dietary fats was evaluated in the third study. Eight-week-old pigs (8 pigs per group) were fed diets containing milkfat, fish oil or cornstarch at an inclusion rate of 10% w/w for 12 weeks. The group receiving the fish oil diet was withdrawn from the study after 6 weeks because the diet was unpalatable. At the end of the feeding period, angioplasty was performed on the left and right external iliac arteries simultaneously in each pig. One artery was randomly assigned to receive an angioplasty catheter with a 10-mm balloon while the contralateral artery was distended to 12 mm. One hour later, the damaged arterial segments were harvested and the size of the thrombi that formed in each artery were estimated with a technique using autologous, radiolabelled platelets and by a morphometric technique. The thrombi that formed in this study were platelet-rich, typical of those found at the site of atheroma rupture in human beings. Five of 32 arterial segments sustained deep injury, indicated by the presence of tears through the internal elastic lamina. Thrombi could also be seen at the site of balloon injury in arteries that did not sustain deep injury, a finding that contrasts with earlier angioplasty studies in pigs, which have shown that thrombi will only form at sites of deep injury. The presence of deep injury, and the length of the tear through the internal elastic lamina, appeared to influence the size of the thrombus that formed at the angioplasty site. Given that the length of the tear was difficult to control with balloon angioplasty, and that thrombi can be identified in the absence of tears, it may be more desirable to avoid deep injury in this model. When the arteries that sustained deep injury were excluded and thrombus size was evaluated by the radiolabelled platelet method, there was some evidence ($p = 0.1$) to suggest that the pigs fed the milkfat diet may have had a greater thrombotic tendency than those fed the cornstarch diet. This model, using autologous radiolabelled platelets for estimating thrombus size, shows promise as a method of evaluating the effects of dietary lipids on thrombosis.

The activity of the coagulation cascade was assessed by determining the activated partial thromboplastin time (APTT), prothrombin time (TT), thrombin time (TT) and factor VII activity and plasma fibrinogen concentrations during the study evaluating the balloon angioplasty model of arterial injury in pigs (described above). These variables were determined at the beginning of the study and 1, 2, 4, 6, 8, 10 and 12 weeks later. There was some evidence ($p = 0.07$) to suggest that the group receiving the milkfat diet had a longer APTT than the group receiving the cornstarch diet. This suggests that the milkfat diet may induce less activity within the intrinsic clotting cascade and/or the common clotting cascade than the carbohydrate diet. This finding therefore, does not explain the tendency for thrombus to be larger at the site arterial injury induced by angioplasty in the pigs receiving milkfat than those receiving cornstarch.

In the final study, the effect of dietary lipids on blood concentrations of markers of thrombosis, platelet indices, white and red blood cell counts and platelet reactivity in pigs was evaluated. The experimental animals and diets were the same as those presented in the second study. Variables evaluated included plasma TAT and fibrinogen concentrations, platelet numbers, mean platelet volume, plateletcrit, white blood cell (WBC) and red blood cell counts and platelet reactivity, assessed by platelet aggregation and by filtragometry. The inclusion of fish oil in the diet of pigs has affected some variables in a way that could be interpreted as 'prothrombotic' compared to the other dietary treatments. The fish oil group had the highest concentrations of TAT and fibrinogen, the highest platelet and WBC count and the greatest plateletcrit. In contrast, platelet function, as assessed by aggregometry, was lowest in the group receiving fish oil. Olive oil appeared to be relatively 'antithrombotic' when compared to the other dietary treatments. The pigs receiving the olive oil had the lowest, or equal lowest, TAT and fibrinogen concentrations, and plateletcrit and WBC counts. Platelet function, as assessed by aggregometry, was the second lowest. This is noteworthy because the consumption of a Mediterranean-style diet, which is rich in olive oil, has been previously been shown to be protective against coronary artery disease. These findings highlight the difficulty associated with using indicators of thrombosis to predict final thrombus size. The establishment of a thrombus end-point in an appropriate animal model is required in order to evaluate the overall thrombogenic potential of dietary fats. Nevertheless, evaluating the effect of dietary lipids on markers of thrombosis, such as those investigated in this study, may provide important insights into mechanisms that lead to thrombosis.

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"It feels like that there are two of us doing this PhD", commented my ever-supportive wife, Rebecca, after one particularly tiresome week. While I was writing in the evening and weekends, she was single-handedly rearing our three preschoolers. Without her love and support, it simply would not have been possible for me to complete this work.

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Chapter 1. Literature review

In the United States of America, the age-adjusted death rate due to heart disease has declined significantly over the last 30 years. Despite this, it is still the leading cause of death, accounting for approximately one-third of total deaths in 1997 (Hoyert *et al*, 1999). Atherosclerosis, complicated by thrombosis, is responsible for the majority of these deaths. The term atherothrombosis has been introduced because of the close link between these two disease processes in the development of ischaemic heart disease (Loscalzo, 1992; Nachman, 1992; Hamsten, 1995).

Recognised as risk factors for coronary heart disease are increased serum LDL cholesterol (LDL-C) concentrations, enhanced platelet reactivity and raised plasma concentrations of markers of haemostasis, such as fibrinogen and factor VII (Fuster *et al*, 1992b). The type and quantity of lipid within the diet has been shown to influence some of these parameters and consequently, the role of nutrition in the prevention of coronary heart disease has been the subject of considerable interest.

Even though well-designed metabolic ward studies performed in human beings can control environmental and dietary variables, many other variables, including the age and genotype of the subject, are difficult to control. Moreover, invasive or long-term metabolic ward studies are impractical in human subjects. For these reasons, the development of a suitable animal model to investigate the role of diet in the prevention of atherothrombosis would represent a major advance. The species of the model should be physiologically similar to human beings, particularly in terms of gut physiology, lipoprotein metabolism, and platelet and haemostatic function. Furthermore, the model should be easily accessible and inexpensive to establish and maintain.

The domestic pig (*Sus domesticus*) may be a suitable model for investigating the effects of dietary lipids on atherothrombosis as it shares many characteristics with human beings. The pig is monogastric and omnivorous and strains of pig with inherited hypercholesterolaemia can develop complicated atherosclerotic lesions by two years of age (Overturf & Loose-Mitchell, 1992). Furthermore, lipoprotein metabolism of humans and domestic pigs has many similarities.

The objective of this thesis were to evaluate the suitability of the pig as a model for studying atherothrombosis and as part of that evaluation, use it to study the effects of dietary lipids on cholesterol metabolism and thrombosis.

The literature review is divided into five sections. In the first section, the morphological characteristics of naturally-occurring atherosclerosis and the contribution of cholesterol to atherosclerosis are described. How the superimposition of a thrombus upon an atherosclerotic lesion contributes to ischaemic heart disease is the subject of section two. Lipoprotein cholesterol metabolism in human beings and pigs is compared in section three. Experimental methods of evaluating platelet function and thrombotic potential, and the effects of dietary lipids on thrombotic potential, are reviewed in sections four and five respectively.

1.1. Atherosclerosis

Atherosclerosis is a chronic disease of the arterial wall. Herbert Stary's (1989) morphological studies in human beings provided evidence that atherosclerosis starts in childhood and generally becomes symptomatic in middle-aged to older people. Stary (1989) described eight types of lesion (I-VIII), with each type characterised by its cells, matrix, architecture and other specific features. Lesion types I to VI are believed to represent the usual sequence of lesion progression with age, while type VII and VIII lesions are variants, which may precede, or follow, type VI lesions (Fuster *et al* 1992a, 1992b; Stary 1989). Lesion types I to III are asymptomatic with types I and II found in infants and type III evolving soon after puberty.

Type I lesions, or initial lesions, cannot be seen macroscopically and are characterised by isolated groups of macrophages distended with intracytoplasmic lipid droplets (foam cells) (Stary, 1989). Foam cells are more abundant in areas where the intima is thickest. Thickening of the intima is believed to be a physiological adaptation to decreased wall shear and/or increased wall tension. Stary (1989) coined the term adaptive intimal thickening to describe this process.

Type II lesions are composed of layers of foam cells within the intima (Stary, 1989). These lesions are also known as fatty streaks because the layers of foam cells are visible grossly through the endothelial surface. Fatty streaks also contain a greater number of macrophages, without lipid droplets, than normal intima. Although the number of smooth muscle cells within the intima is not increased, some of the smooth muscle cells contain lipid droplets. Lipid may also be seen in the extracellular matrix with the aid of electron microscopy. In most people, the majority of type II lesions do not progress, or only progress slowly to more advanced lesions. These progression-resistant lesions are called type IIb lesions. Progression-prone (type IIa) lesions occur at specific sites of adaptive intimal thickening and contain more lipid, macrophages, mast cells and probably more lymphoctyes than progression-resistant lesions.

Type III lesions (preatheromas) are characterised by the accumulation of multiple, microscopically visible pools of lipid and cell debris between layers of smooth muscle cells within the intima (Stary, 1989). Smooth muscle cell death and proliferation are not evident at this stage. These lesions, which occur in teenagers, provide evidence to support the supposition that more advanced lesions (atheromas) develop from the fatty streaks that are seen in children.

Furthermore, preatheromas occur in the same locations as atheromas and fatty streaks. Small, raised atherosclerotic lesions often have caps that are chemically and ultrastructurally akin to fatty streaks and the composition of the deep intimal core of small, raised lesions is similar to those seen in more mature lesions. (Guyton & Klemp, 1994).

The intima of atheromas (type IV lesions) has been eccentrically thickened by aggregates of grossly visible lipid, referred to as the lipid core (Stary, 1989). Despite significant intimal thickening, the cross-sectional area of the lumen generally remains unchanged making these lesions difficult to detect with coronary angiography. Lipid cores consist of degenerating foam cells, incompletely digested lipid droplets (tertiary lysosomes) extruded from smooth muscle cells, macrophages and coalesced lipoproteins that have not been phagocytosed by cells. The lipid core is of sufficient size to displace the smooth muscle cells that are normally present in areas of adaptive intimal thickening. Smooth muscle cells present within the lipid core are attenuated and may have a thick basement membrane. A layer of adaptive intimal thickening overlies the lipid core. This layer can contain macrophages, foam cells, smooth muscle cells, lymphocytes, plasma cells, mast cells and capillaries. The periphery of the atheroma contains more proteoglycan matrix and foam cells but fewer smooth muscle cells than other areas of adaptive thickening. This area is susceptible to fissuring and if this occurs, the atheroma may develop into a complicated (type VI) lesion.

Type V (fibroatheroma) lesions are recognised by the presence of a cap of newly proliferated smooth muscle cells and thick layers of collagen overlying the lipid core (Stary, 1989). The cap is often several times thicker than the lipid core itself. Fibroblasts and neovascularisation may be present around the periphery of the lipid core and in some areas, bleeding may be evident around capillaries. Islands of small blood vessels, connective tissue, macrophages and foam cells can be found in the tunica media adjacent to large fibroatheromas.

Extracellular lipid may also accumulate within the tunica media. With time, fibroatheromas enlarge and the tunica media may become a part of the fibrous component of the lesion. Macrophages, foam cells and lymphocytes often surround the adventitia adjacent to the lesion. As with type IV lesions, fibroatheromas do not obstruct the arterial lumen.

Type VI (complicated) lesions develop from atheromas and fibroatheromas. These lesions are recognised by the presence of fissures (type VIa), haematomas (type VIb) and thrombi (type VIc), or combinations thereof (Stary, 1989). Thrombi may occur on the surface of lesions without fissures or haematomas. Stary (1989) recognised that when thrombi are present the lesions are often thicker due to an increased number of smooth muscle cells and an increased amount of collagen. Large haematomas are the consequence of fissures or erosions that communicate with the arterial lumen while smaller haematomas, which do not communicate with the arterial lumen, are the consequence of haemorrhage from capillaries formed during neovascularisaton. The areas of haemorrhage described in type V lesions are too small to be classified as haematomas. Fissures, haematomas and thrombi may be replaced with collagen. When this occurs, lesions appear as thicker type V lesions and luminal narrowing may be evident.

Some lesions in people over 40 years of age contain a largely quantity of mineralised material. These are termed calcific (type VII) lesions (Stary, 1989). The mineral is calcium phosphate and apatite and it replaces necrotic material and some of the extracellular lipid in the core of the lesion. Although mineralisation is apparent in most advanced lesions (type IV - VI), only when it is a dominant feature are they classified as type VII. Type VII lesions are also characterised by an increased amount of fibrous tissue.

Some atherosclerotic lesions (type VIII or fibrotic lesions) consist almost entirely of dense collagen with little or no lipid (Stary, 1989). The origin of these lesions, which have the capacity to obstruct the lumen of medium-sized arteries, is unknown but they may result from fibrosis of a lesion that has been complicated by a thrombus (type VIc lesion).

From the above descriptions of lesions of atherosclerosis, it is evident that lipid is a large component of many of these lesions. Fuster (1994) has reviewed the purported mechanisms responsible for the accumulation of lipid within these lesions. Firstly, low density lipoprotein (LDL) probably enters the intima of arteries by a receptor-mediated route or, passively when endothelial damage is severe. All the major cell types within the intima are capable of mildly oxidising LDL, which in turn induces the endothelium to express adhesive cell-surface glycoproteins such as E-selectin, vascular cell adhesion molecule-1, or intercellular adhesion molecule-1. Monocytes adhere to the endothelium and gain entry to the intima where they are now called macrophages. Intimal macrophages are responsible for further oxidisation of LDL. Highly oxidised LDL avidly binds to the scavenger receptors of macrophages and enters these cells. As previously mentioned, lipid-laden macrophages are termed foam cells. Extracellular accumulation of lipid within the intima is probably due to foam cell death. Foam cells can release factors such as interleukins and tumour necrosis factor, which recruit further monocytes, thereby perpetuating the lesion.

1.2. Thrombosis

The association between atherosclerotic lesions and mural thrombi was first documented over 100 years ago by von Rokitansky (1852) and has since been the subject of considerable investigation. Prior to 1980, the lack of equipment capable of imaging the vasculature of living subjects hindered the investigation of the link between atherosclerosis, thrombosis and myocardial infarction. This association could only be studied through the examination of post-mortem specimens. Despite the large number of publications that arose from such studies, there was no unifying consensus. This was partly due to the discrepancy in the reported prevalence of arterial thrombosis in infarcted hearts, which ranged from 21% (Branwood & Montgomery, 1956) to 85% (Foord, 1948). In an attempt to maximise the likelihood of finding thrombi within the coronary arteries, Ehrlich and Shinohara (1964) examined serial histological sections of the entire coronary arterial tree of patients who had died of acute myocardial infarction. These workers found that only 50% of infarcted hearts had recent thrombi in their coronary arteries, which lead to the conclusion that "thrombi may follow rather than precede myocardial necrosis". Prior to 1980, this hypothesis was also considered likely by others (Branwood & Montgomery, 1956; Spain & Bradess, 1960), while some postulated that the advanced degree of atherosclerotic narrowing was sufficient on its own to cause myocardial infarction (Edwards, 1956; Hom, 1963).

The inability to examine the coronary circulation within a few hours of myocardial infarction limited the value of post-mortem studies. The advent of coronary angiography made this possible and using this technique, a much higher frequency of total coronary occlusion was detected. Total coronary artery occlusion was detected in 87% of patients who had coronary angiography performed within four hours of developing symptoms of transmural ischaemia (DeWood *et al*, 1980). The incidence dropped to 68% between 6 and 12 hours and to 65% between 12 and 24 hours. Further evidence for the role of thrombosis in the pathogenesis of acute myocardial infarction was provided in the 1980s by studies that used thrombolytic agents such as streptokinase. In these studies, the patency of coronary arteries could be re-established by the intracoronary infusion of this agent in 60-94% of occluded coronary arteries (Brooks, 1983; Rentrop, 1985).

Paradoxically, coronary arterial occlusion and concomitant myocardial infarction is more likely to occur at the site of small to moderate-sized atherosclerotic lesions than large lesions. Two retrospective studies, of patients who had coronary angiography performed before and after a myocardial infarction, have shown that lesions that occluded less than 50% of the arterial lumen were responsible for 66% (Little *et al*, 1988) and 48% (Ambrose *et al*, 1988) of cases of myocardial infarction.

Lesions that occluded less than 70% of the arterial lumen accounted for 78% and 97% of myocardial infarctions. A prospective study has since demonstrated similar results with 85% of infarct-related lesions not being haemodynamically significant at an earlier angiographic study (Webster *et al*, 1990).

There are two possible reasons why mildly to moderately stenotic atherosclerotic lesions (less than 70% stenosis) are more likely to develop thrombotic complications than lesions with a larger degree of stenosis. Firstly, large lesions take longer to develop, thus allowing sufficient time for an effective collateral blood supply to develop (Fuster *et al*, 1992a; Kawai, 1994). Secondly, acute myocardial infarction is generally associated with the formation of an occlusive thrombus at the site of a ruptured atherosclerotic plaque (Kragel *et al*, 1991). Only lipid-rich (fibro)atheromas, which do not impinge significantly on the vessel lumen, are prone to rupturing (Richardson *et al*, 1989; Stary, 1989; Falk, 1992). Once exposed, the lipid core of these lesions is highly thrombogenic. Using an *ex-vivo* extraocorporeal perfusion system, the lipid core of atheromas was found to be at least five times more thrombogenic than the internal matrix of fatty streaks, preatheromas and fibrotic lesions (Femåndez-Ortiz *et al*, 1994). Furthermore, platelet deposition on the collagen-rich matrix of sclerotic plaques was only twice as thrombogenic as normal intimal matrix.

It appears that the increased thrombogenicity of ruptured (fibro)atheromas may be partly due to an increased expression of tissue factor (TF). Tissue factor is a membrane-bound glycoprotein with an extracellular domain that functions as a co-factor for factor VII. Activated factor VII is a protease enzyme that activates factors IX and X. In healthy vessels of human beings, TF is produced by cells of undefined morphology within the tunica media and by adventitial fibroblast-like cells (Wilcox *et al*, 1989). In contrast, TF mRNA and protein is found in foam cells, monocytes adjacent to cholesterol clefts and mesenchymal-appearing intimal cells of atherosclerotic plaques (Wilcox *et al*, 1989). Tissue factor is also found in abundance in the necrotic core of plaques. Activated endothelial cells can export

TF to the subendothelium (Zwaginga *et al*, 1990) and extracellular matrix produced by fibroblasts also contains TF (Grabowski *et al*, 1993).

The importance of TF expression has been highlighted by the reduction in thrombus formation following the use of monoclonal anti-TF antibodies in animal models and in perfusion chamber systems. In a rabbit model of arterial thrombosis, these antibodies have been shown to efficiently block thrombus formation (Jang *et al*, 1992b). Fibrin and platelets are deposited on the surface of human monocytes expressing TF when they are exposed to flowing native blood in a perfusion chamber but when monocytes are incubated with anti-TF antibodies, fibrin deposition is virtually abolished and platelet-rich thrombus formation is reduced by more than 70% (Barstad *et al*, 1995a).

It is now generally accepted that the dynamic interaction between rupture of the atherosclerotic plaque, platelets, the clotting system and arterial spasm results in acute myocardial infarction (Fuster *et al*, 1992a; Jang *et al*, 1992a; Kawai, 1994). Consequently, various therapeutic modalities have been developed for the treatment of coronary artery thrombosis such as plasminogen activators (ie. recombinant tissue-type plasminogen activator), selective thrombin inhibitors (ie. hirudin and argatroban) and platelet inhibitors (ie. anti-glycoprotein IIb/IIIa antibodies) (Jang *et al*, 1992a).

1.3. Lipoprotein metabolism in pigs and human beings

Very low density lipoprotein

Expressed as a percentage of weight, porcine very low density lipoprotein (VLDL) is comprised of 11% cholesterol, 64% triglycerides, 21% phospholipids and 5% protein (Chapman, 1986). In contrast, human VLDL contains almost twice as much cholesterol (19%) but less triglyceride (55%) (Knipping *et al*, 1987; Kane, 1996). Pig serum contains lower concentrations of VLDL than human serum. The fasting serum VLDL concentration in pigs is approximately 50 mg/dL, which accounts for about 11% of porcine lipoproteins in the fasting state (Janado *et al*, 1966).

Nascent VLDL produced by the human liver contains a single copy of apolipoprotein B-100 (apoB 100) as well as apolipoprotein E and the C apolipoproteins (Kane, 1996). Similarly, apoB 100 is the major structural protein of pig VLDL, accounting for approximately 30% of total protein weight (Birchbauer *et al*, 1992). Porcine VLDL also contains lipoproteins resembling human apolipoprotein C-II, apolipoprotein E, and apolipoproteins C-III_1 and C-III_2 (Knipping *et al*, 1975; Birchbauer *et al*, 1992).

Before it is released from the liver, human VLDL contains twice as much phospholipid and much less cholesteryl ester than circulating VLDL. (Kane, 1996). Within the circulation, VLDL gains cholesterol from high density lipoprotein (HDL) in exchange for phospholipid. This is mediated by the activity of phospholipid transfer protein (PLTP) (Kane, 1996).

Low density lipoprotein

Mammals can be characterised as either ‘LDL mammals’ or ‘HDL mammals’ depending on whether LDL or HDL predominates in the lipoprotein profile in the fasting state (Chapman, 1986). In the vast majority of mammals, HDL is the predominant class of lipoprotein (Chapman, 1986). Pigs and human beings are LDL mammals, as are guinea pigs, the Rhinocerotidae and camels (Chapman, 1986).

The concentration of LDL in the serum of fasted pigs is approximately 240 mg/dL, which accounts for about 53% by weight of the total lipoprotein content (Janado *et al*, 1966). Porcine LDL consists of two subfractions: LDL_1 and LDL_2 (Janado *et al*, 1966; Calvert & Scott, 1975). These fractions differ in their size, hydrated density and lipid/protein ratio. While both fractions exhibit β electrophoretic mobility, LDL_1 has a mean diameter of 217 Å and a modal density of 1.035 kg/L (range: 1.03–1.04 kg/L). In contrast, LDL_2 has a mean diameter of 195 Å and a modal density of 1.050 kg/L (Calvert & Scott, 1975). The ratio of LDL_1 to LDL_2, judged by the schlieren patterns and absorbance at 280 nm, is approximately 2:1 (Janado *et al*, 1966; Calvert & Scott, 1975).

The density range of porcine LDL_2, LDL_1 and HDL overlap (Chapman, 1986). These particles however, differ in their flotation rates, which depend on both density and particle size. Janado *et al* employed differential flotation to separate these moieties by layering solvent above solution and adjusting solution density, time and speed of centrifugation so the fastest component reached the uppermost solvent layers (Janado *et al*, 1966). By employing this method, total LDL was recovered at a density of 1.090 kg/L. Calvert and Scott (1975) however, were able to completely separate LDL from HDL by preparative ultracentrifugation at a salt density of 1.063 kg/L. This disparity may reflect methodological differences.

Low density lipoprotein of human beings has been subdivided into at least four different moieties, designated as LDL-I, LDL-II, LDL-III and LDL-IV (Krauss, 1995). The density, size and flotation indices have been categorised for these subclasses. In the human population, there appears to be a bimodal density distribution of LDL (Krauss, 1995). Most human beings have predominantly larger, more bouyant LDL-I and LDL-II particles. This phenotype has been termed 'pattern A'. Approximately 10 – 15% of premenoupasal women and caucasian American males less than 20 years old have a pattern B phenotype where the predominant LDL subclass is the smaller, denser LDL-III particle (Krauss; 1995). The smallest and most dense particles (LDL-IV) are only found in large quantities in humans with severe hypertriglyceridaemia (Krauss, 1995). There has been recent interest in these phenotypes because humans with the 'pattern B' LDL profile have been shown to have a higher risk of developing coronary artery disease (Krauss, 1995).

Apolipoprotein B-100 is the major structural component of porcine and human LDL, accounting for >95% of the total weight of apolipoproteins (Chapman & Goldstein, 1976; Birchbauer *et al*, 1992; Grundy, 1996). Porcine apoB 100 is remarkably similar to its human counterpart. Both behave similarly upon electrophoresis in SDS-polyacrylamide gel and their amino acid composition is markedly homologous (Chapman, 1980). Furthermore, porcine apoB 100 cross-reacts with antiserum to human apoB 100 (Goldstein *et al*, 1971; Chapman & Goldstein, 1976). Human LDL also contains trace concentrations of apolipoprotein C-I, C-II and C-III (Chapman & Goldstein, 1976). The electrophoretic pattern of the tetramethylurea-soluble polypeptides (ie. all non-apolipoprotein B apolipoproteins) of porcine LDL exhibited mobility similar to human apolipoprotein C-I, C-II and C-III. In pigs, approximately half of the porcine apolipoprotein C fraction is apolipoprotein C-II whereas in humans, >80% is apolipoprotein C-III (Chapman & Goldstein, 1976). Furthermore, human LDL contains only a trace amount of apolipoprotein C-II (Chapman & Goldstein, 1976). Apolipoprotein C-II is a cofactor for extrahepatic lipoprotein lipase whereas apolipoprotein C-III is believed to inhibit the activity of lipoprotein lipase (Brewer *et al*, 1996).

High density lipoprotein

The concentration of HDL in the serum of fasted pigs is approximately 160 mg/dL, accounting for 36% of porcine lipoprotein (Janado *et al*, 1966). Porcine HDL is homogenous and dominated by one class of particle, which resembles human HDL_3 (Chapman, 1986). The hydrated density of porcine HDL is between 1.060 and 1.21 and it contains approximately 51% lipid and 43% protein (Janado *et al*, 1966). The major protein moiety of porcine HDL resembles human apolipoprotein A-I (Knipping *et al*, 1975). This apolipoprotein has a very similar molecular weight and amino acid composition to human apolipoprotein A-I (Knipping *et al*, 1975). Furthermore, it can activate lecithin:cholesterol acyl transferase (LCAT) and it cross-reacts immunologically with human apolipoprotein A-I. Lecithin:cholesterol acyl transferase catalyses the esterification of free cholesterol. Porcine HDL also contains a protein that has a similar amino acid composition and molecular weight to human apolipoprotein C-II. Small quantities of a protein similar to apolipoprotein A-II may also be present (Knipping *et al*, 1975). A major difference between pig and human HDL is that there appears to be no, or very low, cholesteryl ester transfer protein (CETP) activity in pig serum (Blum *et al*, 1977; Knipping *et al*, 1987). In human beings, this enzyme is responsible for the flux of cholesteryl ester and triglycerides to and from HDL (Tall & Breslow, 1996).

Unlike porcine HDL, human HDL is heterogenous in its properties and structure. The major subclasses of HDL, HDL_2 and HDL_3, are defined according to their density. The density of HDL_2 is between 1.063 and 1.125 kg/L and it contains approximately 60% lipid and 40% protein. The density of HDL_3 is between 1.125 and 1.210 kg/L and it contains approximately 45% lipid and 55% protein (Tall & Breslow, 1996). The major structural proteins of human HDL are apolipoprotein A-I and apolipoprotein A-II. Apolipoprotein A-I is found in most HDL moieties and it is capable of activating LCAT, a property shared with apolipoprotein A-IV (Tall & Breslow, 1996). Human HDL also contains two important HDL processing enzymes, LCAT and CETP.

High density lipoprotein metabolism

The major role of HDL in lipid metabolism is the transport of cholesterol from peripheral tissues to the liver. This process has been termed "reverse cholesterol transport". Nascent HDL is comprised of apolipoprotein A-I phospholipid discs secreted by the liver and intestine (Brewer *et al*, 1996). As nascent HDL circulates through the bloodstream, it acquires apolipoproteins (A-I, A-II and Cs) and lipid (phospholipid and cholesterol) from chylomicrons and VLDL (Tall & Breslow, 1996). Newly formed HDL also acquires cholesterol from peripheral tissues (Tall & Breslow, 1996). Apolipoprotein A-I mediates the binding of HDL to the cell membrane, allowing sufficient time for cholesterol to diffuse into HDL (Castro & Fielding, 1988). Free cholesterol within HDL is then esterified to cholesteryl ester via the enzymatic action of HDL-associated LCAT (Brewer *et al*, 1996). During this process, HDL changes from a discoid shape to a dense spherical particle termed HDL_3 (Brewer *et al*, 1996). Larger, less dense HDL_2 particles are formed as HDL_3 continues to acquire cholesterol, apolipoprotein and phospholipid (Brewer *et al*, 1996). HDL_3 is regenerated when triglycerides and phospholipids are removed from HDL_2 by the activity of hepatic lipase. In humans, the liver can take up HDL-associated cholesteryl ester via three potential routes.

The most important route for reverse cholesterol transport in human beings is mediated by CETP. In the presence of CETP, cholesteryl ester can be transferred to the triglyceride-rich lipoproteins, VLDL and chylomicrons, in exchange for triglyceride (Tall, 1993). Cholesterol-depleted chylomicrons are termed chylomicron remnants. These are cleared from the plasma by the liver via the chylomicron remnant receptor whereas LDL and VLDL remnants are cleared via LDL receptors (Tall, 1993). The importance of this route is illustrated by the marked accumulation of HDL cholesteryl ester in humans with genetic CETP deficiency (Tall, 1993). In the absence of CETP activity, and with continuing LCAT activity, HDL particles gradually increase in size and acquire apolipoprotein E. Apolipoprotein E is a high affinity ligand for the LDL receptor of the liver and cholesteryl ester may be removed by this mechanism (Kane, 1996). This route is believed to be important in people with genetic CETP deficiency (Tall, 1993).

Unlike healthy humans, the pig has little or no plasma CETP activity (Chapman, 1986; Knipping *et al*, 1987). Despite the lack of CETP, the cholesteryl ester content of pigs (14-19% by weight) and humans (15% by weight) is similar. Porcine HDL contains neither apolipoprotein B nor apolipoprotein E therefore it cannot be cleared via a receptor analogous to the human LDL receptor (Knipping *et al*, 1975). It is evident therefore, that reverse cholesterol transport in this species must be mediated through an alternative, yet to be elucidated, route.

Very low density, intermediate density and low density lipoprotein metabolism

In humans, LDL derives its cholesteryl ester through two routes: via the catabolism of VLDL and via the activity of CETP (see above) (Kane, 1996). VLDL particles have a circulating half-life of 30 to 60 minutes in healthy human beings (Stalenhoef *et al*, 1984). Very low density lipoprotein is subject to lipolysis by lipoprotein lipase, the rate of which is partly regulated by the inhibitory effects of apolipoprotein C-III (Kane, 1996). This apolipoprotein also inhibits endocytosis of newly formed VLDL by the LDL receptor of the liver (Kane, 1996). As core triglycerides are progressively stripped from VLDL, a smaller, spherical particle develops. At the same time, there is loss of C apolipoproteins and surface lipids from VLDL. Apolipoprotein C-II is an important activator of lipoprotein lipase and with its progressive removal, the rate of triglyceride removal from the VLDL particle slows (Mahley *et al*, 1984). The lipoprotein is now called an intermediate density lipoprotein (IDL). It retains its original molecule of apolipoprotein B-100 and some of its copies of apolipoprotein E (Kane, 1996). Two potential fates await IDL; it can be removed from circulation by the liver or it can be metabolised further and become LDL. It is probable that hepatic endocytosis of IDL is governed by the number of copies of apolipoprotein E within each particle (Kane, 1996). Apolipoprotein E is a high affinity ligand for the LDL receptor as it can bind to many sites on the LDL receptor (Mahley *et al*, 1984). Consequently, IDL particles with a larger number of copies of apolipoprotein E are presumably more likely to be removed by this mechanism. The IDL molecules that remain in circulation continue to lose triglyceride via hepatic lipase, an enzyme that plays an essential role in the eventual formation of LDL. Hepatic lipase differs from lipoprotein lipase in three key ways: it does not require apolipoprotein C-II as a co-factor, it removes more phospholipid than lipoprotein lipase and it is activated by apolipoprotein E (Olivecrona & Bengtsson-Olivecrona, 1993). Apolipoprotein E, which binds weakly to IDL stripped of its lipid, is eventually removed. At this point the lipoprotein has become a mature LDL particle (Kane, 1996).

Given the absence of CETP in pig plasma, porcine LDL must attain its cholesteryl ester either via *de novo* synthesis or through the catabolism of VLDL to LDL. There is strong evidence to suggest that porcine LDL cholesteryl ester is synthesised *de novo* by the activity of the LCAT enzyme (Knipping *et al*, 1987; Birchbauer *et al*, 1992). LDL can serve as a substrate for LCAT because it contains sufficient quantities of its principal activator, apolipoprotein A-I, and it may contain quantities of other, albeit less effective, activators (apolipoproteins A-II, A-IV, C-I and E) (Knipping *et al*, 1987). Knipping *et al* showed that when pig serum is incubated *in vitro* at 37°C, there is a reduction in free cholesterol in the VLDL, LDL and HDL fractions with an equimolar gain in cholesteryl ester (Knipping *et al*, 1987). At any point in time, the greatest concentration (60-70%) of newly synthesised cholesteryl ester was within the LDL fraction, indicating that LCAT acts preferentially on porcine LDL. This increase in LDL cholesteryl ester was not due to CETP activity because there was negligible transfer of cholesteryl ester between HDL and LDL in pig serum incubated at 37°C in the presence of the LCAT inhibitor, sodium iodoacetate (Knipping *et al*, 1987).

Perhaps more controversial, is the suggestion that LDL itself can be synthesised *de novo* by the porcine liver. Using radiolabeled VLDL particles, Birchbauer *et al* found that porcine VLDL is rapidly transformed to IDL (Birchbauer *et al*, 1992). The peak appearance of IDL occurred between 50 and 90 minutes after the intravenous injection of radiolabeled VLDL. However, only 11% of the specific activity of VLDL apolipoprotein B was found within the LDL fraction. These workers concluded that 89% of apolipoprotein B was not derived from the catabolism of VLDL and IDL and that this may be due to the large size of porcine VLDL (mean diameter, 550 Å c.f. humans, 350 Å) (Birchbauer *et al*, 1992). Stalenhoef *et al* have shown that when normolipaemic human recipients receive large VLDL particles from lipoprotein lipase-deficient donors, very few of these particles are converted to LDL (Stalenhoef *et al*, 1984). These workers proposed that this may explain why there is relatively little conversion of VLDL to LDL in species with large VLDL particles such as the rat, guinea pig and rabbit. Shames and Havel however, have put forward an alternative hypothesis for the apparent loss of specific activity of the apolipoprotein B of LDL relative to the specific activity in the original VLDL pool (Shames & Havel, 1991). They proposed that the kinetic hypothesis used in previous studies to formulate the concept of *de novo* synthesis does not take into account the heterogeneity of apolipoprotein B in the VLDL of the plasma of many mammals. Shames and Havel proposed a new model, taking into account apolipoprotein B heterogeneity, which explained the previous conclusions without the requirement for *de novo* production of LDL (Shames & Havel, 1991). More work is required to determine whether *de novo* synthesis of LDL does occur in the pig.

The fractional catabolic rate of porcine VLDL (1 hr^{-1}) is faster than that of human beings (0.2 hr^{-1}) (Huff *et al*, 1981; Birchbauer *et al*, 1992). The rate of catabolism of VLDL is, in part, due to the ratio of apolipoprotein E to apolipoprotein C. This ratio appears to be important in regulating the rate of catabolism of triglyceride-rich lipoproteins, possibly by altering the binding of lipoprotein to lipoprotein lipase (Windler *et al*, 1980). The ratio of apolipoprotein E to apolipoprotein C is three times greater in pigs than it is in humans (Birchbauer *et al*, 1992).

1.4. Tests of platelet function and thrombotic potential

The association between platelet aggregation and ischaemic heart disease was documented for the first time in a major population in 1991 in the Caerphilly collaborative heart disease study (Elwood *et al*, 1991). In this study, subjects in the highest quintile for adenosine diphosphate (ADP)-induced primary platelet aggregation were more than twice as likely to have had a previous myocardial infarction. It is therefore not surprising that identification of risk factors for thrombosis has been the focus of considerable research endeavour over the past decade. Unfortunately, there is no 'gold standard' method for assessing the effects of diet and drugs on platelet function and thrombosis. This section describes some of the commonly used tests and discusses their relative strengths and weaknesses. The first part describes *in vitro* methods for evaluating platelet function and thrombotic potential. The second part describes methods that have evaluated the interaction between platelets and biological surfaces using ex *vivo* chambers. The last section describes the important *in vivo* sub-primate models of arterial thrombosis and thrombolysis.

In vitro techniques

As the name suggests, *in vitro* techniques for assessing thrombotic potential require removal of blood from the subject in order to examine its potential to form a thrombus. Such techniques preclude the ability of blood components to interact with the vessel wall under normal rheological conditions, thereby bringing their physiological relevance into question. Despite these significant shortcomings, *in vitro* methods have been, and continue to be, widely used in medical research. *In vitro* techniques have the advantage that they are more readily standardised than ex *vivo* and *in vivo* methods, allowing for better comparison within and between studies.

Born aggregometry

In 1962, Born developed a turbidometric method for assessing the propensity of platelets to aggregate. This method has been used extensively to evaluate the effect of dietary lipids on thrombogenicity (Thorngren e*t al*, 1984; Rand e*t al*, 1986; Kwon e*t al*, 1991; Schoene, 1997). The technique is based on the change in light transmission when platelet clumping occurs (Figure 1.1). The recorder is adjusted so that platelet-poor plasma (PPP) reads at 100% light transmission whereas the initial suspension of platelets (platelet-rich plasma, PRP) reads at approximately 10% light transmission (Kinlough-Rathbone e*t al*, 1983). When a platelet agonist is added to PRP, there is a corresponding increase in light transmission; the increase being proportional to the degree of platelet aggregation. The application of this method assumes that there is a correlation between *in vitro* and *in vivo* aggregation but there are a number of shortcomings.

Firstly, turbidometric methods require the removal of platelets from their physiological milieu. This results in changes in temperature, rheological conditions, removal of the interaction between platelets and blood vessels and other blood cells, and exposure of platelets to non-biological surfaces and solutions. Poor blood sampling technique can result in the formation of small quantities of thrombin, which may be insufficient to result in macroscopic fibrin formation, but may be sufficient to activate platelets (Kinlough-Rathbone *et al*, 1983). In cold temperatures, platelets change from their quiescent disc-like shape to a spherical, activated state (Zucker & Borelli, 1954). A significant negative linear correlation has been found between the total white blood cell count and whole blood platelet aggregation, as measured by impedance aggregometry, with the neutrophil count having the greatest influence (Abbate *et al*, 1986). Eicosanoids produced by leucocytes may modulate platelet aggregation by degrading the platelet agonist, ADP, and by producing platelet inhibitory substances (Abbate *et al*, 1986). Furthermore, the production of neutral proteases (elastase-like protease and chymotrypsin-like protease) by leucocytes alters platelet function (Bykowska *et al*, 1985).

Secondly, the centrifugation step required for the preparation of PRP can result in the selection of a population of smaller platelets, due to the greater sedimentation velocity of larger platelets (Abbate *et al*, 1986). Since smaller platelets aggregate less rapidly than larger ones (Thompson *et al*, 1984), the removal of large platelets may result in underestimation of the aggregability of whole blood. Furthermore, preformed platelet aggregates may be removed.

Thirdly, a platelet agonist must be added to the PRP in order to stimulate platelet aggregation. In some instances, this results in 'non-physiological' responses within platelets. An example of this is the use of the agonist ADP to induce aggregation. In order to prevent coagulation, blood is added to citrate in order to bind calcium ions. When ADP is added to citrated PRP, platelet granules release ADP and serotonin and the arachidonate pathway is activated, resulting in the formation of thromboxane A_2 (TXA_2). This so-called platelet release reaction does not occur under more physiological conditions (Kinlough-Rathbone *et al*, 1983).

Fourthly, a number of end-points can be evaluated when assessing platelet aggregation. Which of these end-points correlates best with platelet aggregation *in vivo* is unknown. A notable example is provided by a study that examined the effect of physiological concentrations of fibrinogen on platelet aggregation (Meade *et al*, 1985). High plasma fibrinogen concentrations appeared to increase aggregability when the aggregation parameter measured was the ED50, the dose of agonist at which aggregation proceeds at half its maximum velocity. In contrast, aggregability decreased when the parameter measured was the estimated maximum response.

Fifthly, platelets can have dichotomous responses to different agonists. Thrombin-induced platelet aggregation was greater in rats fed a diet enriched with butter compared to a low-fat or corn oil-enriched diet (Renaud *et al*, 1970). Conversely, ADP and collagen-induced platelet aggregation was lower in the rats receiving the butter-enriched diet.

Finally, Born aggregometry does not take into account the formation of small platelet aggregates and there is a lack of correlation between aggregate formation and changes in optical density (Satoh *et al*, 1995). A recent advance has been the use of laser technology (Satoh *et al*, 1995). When a laser beam hit platelet aggregates, light is scattered and the intensity and frequency of light scattering provides information on the size and number of aggregates present in a defined area. This method was shown to be more sensitive than traditional

Born aggregometry. Platelet aggregates that formed *in vivo* could also be detected using this technique.

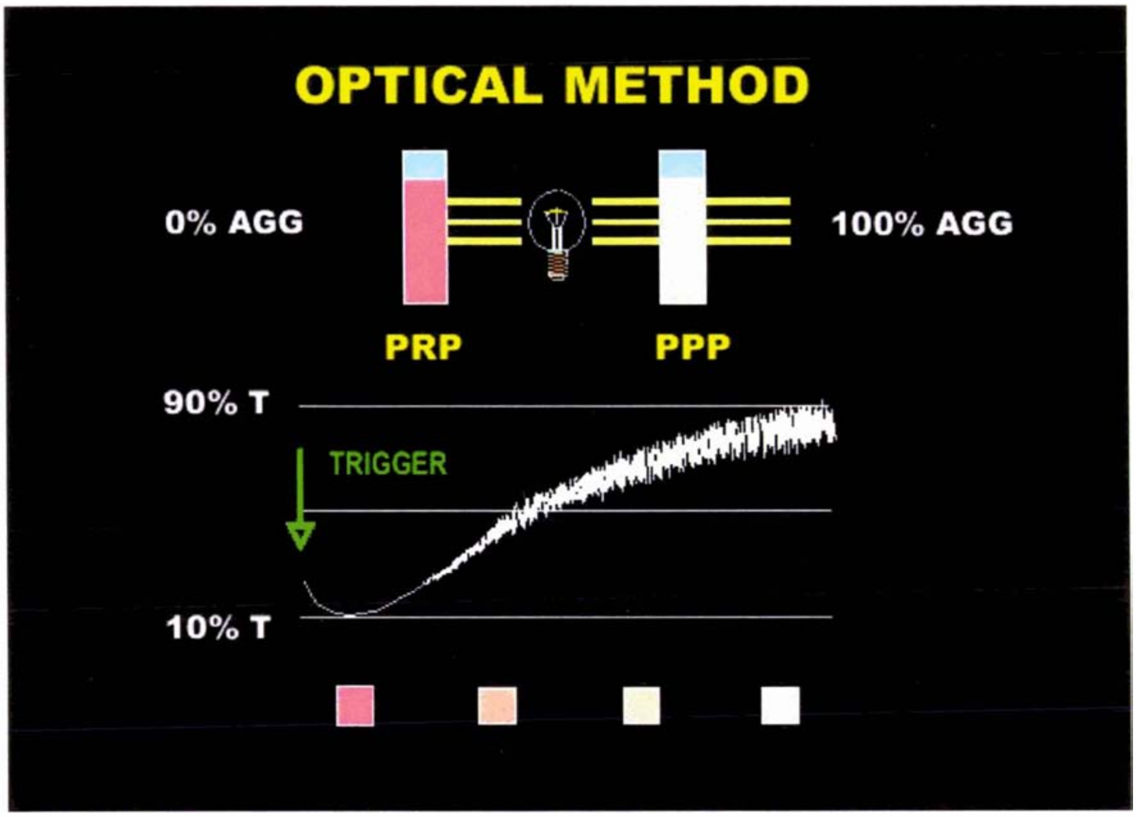

Figure 1.1 A schematic representation of the optical method of aggregometry. Following the addition of a platelet agonist (trigger), platelet aggregates form and light transmission through the sample of platelet-rich plasma increases.

Whole blood impedance aggregometry

The principle of whole blood impedance aggregometry is based on the increase in impedance that occurs between a pair of electrodes following platelet adhesion and aggregation (Figure 1.2) (Cardinal & Flower, 1980). This technique was developed so that whole blood could be used instead of PRP, thereby allowing platelets the opportunity to interact with other blood cells.

The main disadvantage with this technique is that it is impossible to standardise whole blood samples. Consequently, variables other than platelet reactivity may influence results. When PRP is used, the platelet count can be standardised by adding PPP, which allows comparison between subjects. In an effort to overcome this problem, Schreiner and co-workers (1991) have described a mathematical model and developed a software package to adjust for some of the variables inherent in whole blood aggregometry. These variables include the haematocrit, platelet count and the age of the sample (up to 180 minutes old). It is noteworthy however, that these authors did not include the total white blood cell or neutrophil count in their model, a parameter that has been previously shown to influence platelet aggregation (Abbate *et al*, 1986).

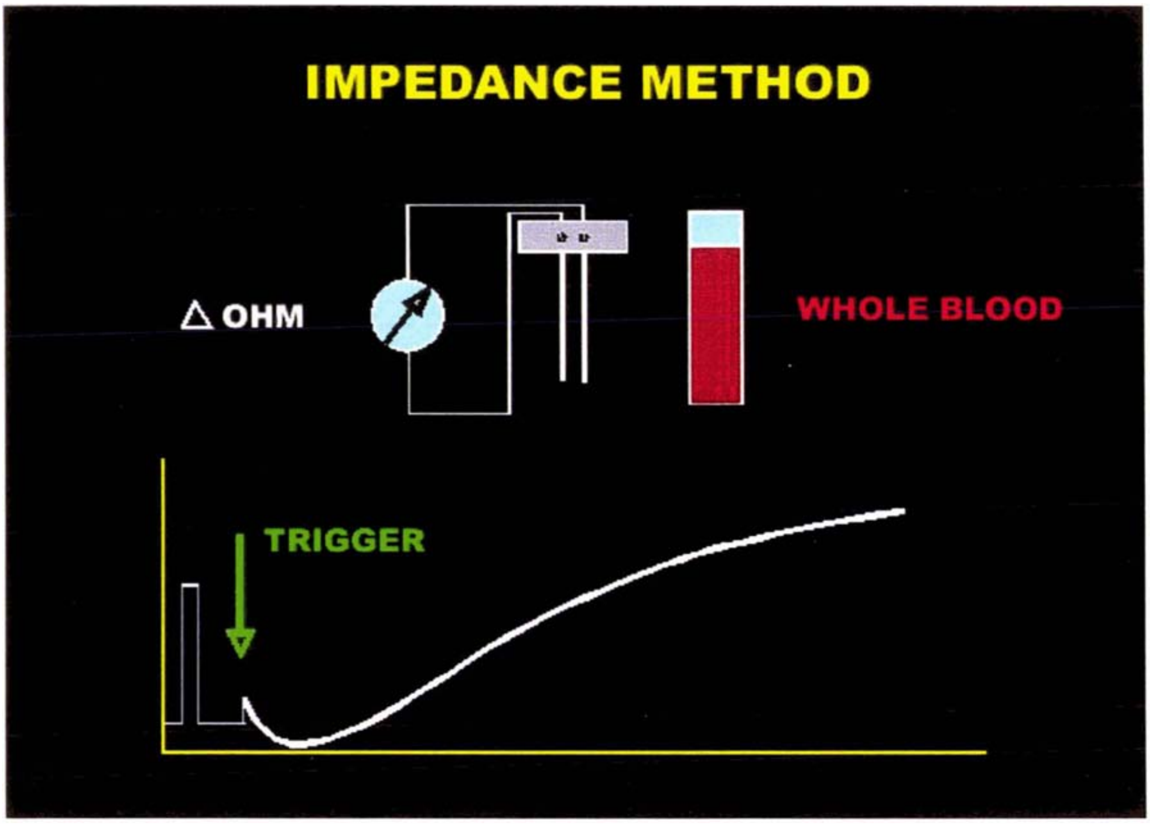

Figure 1.2 A schematic representation of the impedance method of aggregometry. Following the addition of a platelet agonist (trigger), platelets adhere and aggregate to electrodes within the sample and an increase in impedance between the electrodes is detected.

Lumi-aggregometry

'Primary aggregation' is usually defined as aggregation without platelet secretion and it is at least partly reversible (Feinman *et al*, 1985). 'Secondary aggregation' is accompanied by the release of various mediators from platelets and the synthesis of TXA_2. Aggregation *in vitro* can occur maximally without resulting in secondary aggregation and consequently, aggregometry is unable to fully evaluate platelet function. As an example, consider two groups of animals receiving different dietary treatments but with similar aggregometry responses. It could be concluded that there is no difference in the functional state of their platelets. However, the release reaction may have occurred in platelets from individuals in one group but not in the other. This problem provided the impetus for the development of the technique of lumi-aggregometry.

Lumi-aggregometry simultaneously measures platelet aggregation, using a whole blood impedance method, and the production of extracellular adenosine triphosphate (ATP), using a luminescent firefly luciferin-luciferase assay (Feinman *et al*, 1985). Adenosine triphosphate is co-secreted with platelet agonists from dense granules and therefore provides a marker for the platelet release reaction. Luminescence is detected optically and is enhanced by a stable, high-gain photomultiplier tube. The amount of light generated is proportional to the concentration of the ATP present and this is quantitatively determined by direct comparison to a 2 nM ATP standard.

Viscoelastic methods

Viscoelastic technology offers the unique advantage of being able to supply information on coagulation and platelet function in one test. The Sonoclot® Coagulation and Platelet Function Analyzer, manufactured by Sienco® Inc., has been widely used in haemostasis research (Miyashita & Kuro, 1998; Ekback *et al*, 1999; Konrad *et al*, 1999, 2000; Pivalizza *et al*, 1999). The detection mechanism of this analyser consists of a tubular probe that oscillates up and down within a blood sample. Electronic drive and detection circuitry senses the resistance to motion that the probe encounters from the blood sample and generates an analogue electronic signal. The signal is processed by a microcomputer and reported as a 'clot signature' (Figure 1.3).

Following an initial lag phase, there is a steep rise in the clot signal. The time from the beginning of the study to the increase in clot signal is termed the 'liquid phase'. The sudden rise in clot signal indicates an increase in the viscosity and represents initial fibrin formation. The duration of the liquid phase therefore provides a measure of the effectiveness of the coagulation pathways.

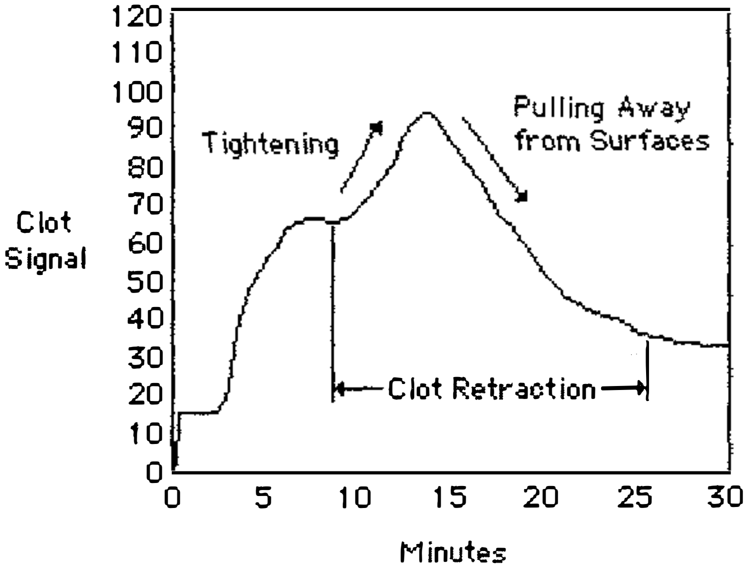

Figure 1.3 A typical clot signal generated by the Sonoclot® Coagulation and Platelet Function Analyzer. Graphic obtained, with permission, from **www.sienco.com**.

The next feature of the clot signature is a rise in the clot signature to a second plateau, which represents polymerisation of fibrin monomers and the formation of a fibrin gel. The extent of fibrin gel formation can be quantitated by measuring the slope of the curve (called the clot RATE) and the height of the clot signal.

The terminal increase in the clot signal is associated with clot retraction. Clot retraction depends on the attachment of actin in the cytoskeleton of platelets to glycoprotein IIb/IIIa receptors, which are attached to extracellular fibrin strands (Marcus, 1996). As clot retraction occurs, it places strain on the probe, resulting in an increase in clot signal. Eventually, the clot pulls away from the walls of the cuvette or the probe resulting in a decline in the clot signature. A sharp, well-defined peak is a qualitative indicator of strong clot retraction. Clot retraction is measured by the time it takes for retraction to occur and the degree of retraction. Platelet function is inversely proportional to the time from the initiation of the study to peak clot signal (Miyashita & Kuro, 1998).

Viscoelastic techniques are also able to detect states of hyperfibrinolysis. Usually, fibrinolysis is not detected by this equipment due to the fact that most tests do not run beyond one hour and clot lysis usually takes several hours. In states where fibrinolytic activity is greater than normal, the clot signal may return to the value attained in the original liquid phase within the time it usually takes to perform the study.

In summary, this unique technique can provide a number of useful indicators of haemostasis in one procedure, thereby rapidly providing useful clinical information. Since the technique uses whole blood, platelet function can be examined in the presence of other blood cells. This is also a disadvantage because it is difficult to exclude the effects of variables other than those attributable to platelet function. A further disadvantage is that the activity of platelets under their normal flow conditions cannot be evaluated with this system.

Filtragometry

Filtragometry was first developed as an *ex vivo* technique for detecting the quantity of preformed platelet aggregates that circulate in human venous blood (Hornstra & Ten Hoor, 1975). The principle of this method is based on the measurement of a pressure difference across a filter when it becomes obstructed with platelet aggregates. A nickel filter, with a diameter of 6 mm and a 20-µm pore size, allows the passage of all normal blood components with the exception of platelet aggregates with a diameter of >20 µm. By means of a syringe pump, blood is drawn directly from the cubital vein through a mixing chamber where it is mixed with heparin. The blood then passes through the filter at a flow rate of 2.25 mL/min. The filter and afferent connecting tube are siliconised and enclosed in a heating block at 37°C. The resultant pressure difference is proportional to the number of circulating platelet aggregates. Since the blood does not undergo centrifugation, the platelets are less likely to undergo artefactual change through mechanical stimulation, handling and storage than the methods previously mentioned.

Scanning electron microscopy showed that the filter is occluded solely by platelet aggregates (Hornstra & Ten Hoor, 1975). Fibrin was not observed. Using latex particles, it was demonstrated that the change in pressure across the filter (ΔP) rises when only 5% of the filter was obstructed. Various parameters describing the ΔP versus time curve were calculated and assessed for repeatability. These included the time taken to reach 5 mmHg (T_a), which corresponds to a 25% blockage of the filter, the slope of the curve, the maximum ΔP, and the time taken for platelet clumps to disaggregate following the infusion of 3.8% trisodium citrate. With the exception of the T_a, the repeatability for these parameters was poor. The T_a has subsequently been used as the measure of platelet aggregability with this technique.

Filtragometry has since been developed as an *in vitro* technique by a Swedish group (Soderback *et al*, 1991; Larsson *et al*, 1992; Broijersen *et al*, 1993). With their modification, blood is drawn into siliconised plastic syringes containing low molecular weight heparin and stored at room temperature in sealed plastic blood bags until required for use. Blood is then drawn from the bag across the filter as described above. As with the *ex vivo* technique, the time taken to reach a ΔP of 5 mmHg was measured. Although the *in vitro* technique does not require the addition of an agonist, ADP was added (final concentration, 0.05 – 0.3 µmol/L) in these studies to reduce the filter occlusion time. Using *in vitro* filtragometry, this group demonstrated that the addition of low concentrations of autologous LDL to whole blood enhances platelet aggregation (Broijersen *et al*, 1993). Platelet aggregation however, was unchanged by the addition of LDL when it was assessed by turbidometric aggregometry. It was hypothesised that the marked platelet aggregation induced by the high ADP concentrations used in aggregometry may have masked any subtle changes in platelet aggregation induced by LDL.

A disadvantage of filtragometry is that it does not take into account variables such as platelet count and white blood cell count, which may have a bearing on the T_a. Nor does it evaluate the interaction between blood vessels and platelets.

Platelet count technique

Based on the observation that circulating platelet aggregates are fixed when exposed to a mixture of formalin and ethylenediaminetetra-acetate (EDTA), Wu and Hoak (1974) described a method that evaluated the number of *in vivo* platelet aggregates. This simple method involves a centrifugation step and an automated platelet count. Venous blood samples (0.5-mL) are drawn into two syringes: one containing buffered EDTA and the other containing buffered EDTA and formalin. Both samples are kept at 22°C for 15 min and then spun at 150 × g for 8 min. The objective of the centrifugation step was to remove the preformed platelet aggregates in the EDTA/formalin sample. The supernatant, PRP, is harvested and platelet counts are performed on both samples. The platelet count ratio is calculated using the following formula:

$$\text{platelet count ratio} = PC_{\text{EDTA/formalin PRP}} \div PC_{\text{EDTA PRP}}$$

where $PC_{\text{EDTA/formalin PRP}}$ is the platelet count in PRP of the EDTA/formalin sample and $PC_{\text{EDTA PRP}}$ is the platelet count in PRP of the EDTA sample.

The lower the ratio, the greater the number of platelet aggregates that have formed *in vivo*. Wu and Hoak (1974) validated this method *in vitro* in citrated blood from a healthy donor and *in vivo* in New Zealand white rabbits. When ADP was added in increasing concentrations to the citrated blood, the platelet aggregate ratio decreased as expected. Arterial blood samples were collected from New Zealand white rabbits before and 7 and 12 min after a continuous intravenous infusion with thrombin (3 rabbits) and saline (3 rabbits). The thrombin infusion resulted in a significant reduction in the platelet aggregate ratio whereas there was no change in the platelet aggregate ratio in the rabbits that received saline.

Using this technique, Wu and Hoak (1974) demonstrated that the platelet aggregate ratio was significantly lower in patients with transient ischaemic attacks, myocardial infarction and 'acute peripheral arterial insufficiency' than in normal subjects. In contrast, there was no difference between normal subjects and patients with stable angina, 'chronic peripheral arterial insufficiency' and hospitalised patients without arterial disease (patient controls). Jakubowski and Ardlie (1978) used the method to show that healthy males had significantly more platelet aggregates when they ate a butter-enriched diet compared to a diet enriched in n-6 polyunsaturated fatty acids.

Despite its simplicity, this method has not been favoured as a method for assessing platelet aggregation. Schoene (1997) has suggested that more data are required to support the physiological relevance of platelet aggregates in circulation. Such doubts over the interpretation of results may explain why the method has not gained widespread acceptance.

Platelet Function Analyzer – 100®

The Platelet Function Analyzer-100® (PFA-100®) is a recent commercial development by Dade Behring Diagnostics Ltd (Miami, USA). In this system, citrated blood flows through a 150-µm diameter aperture in a membrane under high shear stress (5000–6000 s^{-1}) (Kundu *et al*, 1995). The membrane is impregnated with either collagen/ADP or collagen/epinephrine and simulates a damaged vessel wall. The time taken for the aperture to become obstructed with a platelet plug, termed the closure time, is reported.

Deficient von Willebrand factor, and inhibition of glycoprotein Ib or IIb/IIIa receptors with monoclonal antibodies results in prolonged closure times, whereas antibodies directed against fibrinogen have no effect (Kundu *et al*, 1995). This test therefore appears to detect deficiencies in platelet adherence and aggregation (primary haemostasis) rather than defects in coagulation. Ultrastructural analysis of the aggregates that obstruct the aperture showed that they were exclusively composed of platelets (Poujol *et al*, 1998). Although fibrinogen was detected within the aggregate by immunogold staining of ultrathin frozen sections, von Willebrand factor was the most abundant adhesive protein detected.

The PFA-100® closure time is more sensitive to aspirin-induced platelet dysfunction than the template bleeding time (Francis *et al*, 1999). The template bleeding time method involves determining the length of time taken for bleeding to stop after a standardised skin incision has been made. Although widely used as a clinical and research tool, the bleeding time method suffers from several disadvantages (Francis *et al*, 1999). It is highly operator-dependent, it may be normal in the presence of platelet abnormalities, it does not predict bleeding in pre-surgical patients and it can be associated with scarring.

Unlike the *in vitro* methods discussed above, the PFA-100® method evaluates platelet adhesion and aggregation under rheological conditions, which are similar to those found *in vivo*. Although the 'test surface' within the cartridge has been designed to simulate a damaged area of blood vessel, it does not contain the array of adhesive proteins found in the subendothelium of normal or atherosclerotic vessels. Nevertheless, this technology represents a significant advance in the ability to assess *in vitro* platelet function.

Assessment of the interaction between platelets and blood vessels

The methods discussed above do not take into account the platelet-blood vessel interaction. Under normal circumstances, the endothelial lining of blood vessels prevents thrombus formation. Endothelial cells line the luminal surfaces of blood vessels and secrete prostacyclin, a prostanoid that inhibits platelet aggregation (Mann, 1997). If the endothelium is disrupted however, a prothrombotic surface is exposed. Collagen, von Willebrand factor and elastin are ligands for platelet receptors (Mann, 1997) and tissue factor, a co-factor for factor VII, supports coagulation (Wilcox *et al*, 1989). Consequently, the arterial wall plays a crucial role in thrombosis and this has resulted in the development of experimental methods designed to investigate the interaction between blood constituents and vessels.

Annular perfusion chamber

Turitto and Baumgartner (1983) were the first to describe the use of a perfusion chamber to assess the interaction between platelets and blood vessels. Their perfusion chamber consisted of an inner plexiglass rod onto which an everted vessel segment, completely denuded of endothelial cells, was mounted. The rod and vessel were housed in an outer plexiglass shell through which blood was drawn by means of a roller pump. Without having to change the rate of blood flow, the wall shear rate could be adjusted by changing the diameter of the outer plexiglass shell.

This perfusion chamber could be operated as an *in vitro* or as an *ex vivo* system. For the former, previously collected citrated blood was drawn through the perfusion chamber and recirculated. The perfusion chamber and receptacle for the blood were immersed in a water bath at 37°C. For the *ex vivo* system, native blood (containing no anticoagulant) was drawn directly from a venepuncture site, through the perfusion chamber and discarded. The entire perfusion chamber was housed in a water jacket to maintain a constant temperature of 37°C. Following a predetermined perfusion time, the vessel was harvested, processed and platelet adhesion and thrombus size were determined morphometrically (Turitto & Baumgartner, 1983).

Parallel plate perfusion system

In 1983, a perfusion chamber was developed by Sakariassen and co-workers (1983) that allowed for the determination of the thrombotic potential of isolated endothelial cells, their extracellular matrix and collagen. Human vascular endothelial cells, derived from umbilical cord, were isolated and cultured onto glass cover slips that had been pre-coated with gelatin. The endothelial cells could be removed by incubating the coverslips with Triton X-100 (Rohm & Haas Co., Philadelphia), leaving behind the extracellular matrix.

The perfusion chamber comprised two parallel plates, which when screwed together, had a central channel through which blood would flow (Sakariassen *et* *al*, 1983). The top of the perfusion chamber had a fitting that housed the cover slip coated in the test material.

The earliest *in vitro* studies using this technique were performed using citrated blood from healthy volunteers. Perfusion was carried out at 37°C at a constant flow rate (gravitational) of 104 mL/min, which corresponded to a shear rate of 765 s^{-1} (Sakariassen *et al*, 1983). This required reperfusion of blood samples. Compared to the annular perfusion chamber, this system allowed the interaction of platelets with vessel wall components to be studied under well-defined flow conditions. These workers showed that the flow profile with this system was symmetrical at flow rates of 50–150 mL/min and the interaction between platelets and the test surface could be evaluated at a number of shear rates (300-1 100 s^{-1}). Platelet coverage of the test surface was assessed morphometrically or by radiolabelling platelets with 111-Indium (Turitto & Baumgartner, 1983).

The parallel perfusion plate has been used extensively over the last two decades and has been used for performing *ex vivo* studies (Sakariassen *et al*, 1990; Barstad *et al*, 1994, 1995b; Bossavy *et al*, 1999). The system has also been modified to allow for the study of platelet deposition on test surfaces under conditions that simulate high-grade arterial stenosis (Barstad *et al*, 1994). This was achieved by creating a step in the fitting, which houses the coverslip so that it protrudes into the central channel of the chamber. Chambers with stenoses of 60, 80, and 89% of internal diameter were made to create peak wall shear rates of 2 600, 10 500 and 23 000 s^{-1} respectively. Using this model, Barstad *et al* showed that thrombus formation on a stenosed collagen surface is extensive (Barstad *et al*, 1994). At very high shear rates (>10 500 s^{-1}), thrombus composition was heterogenous. A greater proportion of fibrin, erythrocytes and leucocytes were observed within the thrombi compared to those formed at lower shear rates. In general, fibrin formation decreases with increasing shear rate (Turrito *et al*, 1977; Weiss *et al*, 1986) but at very high shear rates, the development of a large occlusive thrombus may result in marked variation in regional shear rates. Consequently, fibrin deposition may occur in areas where shear rates are reduced (zones of recirculation).

Despite making a significant contribution to our understanding of platelet adhesion and aggregation, the parallel plate perfusion chamber has shortcomings. Firstly, a limited range of test materials can be used in the chamber. Atherosclerotic plaques are heterogenous, consisting of foam cells, smooth muscle cells and extracellular matrix (lipid, collagen, proteoglycans, elastin and glycoproteins) (Fuster *et al*, 1992a) and this would be difficult to simulate in this system. Secondly, it is difficult to monitor the kinetics of thrombosis/thromboembolism in this model. This has partly been overcome by choosing predetermined perfusion times and then evaluating the platelet adhesion/aggregation at each time point (Sakariassen *et al*, 1988). This however, does not allow for the evaluation of the dynamics of thrombosis and thomboembolism.

Badimon perfusion chamber

In 1983, Lina Badimon and co-workers (1983) developed a method for evaluating the interaction between platelets and collagen. With this system, autologous platelets were labelled with indium-111-tropolone and injected back into the animal model. The carotid artery was cannulated and blood was drawn with the aid of a roller pump through a warm (37^{o}C) jacket and dripped over 30-mm collagen strips (rabbit Achilles tendon). After a predetermined time interval, the strips were rinsed with saline and platelet deposition was estimated from the radioactivity of the strips.

Although this system introduced the interaction between vessel wall and platelets, it did not take into account haemodynamic consequences of blood flow on thrombus formation such as blood velocity and platelet shear rate and stress.

Recognising this shortcoming, a second perfusion chamber was developed (Badimon,L *et al*, 1986). Plexiglass tubes, with an internal diameter of 1 or 2 mm, were used to mimic arteries. A window, 25 mm long and 1 mm wide, located in the centre of each tube allowed exposure of a 'test surface' to flowing blood. Examples of test surfaces included substrates such as collagen, blood vessel, which had been denuded of endothelium, or atheromatous plaque (Badimon,L e*t al*, 1986; Fernández-Ortiz *et al*, 1994). The substrate was held in place by means of a clamp-like assembly and the apparatus was kept at 37°C by immersion in a continuous circulation, water bath.

As with their earlier system (Badimon,L e*t al*, 1983), autologous platelets labelled with ^{111}In were used (Badimon,L e*t al*, 1986). Under anaesthesia, the carotid artery was cannulated and blood was drawn directly through the perfusion chamber by means of a peristaltic pump. Blood was recirculated back into the pig via a cannula placed in the contralateral jugular vein. By changing both the flow rate of the pump and the internal diameter of the plexiglass tubes, the shear rate on the test surface could be altered between 106 and 3 380 s^{-1}. These shear rates simulate those experienced by large arteries, terminal arterial branches and the microcirculation.

This method was used to show that twice as many platelets are deposited on type 1 collagen than on denuded arterial endothelium (Badimon,L *et al*, 1986). They have since used it to demonstrate that hirudin, a specific thrombin inhibitor, is more efficacious than heparin and/or aspirin at inhibiting thrombus formation on fresh mural thrombus (Meyer e*t al*, 1994). They have also shown that a synthetic α-aminoboronic acid derivative, DuP 714, which is a slow-binding inhibitor of thrombin, inhibited platelet deposition under conditions which mimic mildly stenotic and patent vessels (Badimon,JJ e*t al*, 1994). They have also demonstrated that the lipid core of atheroma is more thrombogenic than fatty streaks, fibrosclerotic lesions and fibrolipid lesions (Fernández-Ortiz *et al*, 1994).

The Badimon perfusion chamber has also been modified to mimic arterial stenosis and this was used to demonstrate that platelet deposition increases with the degree of stenosis, indicating shear-induced platelet activation (Badimon,L & Badimon,JJ 1989). Furthermore, the greatest point of platelet accumulation was shown to be at the apex of the stenosis and not downstream of the apex where blood flow is turbulent.

During these studies, it was recognised that once thrombus reached a critical mass, some of it would break off and travel downstream, mimicking a thromboembolic event. Evidence for this phenomenon was provided by the observation that more platelets would accumulate on some test materials that were exposed to blood at certain flow rates for shorter periods of time. This highlighted a weakness of the Badimon perfusion chamber: it was unable to record the kinetics of thrombus growth and thromboembolism. Recognising this shortcoming, the system was modified to allow for dynamic monitoring of platelet deposition by nuclear scintigraphy (Lassila *et al*, 1990). A gamma camera, with a medium-energy, parallel hole collimator set on the photopeaks for Indium-111, was used to acquire dynamic images of the perfusion chamber every 2 min for a total perfusion time of 50 min. Data were stored in an imaging computer for future analysis.

In summary, the Badimon perfusion chamber is a sophisticated, *ex vivo* system for evaluating thrombotic potential under a range of rheological conditions. This system, however, is not practical for day-to-day evaluation of platelet aggregability in a clinical setting.

In vivo sub-primate models of arterial thrombosis and thrombolysis

Over the past two decades, thrombolytic therapy has unequivocally improved the prognosis for patients suffering from acute myocardial infarction (Lincoff & Topol, 1996). Despite this, it has not been uniformly successful in reestablishing coronary blood flow in all patients (Lincoff & Topol, 1996). Consequently, there has been significant interest in developing *in vivo* animal models, principally as means of testing the effects of thrombolytic drugs and agents.

The most common cause of arterial thrombosis in human beings is ulceration of an atherosclerotic plaque. The ideal *in vivo* animal model should therefore mimic the pathophysiology of atherothrombosis in a species that is biologically as similar to human beings as possible (Runge & Haber, 1991). Creating an atherosclerotic plaque upon which a thrombus can be superimposed in a reproducible manner is time-consuming. As an example, it takes one year for spontaneous vascular lesions to develop in IHLC pigs and a further two years for complex atherosclerotic lesions to develop (Prescott *et al*, 1991). Furthermore, the use of some animal species, such as dogs and rodents, as models for atherosclerosis in human beings has been questioned (Armstrong & Heistad, 1990). It is therefore not surprising that the models of *in vivo* thrombosis that have been used most commonly have not relied on the development of an ulcerated atherosclerotic plaque as the stimulus for thrombus formation.

Despite this, platelet-rich thrombus, which is typically found contiguous with ulcerated atherosclerotic plaque in post-mortem specimens from people who have died from acute myocardial infarction (Friedman & Van der Bovenkamp, 1966), has been simulated in many *in vivo* models. Moreover, spontaneous acute thrombotic reocclusion following successful thrombolysis, a phenomenon that occurs in approximately 13% of patients (Lincoff & Topol, 1996), can be reproducibly created in some models. This has allowed for the evaulation of therapeutic regimens designed to prevent this phenomenon from occurring.

The baboon is an ideal animal for modelling atherothrombosis because its vascular anatomy, platelet structure, coagulation and fibrinolytic systems and its response to drugs closely resemble those of human beings (Harker *et al*, 1991). Consequently, many models of thrombosis or thrombolysis have been described in baboons and these have been reviewed by Harker *et al* (1991). The benefits of using non-human primates have to be weighed up against practical considerations, such as the expense of the model, technical expertise and the availability of test animals. This is of particular importance when there is a requirement to screen a large number of agents for thrombolytic potency. Thus dogs, rats, rabbits, hamsters and pigs have also been widely used as experimental animals.

The large number of *in vivo* models that have been developed is testament to the fact that no single model is suitable for all studies. This review will discuss the most commonly used *in vivo* models, together with their strengths and weaknesses.

The Folts coronary thrombosis model

Folts and co-workers (1976) described a model of thrombus formation in stenosed coronary arteries of open-chest, anaesthetised dogs. A feature of the Folts model is that cycles of thrombus formation (resulting in blood stasis) and thromboembolism (resulting in the re-establishment of blood flow) can be created in a repeatable fashion for at least one hour (Bush & Shebuski, 1990; Folts, 1991). These episodes, termed cyclic flow reductions (CFRs), make this model suitable for evaluating the effect of drugs on thrombosis, since several drugs (or different doses of a single drug) can be evaluated in one animal (Bush & Shebuski, 1990). The model has subsequently been used in pigs, rabbits and monkeys (Folts, 1991).

When the dog is used, a left thoracotomy is performed and either the circumflex or left anterior descending coronary artery is isolated (Bush & Shebuski, 1990; Folts, 1991). Either an electromagnetic or Doppler flow probe is placed proximally on the isolated artery to measure blood flow and then a controlled amount of intimal and medial damage is produced by compressing the artery with vascular or surgical clamps. The degree of intimal damage is crucial to the development of CFRs; they are not seen without intimal damage and the greater the degree of damage, the more frequent the CFRs and the more complete the stasis.

The second crucial stage is the development of stenosis. In most studies, the objective has been to create a so-called 'critical stenosis', which is defined as the degree of arterial narrowing required to abolish reactive hyperaemia following a 20-s occlusion of the coronary artery without affecting coronary blood flow. Reactive hyperaemia is a physiological response that occurs in the downstream coronary tissues as a consequence of coronary artery narrowing. It declines with diminishing vasodilatory reserve prior to the decline in coronary blood flow. A 60-70% reduction in the internal diameter of the artery is usually required before critical stenosis is reached. The stenosis is created by placing an external constrictor, made from Lexan, around the artery. In order to apply the appropriate degree of stenosis, the constrictors are made with a range of internal diameters. The internal diameter of the constrictor is progressively decreased in 0.1-mm increments until critical stenosis is reached. Once the correct constrictor is applied, coronary blood flow starts declining due to thrombus formation and stasis occurs 4 to 12 minutes later. The thrombus is dislodged by flicking the constrictor, thereby restoring blood flow.

The Folts model has been used as a bioassay for *in vivo* platelet activity (Folts, 1991). Endpoints include the frequency of CFRs and measuring the extent to which blood flow is reduced. Since the CFRs are stable for prolonged periods, dose-response curves can be prepared. Furthermore, the onset of action of antithrombotic agents can be evaluated. Using this model, it has been demonstrated that CFRs are abolished within 90 s of administering intravenous boluses of aspirin and monoclonal antibody 7E3, which blocks the platelet glycoprotein IIb/IIIa receptor (Folts, 1991).

A notable disadvantage of this model is that a high level of technical skill is required to ensure that stable CFRs and meaningful data are attained. In addition to the strict experimental conditions required, a number of biological variables also need to be controlled in order to obtain stable CFRs. These include prevention of hypothermia, stable blood gases and pH, platelet count $>100 \times 10^9$/L, and appropriate levels and type of anaesthesia (Folts, 1991). Since each animal serves as its own control and the acute manipulation of CFRs is the endpoint of this model, it is not appropriate for investigating the effect of long-term interventions, such as the effect of diet. Furthermore, because a stable, occlusive thrombus is not formed in this model, it is not ideal for investigating the efficacy of thrombolytic agents.

Thrombogenic inserts

The thrombotic potential of metal implants has been recognised since 1964 when Blair *et al* (1964) reported that spiral wires, made from an aluminium-magnesium alloy, would produce slowly developing occlusive thrombi when inserted into the coronary arteries of dogs that had undergone thoracotomy. To avoid open-chest surgery, thrombogenic metal coils have been positioned with fluoroscopic guidance (Bergmann *et al*, 1983). Angiography and electrocardiography were used to monitor the development of occlusive coronary thrombi but death due to ventricular fibrillation was common. This has limited the application of this technique (Bush & Shebuski, 1990).

In order to prevent fatality associated with coronary occlusion, Bush and Shebuski (1990) developed a femoral artery copper coil model. A significant advantage of this model was that fluoroscopy was unnecessary and a smaller mammal, such as the rabbit, could also be used, thereby reducing expense. In this model, a hollow polyurethane catheter is introduced into the left carotid artery. In almost all instances, advancing the catheter will result in its passage into the descending aorta and, with further advancement, into either femoral artery. A flexible, Teflon-coated guide wire is then inserted through the hollow catheter and the latter is removed. A copper coil is slipped over the guide wire and advanced into the femoral artery. Surgical exposure of the femoral artery allows direct placement of a Doppler flow probe immediately proximal to the copper coil. Thrombotic occlusion occurs 10 to 22 minutes after coil insertion.

Although the mechanism of thrombus formation with these techniques is far removed from the pathophysiology of myocardial infarction, an attractive feature of techniques involving thrombogenic coils is the uniform time to thrombus formation, thrombolysis and reocclusion (Bush & Shebuski, 1990). This model has been mainly used to evaluate various thrombolytic drugs (Bergmann *et al*, 1983; Cercek *et al*, 1986; Leidy *et al*, 1990; Mellott *et al*, 1990) although Bush and co-workers (1990) have evaluated several potential antiplatelet and anticoagulant agents for their ability to delay or prevent thrombotic occlusion following coil insertion.

Thrombogenic inserts have also been used in rats. The aorta-loop model has been extensively used by Homstra to investigate the thrombogencity of dietary fat. In this model, a segment of polyethylene cannula is shaped into a loop under a stream of hot water, siliconised and filled with heparinised saline (500 IU/mL) (Homstra, 1971). The rats are anaesthetised and the abdominal aorta is exposed via a midline laparotomy. The loop is inserted into the aorta between the iliolumbar arteries cranially and the spermatic arteries caudally. The wound is closed in such a way that a part of the loop is exteriorised and the rats are allowed to recover. The loop is examined twice daily for signs of obstruction (decreased surface temperature of the loop and a colour change from light red to blue-black). The time from insertion to obstruction, the obturation time (OT), is typically 5 days, depending on the type of diet the animals receive (Homstra, 1981). Platelet-rich, fibrin-poor thrombi form at the junction between the loop (both proximally and distally) and the vessel wall, presumably due to changes in blood flow and endothelial damage at these sites (Homstra, 1981). The validity of the method was partly established by demonstrating that the intravenous infusion of prostacyclin, a potent inhibitor of platelet adhesion and aggregation, increased the OT by 50%.

Using this model, Homstra has shown that replacing carbohydrate in the diet with sunflower oil resulted in a dose-dependent increase in the OT (Homstra, 1971). Furthermore, increasing the ratio of sunflower oil to hydrogenated coconut oil resulted in an increase the OT. He also showed that there was a negative correlation between the saturated fatty acid content of dietary fat and the OT indicating a prothrombotic effect of dietary saturated fatty acids (Homstra, 1981).

The advantages of this model are that it is relatively inexpensive and a platelet-rich thrombus, typical of that associated with arterial disease in human beings, is formed. This model, however, is of high ethical cost. Besides the surgical intervention, acute, spontaneous, occlusive aortic thrombosis results in considerable pain in the cat (Kittleson, 1998) and it is likely to be similar in the rat. A further disadvantage is that the thrombogenic stimulus is far-removed from the pathogenesis of arterial thrombosis in human beings. It should be noted that there was a poor correlation between platelet aggregation in whole blood and the OT. Compared to a control diet supplying 5% of total energy as sunflower oil (5% en), diets supplying 50% en induced more aggregation in rats, as measured by an impedance method, but paradoxically, the OT was longer (Rand *et al*, 1986, 1988). This anomaly was later demonstrated with other dietary lipids (Homstra & Wierts, 1993).

Thrombin-induced clot model

The thrombin-induced clot model has been used to evaluate the efficacy of thrombolytic drugs and therapeutic regimes designed to prevent acute vascular reocclusion following successful thrombolysis (Bush & Shebuski, 1990). Thrombus is formed by injecting thrombin and autologous blood into an arterial segment that has been isolated between two snares (Bush & Shebuski, 1990). Prior to injection, the endothelium of the arterial segment is intentionally damaged by external compression with blunt forceps. The snares are released 2-5 min later and the thrombus is allowed to age for 1-2 h before the administration of the experimental drugs. In dogs, the left anterior descending coronary artery has been used (Gold *et al*, 1984), while the femoral artery has been used in rabbits (Shebuski *et al*, 1988).

The superimposition of a high-grade stenosis provides a variation to the canine thrombin-induced clot model and simulates the anatomical features occurring in many people with acute myocardial infarction (Ziskind *et al*, 1989; Gold *et al*, 1991). The stenosis is created by applying an external constrictor to the left anterior descending coronary artery at the site of thrombus formation. The constrictor is tightened until blood flow is reduced by 40±10%, equating to a >90% reduction of the original luminal diameter. In the absence of stenosis, spontaneous reocclusion following successful thrombolysis does not occur but in the presence of high-grade stenosis, cycles of reocclusion almost invariably occur (Gold *et al*, 1991). This variation has allowed for the simultaneous investigation of strategies designed to induce thrombolysis and those designed to prevent acute thrombotic reocclusion (Gold *et al*, 1991).

In this model, the initial thrombus is formed from whole blood during arterial stasis and consequently, it is erythrocyte-rich and dissimilar to thrombus that forms contiguous with ulcerated atherosclerotic plaques (Bush & Shebuski, 1990; Gold *et al*, 1991). In the presence of high-grade stenosis however, the thrombus that is formed during reocclusion is platelet-rich and possibly similar to the thrombolysis-resistant material responsible for reocclusion in human beings (Gold *et al*, 1991). Despite this, the thrombus responsible for reocclusion formed in this model does not produce uniform, persistent occlusion, but rather, it results in cycles of occlusion and flow (Gold *et al*, 1991). For this reason, this model is not suitable for the investigation of strategies to overcome reocclusion. Furthermore, the model is not appropriate for evaluating treatment modalities (dietary or drugs) aimed at inhibiting thrombus formation.

Electrically-induced coronary thrombosis

The principle of this method is that current delivered to the coronary arteries via an intravascular wire can induce focal endothelial disruption (Bush & Shebuski, 1990). This in turn induces platelet adhesion and the development of intracoronary thrombosis. Although the placement of the electrode was initially described using fluoroscopic guidance (Salazar, 1961), direct placement of the electrode in open-chest dogs has been used since (Romson *et al*, 1980a, b; Hook *et al*, 1985; Jolly *et al*, 1985; Schumacher *et al*, 1985). Blood flow is recorded directly with an electromagnetic flow probe (Bush & Shebuski, 1990). An external constrictor can also be used to create a fixed coronary artery stenosis in an effort to mimic stenosis secondary to atherosclerosis (Schumacher *et al*, 1985). While earlier applications of this model used continuous electric stimulation throughout the experiment, Benedict *et al* (1986) showed that occlusive thrombi could occur without applying current continuously.

This method allows the investigator to evaluate antithrombotic agents (Romson *et al*, 1980a; Hook *et al*, 1985) and the efficacy of thrombolytic agents (Schumacher *et al*, 1985). Depending on the experimental design, various end-points can be monitored with this technique: blood flow, thrombus mass at the site of injury, myocardial infarct area, and myocardial infarct mass (Romson *et al*, 1980a, b; Hook *et al*, 1985; Jolly *et al*, 1985; Schumacher *et al*, 1985). The thrombus formed with this technique is platelet-rich, typical of that observed at the site of plaque rupture in humans (Bush & Shebuski, 1990).

Arterial eversion graft model

The adventitial layer of blood vessels is rich in collagen and tissue factor, the latter also being present in large amounts in atherosclerotic plaques (Wilcox *et al*, 1989). With this knowledge, Gold and co-workers (1991) developed the arterial eversion graft models in dogs and rabbits. A left thoracotomy is performed and a Doppler flow probe is placed around the circumflex coronary artery proximal to the planned graft site (Gold *et al*, 1991). A stenosis is created by placing a 2-mm-wide plastic tie around the artery distal to the planned graft site. The plastic tie is tightened in order to reduce blood flow to 40±10% of baseline flow. This corresponds to a >90% reduction in luminal diameter. A 1-cm arterial segment is excised between two microvascular clamps, stripped of excessive adventitia, everted and reinserted by end-to-end anastamosis. Within 5 min of removing the microvascular clamps, a persistent occlusion of the graft segment occurs in 80% of dogs. Complete occlusion is confirmed by angiography and documentation of an arterial flow rate less than 0.5 mL/min. Experimental drugs are administered after 30 min of complete, stable occlusion. The time to reperfusion and reocclusion can subsequently be assessed.

A very similar technique using the femoral artery has been described in rabbits (Gold *et al*, 1991). The advantage of the rabbit model is that it is technically less demanding and less expensive (Haber *et al*, 1991).

The thrombus formed with this model is platelet-rich and therefore resembles the type of thrombus seen in naturally occurring coronary artery disease (Gold *et al*, 1991). A further advantage with this model is that following successful thrombolysis, a stable, occlusive thrombus will form, unlike the Folts model and the thrombin-induced clot model (Gold *et al*, 1991; Haber *et al*, 1991). It is therefore appropriate to use this model when the objective is to investigate the efficacy of therapy directed at preventing acute thrombotic reocclusion. Its disadvantages is that it is technically difficult and labour intensive (Haber *et al*, 1991) and it is not suitable for examining methods of preventing the formation of the initial occlusive thrombus.

1.5. The effect of dietary lipids on thrombosis

Mechanisms of action

It is now well established that dietary lipids influence the fatty acid composition of platelet phospholipids (Dyerberg & Bang, 1979; Siess *et al*, 1980a; Vas Dias *et al*, 1982; Renaud *et al*, 1986a; Kwon *et al*, 1991). Phospholipid fatty acids are the precursors of eicosanoids, which, through their hormone-like action, affect platelet aggregation, vascular permeability, vascular tone and blood pressure (Gerster, 1995). Eicosanoids of series one and two are derived from arachidonic acid (AA) and eicosapentaenoic acid (EPA) respectively. The formation and function of these eicosanoids, derived from platelets and vascular endothelium, are shown in Figure 1.4.

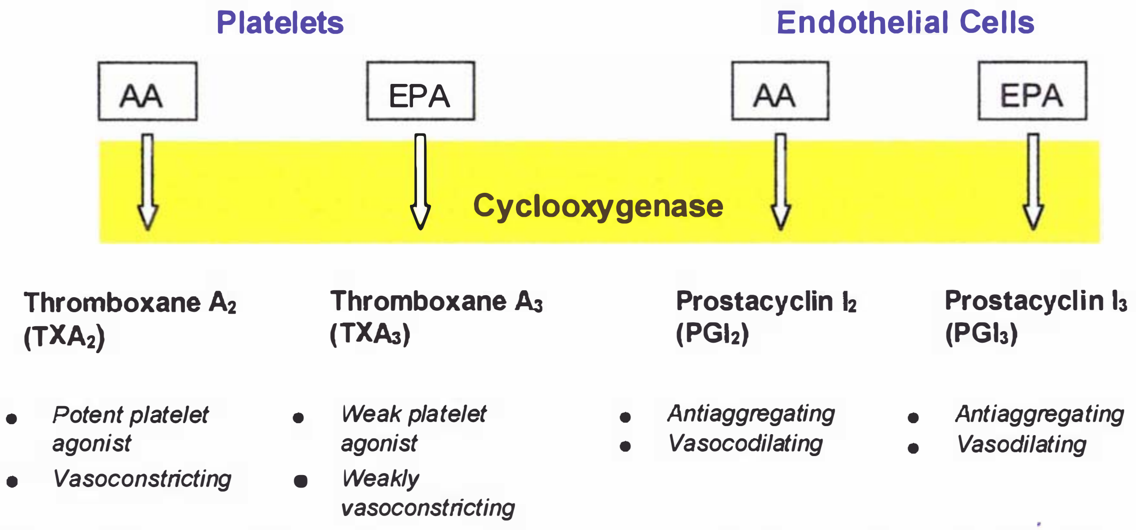

Figure 1.4 Simplified diagram of the pathways of eicosanoid production from arachidonic acid and eicosapentaenoic acid and their biological effects.

In 1979, Dyerberg and Bang compared bleeding times, platelet aggregation and the platelet phospholipid fatty acid composition of Eskimos with age- and sex-matched Danish controls. Eskimos had significantly longer bleeding times, reduced platelet aggregability and higher concentration of EPA in their platelet phospholipids, which mirrored their plasma lipid concentrations. They proposed that EPA is "converted by vascular wall tissue to an anti-aggregatory prostacyclin" and "partial dietary substitution of AA by EPA may reduce the incidence of thrombotic disorders, including myocardial infarction". There are now a number of lines of evidence to support their thesis, which is illustrated in Figure 1.5. The platelet phospholipid ratio of EPA:AA increased in healthy men given a mackerel diet for one week (Siess *et al*, 1980a). In this study, a negative correlation existed between the EPA:AA ratio and platelet aggregation in PRP and thromboxane B_2 (TXB_2) formation from collagen-stimulated platelets. In rabbits, changes in platelet aggregation also reflect the ratio of EPA to AA in platelet phospholipids, which in turn reflects the n-3 polyunsaturated fatty acid composition of the diet (Vas Dias *et al*, 1982). In further support of Dyerberg and Bang's thesis, a number of end-points of platelet aggregation (the degree of aggregation at 6 min, the maximum aggregation time and ATP secretion) have been positively correlated with AA and negatively correlated with EPA concentrations within platelet phospholipids (Renaud *et al*, 1986a; Kwon *et al*, 1991).

Other studies have shown that supplementing the diet of human beings with fish oil can alter eicosanoid production. Fish oil consumption results in a reduction in thromboxane A_2 (TXA_2) released by stimulated platelets in favour of EPA-derived thromboxane A_3 (TXA_3) (Siess *et al*, 1980b; Hornstra *et al*, 1990; Kinsella *et al*, 1990). Thromboxane A_3 is only a weak platelet agonist and occupies platelet receptors normally occupied by the more potent platelet agonist, TXA_2 (Kinsella *et al*, 1990). Furthermore, in human beings, fish oil consumption enhances the excretion of prostacyclin I_3 (PGI_3) but not at the cost of prostacyclin I_2 (PGI_2) production (von Schacky *et al*, 1985; Hornstra *et al*, 1990; Kinsella *et al*, 1990). Both PGI_3 and PGI_2 have vasodilatory effects. The functional corollary of this shift in eicosanoid production is inhibition of platelet aggregation and promotion of vasodilation.

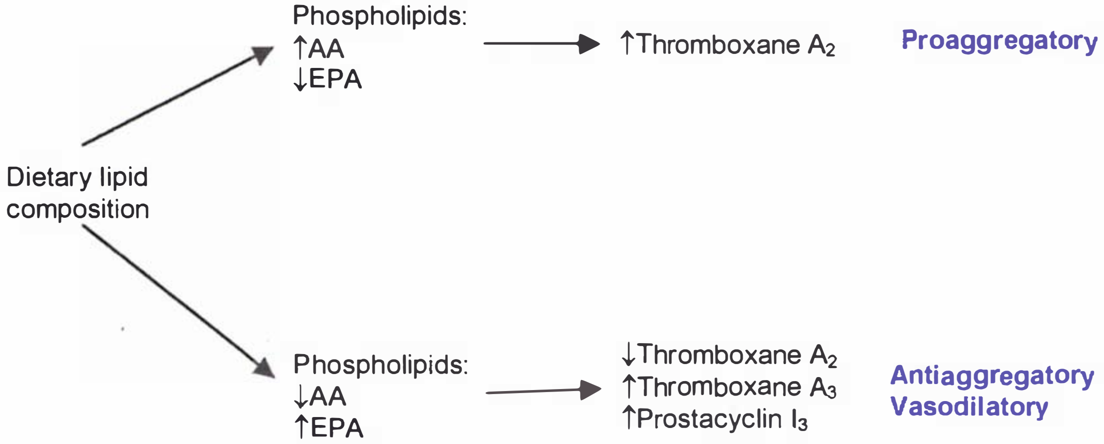

Figure 1.5 Putative mechanism by which dietary fatty acids can affect platelet and endothelial function.

Dietary n-3 fatty acids increase the ratio of EPA to AA within tissue phospholipids in a number of ways. Firstly, fish oil inhibits Δ6- and Δ5-desaturase activity thereby reducing the conversion of linoleic acid (LA, 18:2 n-6) to AA (Brenner, 1974; Garg *et al*, 1988; Christiansen *et al*, 1991). Secondly, LA and α-linolenic acid (ALA, 18:3 n-3) employ a common enzyme pathway for desaturation and elongation and an excess of either fatty acid leads to suppression of elongation and desaturation of the other (Holman, 1964). This mechanism is exemplified by a study that examined the effect of supplementing the diets of vegans and omnivores with linseed oil, a rich source of ALA. In omnivores, the platelet phospholipid concentration of EPA doubled, whereas no increase in the platelet phospholipid concentration of EPA was seen in vegans (Sanders & Younger, 1981). The high intake of LA by vegans excluded ALA desaturation and hence their phospholipid concentrations of EPA did not increase. A third mechanism is that ALA competitively excludes LA from binding to Δ6-desaturase (Brenner, 1974). Δ6-desaturase is the rate-limiting step in the desaturation and elongation of LA to AA and ALA (Brenner, 1974). The affinity for Δ6-desaturase and the subsequent rate of desaturation, is highly dependent upon the number of double bonds within the substrate (Brenner, 1974). Consequently, Δ6-desaturation is low for oleic acid (18:1 n-9), higher for LA, which has two double bonds, and the highest for ALA, which contains three double bonds.

As well as their effects on the platelet phospholipid EPA:AA, dietary fish oils appear to inhibit platelet aggregation by other, more direct mechanisms. Eicosapentaenoic acid and docosahexaenoic acid (DHA, 22:6 n-3) inhibit TXA_2-induced platelet aggregation and vascular smooth muscle contractility (Croset *et al*, 1988) by interfering with the binding of TXA_2 to its receptor (Croset *et al*, 1988; Swann *et al*, 1989). Furthermore, these long chain n-3 polyunsaturated fatty acids show a high affinity for PGH synthase (cyclooxygenase type 1) thereby competitively excluding AA from binding to this enzyme (Corey *et al*, 1983; Rao *et al*, 1983). PGH synthase converts AA to prostaglandin H_2 (PGH_2) prior to conversion to TXA_2. Docosahexaenoic acid and EPA have been shown to inhibit PGH synthase expression (Achard *et al*, 1997). Despite these experimental observations, a nutritional study in healthy male subjects has found that the concentrations of DHA and docosapentaenoic acid (22:5 n-3) within platelet phospholipids were positively correlated with end-points used to assess platelet aggregation (Kwon *et al*, 1991).

There is experimental evidence to suggest that compared to polyunsaturated fatty acids, dietary saturated fatty acids, particularly 14:0, 16:0 and 18:0, have prothrombotic effects (see discussion below). Given the differences in melting points between saturated and polyunsaturated fatty acids with an equal number of carbon atoms, interest was stimulated in examining whether platelet membrane fluidity influences platelet function. Rendering platelet membranes more fluid by incubating them *in vitro* with a cyclopropyl fatty acid ester (A2C) has been shown to reduce platelet aggregation with thrombin, ristocetin and collagen (Vlasic *et al*, 1993). However, platelet membrane fluidity *in vivo* appears to be under tight homeostatic control and relatively resistant to changes in the ratio of dietary saturated to unsaturated fatty acids (Heemskerk *et al*, 1989). In rats, large quantities of dietary polyunsaturated fatty acids (either 50% en marine fish oil or 50% en sunflower oil) resulted in greater membrane fluidity than a 5% en sunflower oil control diet (Rand *et al*, 1986, 1988). Both test diets lowered arterial thrombosis tendency more than the control, but it is not possible to conclude that increased membrane fluidity was directly responsible for this effect. Other variables that are known to affect platelet aggregation, such as the platelet membrane fatty acid composition, would also be altered in such a study.

Can the apparent prothrombotic effects of saturated fats be explained by changes in platelet phospholipid concentrations in AA and EPA and subsequent eicosanoid production? In healthy humans, 5,8,11-eicosatrienoic acid (20:3 n-9) is the only platelet phospholipid fatty acid positively correlated with dietary saturated fatty acid intake (Renaud *et al*, 1981, 1986b). This fatty acid is principally derived from 18:0 by desaturation and elongation (Sprecher, 1983) and of all the fatty acids within platelet phospholipids, it correlates most strongly with the clotting activity of platelets and thrombin-induced platelet aggregation in human beings (Renaud *et al*, 1986b) and rats (McGregor *et al*, 1980). On first appearances, this observation appears anomalous because 5,8,11-eicosatrienoic acid is neither a precursor to series one nor series two eicsoanoids. Lagarde and co-workers showed however, that 20:3 n-9 is metabolised by platelets to 12-hydroxy-5,8,10-eicosatrienoic acid and that this hydroxylated fatty acid has potent platelet aggregating ability (Lagarde *et al*, 1983, 1985). Moreover, when 5,8,11,14-heneicosatetraynoic acid, a compound known to inhibit the lipoxygenase pathway, was added to PRP, the potentiating effect of 5,8,11-eicosatrienoic acid on platelet aggretation was inhibited. These findings suggested for the first time that metabolites of the lipoxygenase pathway might also regulate platelet function.

The *in vitro* addition of oleic acid to isolated platelets inhibits platelet aggregation induced by platelet activating factor and serotonin release (Nunez *et al*, 1990). Rather than acting as a precursor for eicosanoid synthesis, oleic acid inhibits transmembrane signal transduction by decreasing the synthesis of phosphatidylinositol 4,5-biphosphate (PIP_2). Cleavage of PIP_2 by the action of phospholipase C leads to the generation of diacylglycerol, which is an activator of protein kinase C and inositol 1,4,5-trisphosphate (IP_3), which induces the release of calcium from platelets' dense tubular system (Marcus, 1996). Diacylglycerol and calcium are considered secondary messenger molecules and initiate a series of events leading to platelet activation (shape change and release of agonists).

Effects of dietary lipids on platelet aggregation and thrombosis: experimental evidence

Using the aorta loop model in rats, Hornstra and co-workers have published widely on the effect of dietary lipids on arterial thrombosis (Hornstra, 1971, 1972, 1981, 1989b, c, d; Hornstra & Vendelmans-starrenburg, 1973; Hornstra & Lussenberg, 1975; Hornstra & Lussenburg, 1975; Rand *et al*, 1986, 1988; Heemskerk *et al*, 1989, 1991; Hornstra & Wierts, 1993; Hornstra *et al*, 1993). This work led to the conclusion that thrombogenicity is inversely proportional to the ratio of polyunsaturated to saturated fatty acids within the diet, whereas monounsaturated fatty acids have a neutral effect (Hornstra, 1989a). Replacing carbohydrate in the diet with sunflower oil resulted in a dose-dependent increase in the OT of the aorta loop (Hornstra, 1971). Increasing the ratio of sunflower oil to hydrogenated coconut oil resulted in an increased OT. A negative correlation between the saturated fatty acid content of dietary fat and the OT indicates a prothrombotic effect of dietary saturated fatty acids (Hornstra, 1981). Paradoxically, a positive correlation has been found to exist between the OT and the extent of whole blood platelet aggregration (Hornstra & Wierts, 1993). Whether the OT or whole blood aggregation data is a better predictor of arterial thrombotic potential in human beings is unknown. The aorta loop method was developed to evaluate thrombotic potential of dietary lipids under arterial flow conditions but the mechanism that initiates thrombus formation differs markedly from that which causes ischaemic heart disease.

Despite this anomaly, Hornstra's observations have generally been supported by other studies. A diet enriched in saturated fats (provided by lard) fed to pigs resulted in more extensive platelet aggregation (assessed by maximum aggregation using ADP as the agonist) than a diet enriched in n-3 polyunsaturated fatty acids (Verdouw *et al*, 1989). Thrombin-induced platelet aggregation (PRP) was greater when rats received either a butter or stearic acid-enriched diet than a diet containing either corn oil or oleic acid (Renaud *et al*, 1970). In healthy human beings, ATP release from activated platelets was significantly lower after feeding a diet enriched with canola oil for 8 weeks than a diet enriched with butter, which has a higher saturated fatty acid content (Kwon *et al*, 1991). ATP is co-secreted with ADP from platelet dense bodies and its concentration in the fluid surrounding activated platelets is proportional to the extent of the platelet release reaction (Feinman *et al*, 1985). There is also epidemiological evidence to suggest that the dietary intake of saturated fatty acids is positively and significantly correlated to thrombin-induced platelet aggregation (Renaud *et al*, 1986b).

The ratio of total dietary polyunsaturated to saturated fatty acids oversimplifies the complex interplay between fatty acids within dietary lipids. Recognising this, Ulbricht and Southgate (1991) proposed an index of thrombogenicity:

$$\frac{aS}{b(\text{oleic}) + c(\text{MUFA}) + d(n\text{-}6) + e(n\text{-}3) + n\text{-}3/n\text{-}6}$$

where: S = 14:0, 16:0 and 18:0
MUFA = monounsaturated fatty acids other than oleic acid
n-6 = n-6 polyunsaturated fatty acids
n-3 = n-3 polyunsaturated fatty acids
a, b, c, d are constants with a=1; b, c and d = 0.5; and e=3.

This formula took into account the relatively greater antithrombotic effect of n-3 polyunsaturated fatty acids compared with n-6 polyunsaturated fatty acids and the greater thrombotic tendency of saturated fats with 14, 16 or 18 carbon atoms. Monounsaturated fats, other than oleic acid, and n-6 polyunsaturated fatty acids were treated equally by this formula.

There is experimental evidence to support the thrombogenicity index proposed by Ulbricht and Southgate (1991). Omega-3 polyunsaturated fatty acids appear to be less thrombogenic than n-6 polyunsaturated fatty acids and have been weighted accordingly. The effects of fish oil supplementation on thrombotic potential have been discussed above. Feeding commercial rabbit food for 60 days with 60g/kg linseed oil or fish oil, resulted in less platelet aggregation induced by thrombin and collagen than diets containing the same quantity of corn oil and coconut oil (Vas Dias *et al*, 1982). Linseed oil and fish oil provide good sources of 18:3 n-3 and long-chain n-3 polyunsaturated fatty acids respectively, while corn oil and coconut oil are rich sources of 18:2 n-6 and saturated fats respectively. Interestingly, ADP-induced platelet aggregation was significantly reduced only in rabbits receiving fish oil.

There is also evidence to suggest that not all saturated fatty acids are equally thrombogenic. Based on studies in rats, the longer chain saturated fatty acids, 14:0 (myristic acid), 16:0 (palmitic acid) and 18:0 (stearic acid), correlate more strongly with thrombin-induced platelet aggregation (McGregor *et al*, 1980) and the OT of Hornstra's aorta loop than other fatty acids (Hornstra, 1973; Hornstra & Lussenberg, 1975). Palm oil however, which comprises approximately 50% saturated fatty acids, the majority of which is palmitic acid, has an antithrombotic effect in rats. Compared with a control diet of 5% en sunflower seed oil, a diet containing 50% en palm oil reduced TXA_2 release by collagen-activated platelets and tended to increase the OT of Hornstra's aorta loops (Rand e*t al*, 1988). This effect of palm oil may be due to its high content of monounsaturated fatty acids (~40%) and LA (10%). Palm oil has a low index of thrombogenicity (0.49) using Ulbricht and Southgate's formula, compared with an index of 0.37 for fish oil and 3.09 for coconut oil. In human beings, platelet phospholipid concentrations of palmitic acid (16:0) and stearic acid (18:0) have been shown to be negatively correlated, albeit weakly, with platelet aggregability, suggesting that some dietary saturated fatty acids may in fact have an inhibitory effect on platelet function (Renaud *et al*, 1986b; Kwon *et al*, 1991). This relationship was observed despite a significant positive correlation with total platelet phospholipid saturated fatty acid content (Renaud *et al*, 1986b).

Ulbricht and Southgate (1991) also included the ratio of dietary n-3:n-6 polyunsaturated fatty acids in the denominator of their index of thrombogenicity. This ratio, as well as the absolute concentrations of these PUFAs, has a bearing on prostacyclin production. The mechanisms by which this ratio can influence platelet phospholipid concentrations of EPA and AA have been discussed above. A proportional increase in EPA concentrations at the expense of AA in tissue phospholipids has been noted in rats fed diets containing increasing amounts of butter, but not lard (O'Dea *et al*, 1988). This occurred despite the fact that both dietary fats contained similar concentrations of ALA, the precursor to EPA. However, the ratio of ALA to LA was higher in the butter-fed rats. Since ALA and LA compete for desaturation and elongation (Holman, 1964) and ALA is a more effective competitor for the desaturase enzyme than LA (Brenner, 1974), a higher dietary n-3:n-6 ratio may have facilitated the synthesis of EPA at the expense of AA. Since AA is the precursor to series 2 eicosanoids, it was not surprising that the EPA:AA ratio within tissue phospholipids was negatively associated with *in vitro* PGI_2 production by aortic rings. However, there was no effect of diet on platelet aggregation and thromboxane B_2 concentrations by stimulated platelets. Given that the TXB_2 concentrations produced by stimulated platelets was not significantly different from TXB_2 concentrations within platelet poor plasma 'blanks', the sensitivity of the assay in this study is questionable.

A further example of the impact of the dietary n-3:n-6 ratio is provided by a study that compared the effects of feeding soybean oil and canola oil to rats (Piché &

Mahadevappa, 1990). The soybean and canola oil diets contained very similar concentrations of ALA yet significantly more EPA and DHA were incorporated into platelet phospholipids when canola oil was fed. However, the ratio of n-3:n-6 fatty acids in the canola oil diet was approximately 2.5 times greater in the canola oil diet compared to the soybean oil diet. Unfortunately, platelet aggregation was not evaluated in this study so the functional corollary of this difference in the platelet phospholipid fatty acid composition cannot be evaluated.

A potential weakness of Ulbricht and Southgate's thrombogenicity index, is that it treats all n-3 polyunsaturated fatty acids as equally antithrombogenic. Yet in human beings, relatively small concentrations of dietary EPA and DHA, provided by a fish oil concentrate, are incorporated into platelet phospholipids in greater concentrations than much larger concentrations of ALA, provided by linseed oil (Sanders & Younger, 1981). Although platelet aggregation was not performed in this study, epidemiological evidence (Dyerberg & Bang, 1979) and results from other studies have shown a functional correlation between EPA and reduced platelet function (Siess *et al*, 1980a, b; Hornstra *et al*, 1990).

There is strong evidence for an association between a Mediterranean-style diet, in which olive oil is the principal source of fat, and protection from cardiovascular disease (Keys, 1980; Ferro-Luzzi & Branca, 1995; Majem-Serra *et al*, 1995). Although there are many components of the Mediterranean diet that may contribute to the low rate of coronary heart disease, the high oleic acid content of this diet has prompted investigations into the potential antithrombotic activity of this fatty acid. Aorta loop studies in rats suggest that oleic acid has less thrombotic potential than long-chain saturated fatty acids, but is more thrombotic than LA (Hornstra & Lussenberg, 1975). Salonen *et al* did not find any difference in platelet aggregation or thromboxane production *in vivo* between individuals who had their usual diets supplemented with MAXEPA® (supplying almost 3 g of n-3 fatty acids daily) and those receiving the same volume of an olive oil placebo, for 12 weeks (Salonen *et al*, 1987). It is of interest that compared to the subjects' usual diet, both oils reduced secondary aggregation, indicating that the antithrombotic effect of olive oil, which is a rich source of oleic acid, is similar to that of concentrated marine fish oil. In contrast, Burri and co-workers (1991), under well-controlled metabolic ward conditions, found that a diet enriched with oleic acid induced greater platelet aggregation in healthy men than the same basal diet enriched with LA. Notably, significant changes in aggregability occurred only after day 63 on the diet. This is markedly longer than the time required for diets containing fish oil to induce significant changes in platelet function diets, which can occur as early as one week after supplementation (Siess *et al*, 1980a).

In summary, the type of dietary lipid appears to influence platelet function and thrombotic potential. Dietary fatty acids can influence the ratio of series 2 and series 3 eicosanoids produced by vascular endothelium and platelets, but this is not the only mechanism through which dietary lipids can affect thrombus size. Generally, dietary fats rich in long-chain saturated fatty acids appear to have greater thrombotic potential than those containing *cis*-monounsaturated fats and n-6 polyunsaturated fatty acids, which in turn have greater thrombotic potential than n-3 polyunsaturated fatty acids, particularly those sourced from marine fish oils. This however does not take into account the complex interplay between fatty acids within lipids, such as the ratio of n-6 to n-3 polyunsaturated fatty acids. Nor does it consider other components of dietary lipids such as antioxidant status or concentrations of other components, such as phytosterols, which may influence haemostasis.

1.6. References

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Chapter 2. Determination of fasting and postprandial lipoprotein cholesterol concentrations in pigs: a comparison of methods

2.1. Introduction

In 1972, Friedewald *et al* proposed a formula for estimating the fasting serum LDL-C concentration in human beings. Laboratories adopted the Friedewald formula because it avoided the laborious, time-consuming ultracentrifugation steps required to isolate LDL-C. The formula requires the measurement of three parameters: the fasting total serum cholesterol concentration, the serum triglyceride concentration and the high-density lipoprotein cholesterol (HDL-C) concentration (Figure 2.1). Fasting total cholesterol and triglyceride concentrations are determined by enzymatic methods. In human beings, HDL-C concentrations are measured in the supernatant following precipitation of apolipoprotein-B containing lipoproteins (predominantly LDL and very low-density lipoprotein (VLDL)}, or by direct assay (Sugiuchi *et al*, 1995).

$$[\text{LDL-C}] = [\text{Total cholesterol}] - [\text{HDL-C}] - ([\text{Triglycerides}] \times 0.46)$$

Figure 2.1 The Friedewald formula. The triglyceride concentration multiplied by 0.46 provides an estimate of the very low-density lipoprotein cholesterol concentration of the sample. [LDL-C], low-density lipoprotein cholesterol concentration; [HDL-C], high-density lipoprotein cholesterol concentration. All concentrations are in mmol/L.

In human subjects, this formula reliably estimates serum LDL-C concentrations provided it is applied to fasting blood samples, the triglyceride concentration is less than 4.5 mmol/L and the patient is not suffering from dysbetalipoproteinaemia (Friedewald *et al*, 1972; Rifai *et al*, 1992). Following a meal, a large proportion of circulating triglycerides is found within chylomicrons. In this circumstance, the Friedewald formula will tend to overestimate the VLDL cholesterol (VLDL-C) concentration and underestimate the LDL-C concentration. Individuals with dysbetalipoproteinaemia have VLDL that is abnormally enriched with cholesterol relative to triglyceride and consequently, the Friedewald formula will tend to underestimate VLDL-C concentrations and overestimate LDL-C concentrations (Rifai *et al*, 1992)

Validation of the Friedewald formula on porcine samples would confer similar benefits to those experienced in medical laboratories and allow evaluation of large numbers of samples inexpensively and easily. The principle objective of this study was therefore to determine whether cholesterol fractions in pig serum could be estimated without using the ultracentrifugation procedure. A second objective was to determine the effect of recent feeding of pigs on their serum lipoprotein cholesterol concentrations.

2.2. Materials and methods

Experimental procedure

Five, 8-week-old Large White male pigs were used in this study. Blood samples were collected from the pigs following an overnight fast and 2 hours after they had been given *ad-libitum* access to commercial grower ration. The blood samples were collected from the right brachiocephalic or jugular vein, allowed to clot and centrifuged at 2 250 x g for 10 min (11105 rotor, Centra -3C centrifuge, International Equipment Co, Needham Heights, MA, USA). Serum was harvested and refrigerated until analysis, which occurred within 24 hours of blood collection.

Analytical methods

Determination of lipoprotein cholesterol concentrations by sequential ultracentrifugation

The lipoprotein fractions were prepared using an ultracentrifuge (Centrikon T-2070, Kontron Instruments, Zurich, Switzerland, fitted with either a TFT 80.4 or TFT 45.6 rotor). Ultracentrifugation was performed as described by Mills *et al* (1984). Initially, serum was overlaid with NaCl solution (0.196 molal, density 1.006 kg/L at 20°C). The sample was centrifuged for 15 hours at 117 000 × g at 20°C and the supernatant was collected by a tube-slicing technique. The infranatant was then adjusted to a density of 1.063 kg/L using NaBr solution (4.778 molal, 1.3199 kg/L at 20°C) and overlaid with NaBr solution (0.844 molal, 1.063 kg/L at 20°C). The sample was centrifuged for 20 hours at 191 500 × g and the supernatant was collected. A second supernatant layer was also collected by tube slicing. The remaining infranatant was then adjusted to a density of 1.21 kg/L using NaBr solution (7.593 molal, 1.4795 kg/L at 20°C), overlaid with NaBr solution (2.973 molal, 1.21 kg/L at 20°C) and centrifuged for a further 21.5 hours at 205 000 × g. The supernatant was harvested. All solutions contained EDTA (1 mmol/L) and azide (2 mmol/L).

Each fraction was weighed and the concentration of cholesterol determined by the enzymatic method of Roche (formerly Boehringer Mannheim) using cholesterol esterase, cholesterol oxidase and peroxidase. The lipoprotein content of each fraction was determined qualitatively by electrophoresis on agarose gel using a barbitone buffer (76 mmol/L, pH 8.6) and staining with Fat Red.

Determination of lipoprotein cholesterol concentrations by simple methods

HDL cholesterol (HDL^{ppt})

ApoB-containing lipoproteins (LDL and VLDL) were precipitated from 1 mL of serum at 4 °C by the addition of 50 μL of sodium heparin at a concentration of 4 000 USP/mL and 100 μL of 1 mol/L $MnCl_2$ (Farish & Fletcher, 1983). Precipitation methods for determining the HDL content of pig sera have been previously validated (Calvert & Scott, 1975; Knipping *et al*, 1975). After mixing, the sample was allowed to stand for 30 min at 4°C and then centrifuged at 15 000 × g for 5 min in a microcentrifuge. The supernatant was removed and its cholesterol concentration was determined by the automated enzymatic method described above using a cholesterol reagent modified by the further addition of EDTA to a final concentration of 8 mmol/L.

VLDL cholesterol ($VLDL^{trig}$)

The ratio of the mass of triglyceride to that of cholesterol in porcine VLDL is approximately 9:1 (Fidge, 1972), corresponding to a molar ratio of 4:1. Consequently, in this study, the molar concentration of VLDL-C was estimated by multiplying the serum triglyceride concentration by 0.25. The serum triglyceride concentration was determined by the enzymatic method of Roche (formerly Boehringer Mannheim) using lipase, glycerol kinase, glycerol phosphate oxidase and peroxidase.

LDL cholesterol (LDL^{friede})

LDL cholesterol was determined by applying a modification of the Friedewald formula (Figure 2.2).

[LDL-C] = [Total cholesterol] - [HDL-C] - ([Triglycerides] x 0.25)

Figure 2.2 The modified Friedewald formula used in this study. The triglyceride concentration multiplied by 0.25 provided an estimate of the very low density lipoprotein cholesterol concentration. [LDL-C], low-density lipoprotein cholesterol concentration; [HDL-C], high-density lipoprotein cholesterol concentration. All concentrations are in mmol/L.

Expression of results

The total cholesterol concentration was determined directly from the serum samples by the enzymatic method outlined above ($chol^{total}$) and from the data obtained from sequential ultracentrifugation ($chol^{sum}$). The latter method involved weighing the VLDL, LDL and HDL fractions, determining the volume and number of moles of cholesterol in each and expressing the result as a concentration relative to the volume of the original sample. The proportion of cholesterol recovered from the fractions was then calculated by dividing $chol^{sum}$ by $chol^{total}$.

The raw VLDL, LDL and HDL-C concentrations obtained from sequential ultracentrifugation were corrected for cholesterol recovery by dividing the raw concentrations by the proportion recovered.

The difference between the serum LDL-C concentration obtained by applying the Friedewald formula (LDL^{friede}) and the serum LDL-C concentration determined by sequential ultracentrifugation (LDL^{fuge}) was calculated and expressed as a percentage of the LDL^{fuge} (Figure 2.3). This was performed on both the raw data and the recovery-adjusted data. Similar differences were calculated for the HDL-C and VLDL-C concentration data.

Student's two-tailed t-test for paired samples was used to evaluate the effect of feeding on the total cholesterol, LDL-C, VLDL-C and HDL-C concentrations. A p-value of less than 0.05 was considered significant.

$$\text{LDL\% difference} = (\text{LDL}^{\text{friede}} - \text{LDL}^{\text{fuge}}) \div \text{LDL}^{\text{fuge}} \times 100$$

Figure 2.3 Formula used to calculate the percent difference between the LDL cholesterol concentration as determined by sequential ultracentrifugation and derived from the Friedewald formula. This was also performed on the HDL and VLDL cholesterol data.

2.3. Results

After removal of the supernatant fraction floating at a density of 1.006 kg/L, electrophoresis of the floating layer obtained at a density of 1.063 kg/L showed only a prominent β-migrating band. The layer immediately below contained significant quantities of cholesterol and triglyceride and had an identical electrophoretic pattern. It was concluded that these layers were respectively LDL_1 and a dense subfraction of LDL, possibly LDL_2. For analytical purposes they were combined. Electrophoresis of the floating layer obtained following ultracentrifugation at a density of 1.21 kg/L showed only an α-migrating band, consistent with HDL and was free of contaminating species.

Approximately 86% of the total serum cholesterol was recovered during sequential ultracentrifugation from each sample (Table 2.1). Duplicate Chol^{sum} fractionations gave a coefficient of variation of 1.4%. Coefficients of variation for the individual $\text{VLDL}^{\text{fuge}}$, LDL^{fuge} and HDL^{fuge} fractions were 6.8%, 2.0% and 2.5% respectively.

LDL cholesterol concentrations, estimated by the modified Friedewald formula, were consistently greater than the raw concentrations derived from sequential ultracentrifugation (Table 2.2). Correcting the LDL^{fuge} data for cholesterol recovery greatly improved the agreement between the methods. Serum HDL-C concentrations, derived from the precipitation method, agreed closely with the concentrations determined following sequential ultracentrifugation (both raw and corrected) (Table 2.3). The HDL^{ppt} was marginally higher than the raw HDL^{fuge} but marginally lower than the corrected HDL^{fuge}.

Table 2.1 Mean total cholesterol concentration (Chol^{total}) determined enzymatically and the mean cholesterol concentration determined by summation of the cholesterol content of the VLDL, LDL and HDL fractions following sequential ultracentrifugation (Chol^{sum}).

	Chol^{total} *mmol/L*	Chol^{sum} *mmol/L*	Recovery[1] %
Fasting	2.21 (0.17)	1.90 (0.18)	0.86 (0.03)
Fed	1.92 (0.16)	1.64 (0.13)	0.85 (0.01)
p-value[2]	0.03	0.03	

[1] Recovery refers to the amount of cholesterol recovered following sequential ultracentrifugation expressed as a proportion of the Chol^{total}.
[2] The p-value is the level of statistical significance for the comparison between the fasting and fed data.
Standard deviations are shown in brackets.

There was significant disparity between the methods used to determine the VLDL-C concentration (Table 2.4). The VLDL-C concentration, calculated from the serum triglyceride concentration, was 2½ times greater than the raw VLDL-C concentrations, derived from sequential ultracentrifugation. The agreement improved when the raw data was corrected for cholesterol recovery. There was closer agreement between the methods in the postprandial samples than in the fasting samples.

Table 2.2 Mean serum LDL cholesterol concentrations prepared from sequential ultracentrifugation (LDL^{fuge}) and the mean serum LDL cholesterol concentrations derived from the modified Friedewald formula (LDL^{friede}). Ultracentrifuged values, which have been corrected for recovery, are also shown.

	LDL^{friede} *mmol/L*	LDL^{fuge} raw *mmol/L*	LDL^{fuge} corrected *mmol/L*	Difference raw[1] %	Difference corrected[1] %
Fasting	1.34 (0.11)	1.13 (0.13)	1.31 (0.11)	18.6 (7.3)	4.2 (2.4)
Fed	0.90 (0.11)	0.76 (0.08)	0.89 (0.09)	18.1 (4.0)	2.9 (1.7)
p-value[2]	0.002	0.002	0.001		

[1] Difference (raw and corrected) is the mean absolute difference between the LDL^{friede} and LDL^{fuge}, expressed as a percentage of the LDL^{fuge}.
[2] The p-value is the level of statistical significance for the comparison between the fasting and fed data.
Standard deviations are shown in brackets.

Regardless of the method of determination, the total cholesterol concentration and the LDL-C concentration were significantly lower in the postprandial samples than in the fasted samples. Conversely, the HDL-C was significantly higher in the fasted samples than in the postprandial samples. Feeding did not significantly change $VLDL^{trig}$ but it resulted in a doubling of the $VLDL^{fuge}$.

Table 2.3 Mean serum HDL cholesterol concentrations prepared from sequential ultracentrifugation (HDL^{fuge}) and the mean serum HDL cholesterol concentrations derived following the precipitation of apolipoprotein B-containing lipoproteins (HDL^{ppt}). Ultracentrifuged values, which have been corrected for recovery, are also shown.

	HDL^{ppt} *mmol/L*	HDL^{fuge} raw *mmol/L*	HDL^{fuge} corrected *mmol/L*	Difference raw[1] %	Difference corrected[1] %
Fasting	0.78 (0.06)	0.74 (0.05)	0.84 (0.07)	5.4 (3.8)	7.8 (4.5)
Fed	0.91 (0.07)	0.83 (0.06)	0.97 (0.08)	10.1 (1.7)	5.8 (1.6)
p-value[2]	0.03	0.05	0.06		

[1] Difference (raw and corrected) is the mean absolute difference between the HDL^{ppt} and HDL^{fuge}, expressed as a percentage of the HDL^{fuge}.
[2] The p-value is the level of statistical significance for the comparison between the fasting and fed data.
Standard deviations are shown in brackets.

Table 2.4 Mean serum VLDL cholesterol concentrations prepared from sequential ultracentrifugation ($VLDL^{fuge}$) and the mean serum VLDL cholesterol concentrations calculated by dividing the serum triglyceride concentration by four ($VLDL^{trig}$). Ultracentrifuged values, which have been corrected for recovery, are also shown.

	$VLDL^{trig}$ *mmol/L*	$VLDL^{fuge}$ raw *mmol/L*	$VLDL^{fuge}$ corrected *mmol/L*	Difference raw[1] %	Difference corrected[1] %
Fasting	0.10 (0.02)	0.03 (0.01)	0.04 (0.01)	210 (74)	170 (73)
Fed[2]	0.11 (0.04)	0.06 (0.02)	0.07 (0.02)	98 (29)	67 (27)
p-value[3]	0.5	0.009	0.009		

[1] Difference (raw and corrected) is the mean absolute difference between the $VLDL^{trig}$ and $VLDL^{fuge}$, expressed as a percentage of the $VLDL^{fuge}$.
[2] Fed data represents the cholesterol concentration of the combined VLDL/chylomicron fraction.
[3] The p-value is the level of statistical significance for the comparison between the fasting and fed data.
Standard deviations are shown in brackets.

2.4. Discussion

This study has shown that porcine serum HDL-C and LDL-C concentrations, as determined by the precipitation method and the modified Friedewald formula respectively, agree well with concentrations determined by sequential ultracentrifugation. With the exception of the fasting HDL-C concentration, the agreement was better when the ultracentrifuged concentrations were corrected for cholesterol recovery. Conversely, for both the fasting and postprandial samples, there was poor agreement between the $VLDL^{trig}$ and the $VLDL^{fuge}$ cholesterol concentrations. This occurred despite the fact that the Friedewald formula was modified to reflect the higher triglyceride:cholesterol ratio in the VLDL of pigs compared to human beings. While only small amounts of VLDL (30–80 mg/dL) are to be expected in pig serum (Knipping *et al*, 1975) any loss of VLDL during tube-slicing would lower the concentration of $VLDL^{fuge}$ even further. Repeating fractionation with larger samples, which should allow for better recovery, might reveal the significance of such losses. However, since the quantity of cholesterol contained within the VLDL fraction is <5% of the total cholesterol concentration, any error inherent in either of the methods used to determine the VLDL-C concentration had little bearing on the agreement between LDL^{friede} and LDL^{fuge}.

Since neither of the methods for VLDL-C determination used in this study takes into account postprandial chylomicronaemia, the VLDL-C concentration after feeding is effectively the cholesterol concentration in both the VLDL and chylomicron fractions. The mean $VLDL^{fuge}$ doubled but the $VLDL^{trig}$ remained unchanged after feeding. Luhman *et al* (1992) found that the cholesterol concentration of a VLDL/chylomicron fraction in pigs tended to be greater four hours after feeding than after a 12-hour fast, which is consistent with the observation of a significant increase in the $VLDL^{fuge}$ following feeding in the current study. Conversely, Luhman *et al* (1992) found that compared to fasting samples, the plasma triglyceride concentration was significantly lower four hours after feeding but there was no difference in the plasma triglyceride concentration one hour after feeding. Given that in the current study, the $VLDL^{trig}$ is determined from the serum triglyceride concentration, the results of Luhman *et al* (1992) may explain why the $VLDL^{trig}$ was not affected by feeding. Consequently, the use of the serum triglyceride concentration to determine VLDL/chylomicron cholesterol concentrations should be avoided on postprandial samples.

The raw and corrected sequential ultracentrifugation data have been presented because both sets of values are inherently subject to error. The quantity of cholesterol recovered from the various lipoprotein fractions following sequential ultracentrifugation was approximately 15% less than the quantity of cholesterol determined by assay of the original sample. This 'loss' of cholesterol is recognised as a shortcoming of lipoprotein preparation following extensive ultracentrifugation (Janado *et al*, 1966; Cohn *et al*, 1988) and consequently, the raw data may underestimate the actual cholesterol concentration in each of the lipoprotein fractions. By correcting for recovery however, it is assumed that the cholesterol loss following sequential ultracentrifugation is the same in each lipoprotein fraction. This may not be the case and for some classes of lipoprotein, correction may have led to overestimation of the actual cholesterol concentration.

The density range of porcine LDL_2 has been shown to overlap the lower density limit of porcine HDL (Janado *et al*, 1966). This has resulted in the employment of differential flotation to separate LDL_2 from HDL (Janado *et al*, 1966). In the current study, contamination of the LDL layer with HDL (and vice versa) was not detected by electrophoresis. The second layer that was harvested following ultracentrifugation at a density of 1.063 kg/L might have contained the LDL_2 subfraction, thereby preventing HDL contamination.

Feeding significantly reduced the serum total cholesterol and LDL-C concentrations but increased the HDL-C concentration. This is in contrast to the findings of Luhman *et al* (1992) who found that feeding had no effect on these parameters in pigs. In human beings, the postprandial HDL cholesteryl ester concentration appears to be inversely related to the postprandial rise in plasma triglyceride concentrations (Tall, 1986). Despite an increase in LCAT activity within HDL postprandially, the presence of increased levels of substrate for CETP results in a net transfer of cholesteryl ester from HDL to triglyceride-rich lipoproteins (Rose & Juliano, 1979). In contrast to human beings, pigs lack CETP activity (Chapman, 1986; Knipping *et al*, 1987), which may explain why an increase in the postprandial HDL-C concentration was observed. Cohn *et al* (1988) found that LDL-C concentrations in human beings were significantly lower 3 and 6 hours after eating a fat-rich meal. This was associated with a decrease in the plasma concentration of apolipoprotein B and the authors hypothesised that the postprandial rate of catabolism of LDL exceeded its synthesis. Although apolipoprotein concentrations were not measured, a similar mechanism may explain the decrease in LDL-C concentrations postprandially in the pigs in this study.

In summary, this study has demonstrated that a precipitation method for determining the HDL-C concentration, and a modification of the Friedewald formula for estimating the LDL-C concentration, closely approximate values obtained from sequential ultracentrifugation in porcine serum samples. This methodology was applied in the next study in order to evaluate the effect of dietary lipids on porcine LDL-C and HDL-C concentrations. Conversely, the VLDL-C concentration, determined by multiplying the triglyceride concentration by 0.25, did not agree with concentrations determined by sequential ultracentrifugation. In order to address this issue, the same study should be repeated on large-volume or pooled blood samples. This study has also demonstrated that like human beings, porcine LDL-C concentrations decrease postprandially, but unlike human beings HDL-C concentrations increase postprandially. This latter phenomenon may be due to the lack of CETP activity in porcine blood.

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Chapter 3. Serum lipoprotein cholesterol and triglyceride concentrations in pigs fed diets containing fish oil, milkfat, olive oil and coconut oil.

3.1. Introduction

Dietary fat can have a profound effect on serum lipoprotein cholesterol and triglyceride concentrations and atheroma formation in human beings (Harris, 1989; Grundy & Denke, 1990; Gurr, 1991). This has resulted in the development of animal models to examine the effects of diet on lipoprotein metabolism and atherosclerosis (Overturf & Loose-Mitchell, 1992). The pig has been considered a suitable model for studying lipoprotein metabolism and atherosclerosis because the major lipoprotein class of both pigs and human beings is LDL and there is remarkable homology in the lipoprotein composition of the two species (Chapman & Goldstein, 1976; Chapman, 1986). Despite these similarities, there are several important differences. Firstly, there is little CETP activity in the blood of pigs (Chapman, 1986, Knipping *et al*, 1987). Secondly, there is evidence to suggest that most of the LDL apolipoprotein B of pigs is synthesised *de novo*, rather than originating from VLDL catabolism (Birchbauer *et al*, 1992). Thirdly, porcine LCAT prefers LDL-derived cholesterol rather than HDL-derived cholesterol as its substrate for cholesteryl ester formation (Knipping *et al*, 1987).

The objective of this study was to determine lipoprotein cholesterol and triglyceride concentrations of pigs fed diets containing concentrated marine fish oil, milkfat, olive oil or coconut oil at an inclusion rate of 4% w/w for the purpose of developing the pig as a model for human lipid research.

3.2. Materials and methods

Experimental animals

Thirty entire male pigs, comprising of six sets of five littermates were used in the study. The pigs, which were all of the same genotype (Large White (LW) × Landrace first-cross dams and LW × Duroc first-cross sires), were obtained from the Pig Research Unit, Massey University. All procedures involving animals in this study were conducted with the approval of, and under the guidelines established by, the Massey University Animal Ethics Committee.

Experimental diets

There were five dietary treatments with 6 pigs per treatment. The composition of the experimental diets is shown in Table 3.1. Barley was the main source of digestible energy and lactic casein and soy protein isolate were the main sources of crude protein. Sunflower seed oil was added to all diets to ensure adequate concentrations of LA, an essential fatty acid. The experimental diets were stored at room temperature and were used within 10 days of making.

Apparent digestible energy, crude protein, lysine and macro-elements in the experimental diets were calculated using a least-cost diet linear programme (Moughan, 1982), which uses a nutrient composition database compiled by staff at the Monogastric Research Centre, Massey University, Palmerston North. Adequacy of the nutrient balance was assured by comparison with the requirements for growing pigs (Braude *et al*, 1981). The experimental diets contained (per kg, as fed): 230 g crude protein, 14.2 g lysine, 11 g calcium, 8 g phosphorus, 3 g sodium, 3 g potassium and 2 g chloride. The carbohydrate diet and the diets containing fat provided 12.2 and 13.1 mJ/kg apparent digestible energy (ADE) respectively. The lysine:ADE ratio was 1.1.

Table 3.1 Ingredient composition of the experimental diets.

Ingredient	Dietary Treatment				
	Carbo[1]	Fish	Milkfat	Olive	Coconut
			(g/kg, as fed)		
Barley	540.00	540.00	540.00	540.00	540.00
Lactic casein[2]	100.00	100.00	100.00	100.00	100.00
Soy protein isolate[3]	100.00	100.00	100.00	100.00	100.00
Wheat bran	97.75	97.75	97.75	97.75	97.75
Limestone	17.45	17.45	17.45	17.45	17.45
Calcium hydrogen phosphate[4]	17.43	17.43	17.43	17.43	17.43
Trisodium orthophosphate[5]	10.30	10.30	10.30	10.30	10.30
Sunflower oil[6]	10.00	10.00	10.00	10.00	10.00
Vitamin/trace element premix[7]	5.00	5.00	5.00	5.00	5.00
Salt	1.95	1 95	1.95	1.95	1.95
Endox[8]	0.13	0.13	0.13	0.13	0.13
Corn starch	100.00	60.00	60.00	60.00	60.00
Concentrated marine fish oil[9]		40.00			
Anhydrous milkfat[10]			40.00		
Extra virgin olive oil[11]				40.00	
Hydrogenated coconut oil[12]					40.00

[1] Carbohydrate diet
[2] Spec 100, Tui Milk Products, Palmerston North, New Zealand
[3] Supro 590, Columbit (New Zealand) Ltd, Auckland, New Zealand
[4] Technik Products, Auckland, New Zealand
[5] Pacific Raw Materials, Auckland, New Zealand
[6] Davis Trading Co. Ltd, Palmerston North, New Zealand
[7] Commercial pig starter vitamin and trace element premix (kindly donated by Nutritech International Ltd, Auckland, New Zealand) supplied in the finished diet (per kg): 18 000 IU vitamin A, 2 200 IU vitamin D, 50 mg vitamin E, 2.5 mg vitamin K, 1.5 mg vitamin B1, 4 mg vitamin B2, 3 mg vitamin B6, 0.03 mg vitamin B12, 0.5 mg folic acid, 12 mg pantothenic acid, 0.075 mg biotin, 22 mg niacin, 150 mg choline, 20 mg vitamin C, 0.5 mg cobalt, 1 mg iodine, 125 mg copper, 100 mg iron, 45 mg manganese, 120 mg zinc and 0.3 mg selenium.
[8] Technik products, Auckland, New Zealand
[9] Scotia Pharmaceuticals Ltd, Carlisle, Scotland. The concentrated marine fish oil contains 30% v/v eicosapentaenoic acid (20:5, n-3) and 20% docosahexaenoic acid (22:6, n-3) with a total long chain omega-3 content of 60%.
[10] New Zealand Dairy Board, Wellington, New Zealand
[11] Salat brand, Davis Trading Co. Ltd, Palmerston North, New Zealand
[12] Confectionery fat 92, Abels Ltd, Auckland, New Zealand

No attempt was made to ensure that each diet contained the same level of cholesterol or antioxidants since the objective of the study was to determine the effects of dietary fats, rather than the effects of individual fatty acids, on lipid metabolism in pigs.

Experimental procedure

At one week of age, pigs from each litter were randomly assigned to one of the five dietary groups. The piglets were then housed together and fed identical diets (commercial weaner diet) until the start of the trial at 8 weeks of age. Seventy-two hours prior to the study, each group of pigs was transferred to a separate pen within the test-house to ensure that the pigs had sufficient time to become accustomed to individual feeding.

The test diets were fed for 21 days. Each pig was given 10% of their metabolic bodyweight ($kg^{0.75}$) daily and this quantity was recalculated weekly. Approximately half of their daily ration was offered at 0900 hours and the remainder at 1600 hours. If food was refused at the morning meal it was offered again with the afternoon meal. The quantity of food remaining at the end of each day was recorded. Water was available *ad libitum* from drinking nozzles situated in the races.

Blood samples (10 mL) were obtained from the right brachiocephalic or jugular vein on the first and the last day of the study. All pigs had been fasted overnight prior to blood sampling.

To aid restraint for bleeding, the pigs were anaesthetised with a combination of two agents: xylazine hydrochloride (various suppliers) and Zoletil 100® (Virbac Laboratories, France), which contains zolazepam hydrochloride and tiletamine hydrochloride in equal parts. The Zoletil 100® and xylazine hydrochloride were mixed in the same syringe and administered at dose rates of 4.0 mg/kg and 2.2 mg/kg respectively by deep intramuscular injection into the neck. The pigs were allowed to recover from anaesthesia in a deep bed of straw.

Analytical methods

The blood was allowed to clot and then immediately centrifuged at 1600 × g for 10 min. The serum was collected and stored at 4°C until the cholesterol and triglyceride concentrations could be determined. Analysis was performed within 7 days of blood collection in batches according to litter.

Serum cholesterol concentrations were determined using the enzymatic method of Boehringer Mannheim using cholesterol esterase, cholesterol oxidase and peroxidase. Serum triglyceride concentrations were determined using the enzymatic method of Boehringer Mannheim using lipase, glycerol kinase, glycerol phosphate and peroxidase. Low density lipoprotein and HDL-C concentrations were determined using the modified Friedewald formula described in Chapter 2.

Statistical analysis

Analysis of variance was used to compare the effect of diet on each variable. The concentration of each variable at the beginning of the study was used as a covariate and the statistical model considered blocking effects for each litter of pigs. The correlations between the mean serum total cholesterol concentration and both the mean serum HDL-C and LDL-C concentrations across all groups were determined.

3.3. Results

There were no food refusals by any pigs during the study. The weight gain of the pigs in the carbohydrate group was less than that of the milkfat and coconut oil groups (Table 3.2).

Table 3.2 Mean weight gain (in kilograms) of each group of pigs during the 21-day study.

Variable	Dietary Treatment					LSD[2]	DF[3]
	Carbo[1]	Fish	Milk	Olive	Coconut		
Weight gain	10.52^a	12.12^{ab}	12.98^b	12.17^{ab}	12.50^b	1.8	19

[1] Carbohydrate diet
[2] Least significant differences of the means at the 5% level.
[3] Degrees of freedom.
Differing letter superscripts denote significant differences between groups at the 5% level.

The mean serum lipid concentrations of the pigs on day 21 are shown in Table 3.3. The total cholesterol concentration was significantly higher in the group of pigs receiving coconut oil than the pigs receiving either carbohydrate or fish oil. Serum triglyceride concentrations were significantly higher in the milkfat and coconut oil groups than in the other groups. The coconut oil, milkfat and olive oil groups had significantly higher serum concentrations of HDL-C than the fish oil and carbohydrate groups. There were no significant differences between groups in LDL-C concentration at day 21. The LDL-C:HDL-C ratio was significantly higher in the pigs receiving fish oil compared to all other groups. The LDL-C:HDL-C ratio was significantly higher in the pigs receiving the carbohydrate diet compared to the pigs receiving the milkfat, coconut oil and olive oil diets.

Table 3.3 Mean serum concentrations of lipids at day 21.

Variable	Dietary Treatment					LSD[2]	DF[3]
	Carbo[1]	Fish	Milk	Olive	Coconut		
T. chol[4]	1.95^a	1.98^a	2.19^{ab}	2.13^{ab}	2.34^b	0.25	19
TG[5]	0.29^a	0.28^a	0.40^b	0.33^a	0.47^b	0.07	24
HDL-C[6]	0.69^a	0.61^a	0.93^b	0.93^b	1.00^b	0.09	19
LDL-C[7]	1.13^a	1.29^a	1.15^a	1.14^a	1.22^a	0.19	20
LDL-C:HDL-C[8]	1.67^b	2.05^a	1.14^c	1.15^c	1.12^c	0.24	20

[1] Carbohydrate diet
[2] Least significant differences of the means at the 5% level.
[3] Degrees of freedom.
[4] Total serum cholesterol concentration (mmol/L).
[5] Total serum triglyceride concentration (mmol/L).
[6] High density lipoprotein cholesterol concentration (mmol/L).
[7] Low density lipoprotein cholesterol concentration (mmol/L).
[8] Ratio of LDL cholesterol concentration to HDL cholesterol concentration.
Differing letter superscripts denote significant differences between groups at the 5% level.

There was a moderate correlation between the serum total and HDL-C concentrations ($r^2=0.59$, $p=0.0001$) and a weak correlation between the serum total and LDL-C concentrations ($r^2=0.32$, $p=0.0012$) at the end of the study.

3.4. Discussion

This study has demonstrated that dietary fats that have a hypercholesterolaemic effect in pigs raise serum HDL-C concentrations rather than serum LDL-C concentrations. This finding contrasted with responses in human beings where changes in the total serum cholesterol concentration, associated with substituting one type of dietary fat (or carbohydrate) with another, are predicted to be strongly associated with changes in serum LDL-C concentration (Katan *et al*, 1994). Furthermore, substituting saturated fatty acids with carbohydrate, monounsaturated fatty acids and polyunsaturated fatty acids is predicted to result in a decrease in serum total and LDL-C concentrations (Zanni *et al*, 1987; Katan *et al*, 1994). In humans, this is believed to be partly due to the effect of ACAT, a key hepatic enzyme in cholesterol metabolism. This enzyme esterifies free cholesterol to cholesteryl ester within the cytoplasm of hepatocytes but it prefers unsaturated fatty acids, rather than saturated fatty acids, as the substrate for esterification (Levy, 1995). Diets high in unsaturated fats result in increased ACAT activity and a decrease in the hepatic pool of free cholesterol (Levy, 1995). Low levels of free cytosolic cholesterol increases the transcription of the LDL receptor gene. The net result is an increase in the number of hepatic LDL receptors and a concomitant decrease in the serum LDL concentration (Goldstein & Brown, 1990). Why there was no effect of diet on the serum LDL-C concentration in the present study is open to speculation. A possible explanation is that the ACAT of pigs is less specific and can use a wider range of fatty acids than the human counterpart. Alternatively, the regulation of porcine LDL receptor transcription may be less dependent upon hepatocellular free cholesterol concentrations.

Differences in the metabolism of LDL between pigs and human beings may also have a bearing on the lack of association between the serum total and LDL-C concentrations. Firstly, porcine plasma lacks CETP activity and consequently, very little HDL cholesteryl ester is transferred to the LDL fraction (Knipping *et al*, 1987). Secondly, unlike humans, only a small percentage (11%) of VLDL apolipoprotein B in pigs is converted into LDL and there is evidence to suggest that much of the cholesteryl ester within porcine LDL is synthesised *de novo* by the activity of LCAT (Knipping *et al*, 1987; Birchbauer *et al*, 1992). However, if this was the major determinant of the LDL-C concentration in pigs, one would have predicted a lower serum LDL-C concentration in the pigs fed fish oil because n-3 fatty acids are poor substrates for the LCAT enzyme (Kane, 1996). It is possible that the decreased activity of LCAT in pigs fed fish oil may have been counteracted by an increase in the conversion of VLDL to LDL and a lower fractional catabolic rate of LDL (Huff & Telford, 1989; Huff *et al*, 1993).

Another possible explanation for the lack of a dietary effect on serum LDL concentration is that the concentration of dietary fat, and/or the length of time the diets were fed, was not sufficient to induce a difference in LDL-C concentrations. This is unlikely given that significant changes were induced in the other lipid fractions.

In previous studies, there has been no consensus on the effects of dietary fat on the serum LDL-C concentration in pigs. Faidley *et al* (1990) reported no significant differences in the serum LDL-C concentration when pigs were fed for 4 weeks on diets containing tallow, soybean oil, or a combination thereof. Conversely, Walsh Hentges *et al* (1985) found that feeding beef tallow (17% DM) for four weeks significantly lowered serum LDL-C and raised HDL-C concentrations when compared to a soybean diet. In both of these studies, cholesterol was added to ensure each diet had an equivalent cholesterol concentration. Groot *et al* (1989) found that pigs fed a diet high in n-3 fatty acids (21% energy mackerel oil) for 8 weeks had significantly lower total, LDL, HDL and VLDL-C concentrations than pigs receiving lard. It should be noted that in the first two studies, sequential ultracentrifugation was used to separate the lipoprotein fractions. Since the density range of porcine LDL_2 overlaps with that of HDL, it is possible that LDL_2 could have been lost to the HDL fraction using this technique (Janado *et al*, 1966; Chapman 1986). No attempts were made to isolate apolipoprotein B-containing lipoproteins in the HDL fraction and this is a potential source of error in these studies.

The total cholesterol and HDL-C concentrations were significantly higher in pigs fed the diet containing coconut oil compared to those receiving either carbohydrate or fish oil. This was not surprising, as previous studies in animals (Carlson & Kottke, 1990) and human beings (Fisher *et al*, 1983; Mattson & Grundy, 1985; Zanni *et al*, 1994) have shown that total cholesterol concentrations rise when saturated fat (especially coconut oil) replaces monounsaturated fat, polyunsaturated fat or carbohydrate. Furthermore, substituting carbohydrate or n-6 polyunsaturated fat with saturated fat results in an increase in the concentration of HDL-C in man and animals (Fisher *et al*, 1983; Chong *et al*, 1987; Quig & Zilversmit, 1989; Carlson & Kottke, 1990; Katan *et al*, 1994). Coconut oil has been shown to raise HDL-C concentrations more profoundly than other sources of saturated fat (Quig & Zilversmit, 1989; Carlson & Kottke, 1990). Trilaurin appears to be the triglyceride in coconut oil responsible for this effect (Carlson & Kottke, 1990). In the present study, the lack of difference in the total cholesterol concentration between the groups receiving coconut oil and olive oil is unable to be explained.

The quantity of cholesterol within HDL depends on the activity of LCAT, which in turn depends on the composition of phosphatidylcholine within HDL (Pownall *et al*, 1985; Jonas *et al*, 1987; Subbaiah & Monshizadegan, 1988; Parks *et al*, 1989; Thornburg *et al*, 1995). Phosphatidylcholine donates a fatty acid during the esterification of cholesterol to cholesteryl ester, a reaction catalysed by LCAT. This enzyme preferentially uses either oleic acid or linoleic acid at the sn-2 position of HDL phosphatidylcholine (Subbaiah & Monshizadegan, 1988; Thornburg *et al*, 1995). Thornburg *et al* (1995) found that monkeys receiving diets enriched in n-3 fatty acids have HDL phosphatidylcholine with either eicosapentaenoic acid or docosahexaenoic acid at the sn-2 position. This is a poor substrate for LCAT and consequently, the HDL-C concentration is lower in these animals than in those receiving a diet enriched with monounsaturated or saturated fatty acids (Kane, 1996). The HDL of monkeys fed diets enriched in n-6 polyunsaturated fatty acids also had lower HDL-C concentrations than monkeys fed diets supplemented with saturated fat (Thornburg *et al*, 1995). In this instance however, it is not due to the position of fatty acids within the phosphatidylcholine. These authors observed that when n-6 polyunsaturated fatty acids are fed, there is a lower HDL phospholipid concentration and they speculated that this reduces the quantity of substrate for LCAT activity. It was therefore not surprising that in the current study, the pigs fed fish oil had a lower HDL-C concentration than the pigs receiving milkfat, olive or coconut oil.

The pigs receiving fish oil had a significantly lower serum triglyceride concentration than those receiving either milkfat or coconut oil. This finding is in agreement with previous studies, which have shown that n-3 fatty acids lower the triglyceride and plasma VLDL apolipoprotein B concentrations in animals and man (Herold & Kinsella, 1986; Groot *et al*, 1989; Harris, 1989; Huff & Telford, 1989; Katan *et al*, 1994). Huff and Telford (1989) have demonstrated that the fractional catabolic rate of VLDL in miniature pigs, whose diets had been supplemented with fish oil, was almost twice that of pigs fed diets supplemented with corn oil. The same study showed that fish oil supplementation has a striking effect on the source of LDL apolipoprotein B. Low density lipoprotein apolipoprotein B derived from VLDL catabolism was increased but the *de novo* synthesis of LDL apolipoprotein B was reduced; the net result was a reduction in LDL apolipoprotein B synthesis. However, the fractional catabolic rate of LDL was reduced by fish oil and this was later shown to be due to a significant decrease in LDL receptor activity (Huff *et al*, 1993). Overall, the pool size of LDL apolipoprotein B decreased due to the decrease in synthesis being greater than the decrease in catabolism. Despite the reduction in LDL apolipoprotein B, Huff and Telford did not find a significant difference in the LDL-C concentrations between the fish oil and corn oil groups (Huff & Telford, 1989). Similarly, no significant difference in the LDL-C concentration was observed across treatment groups in the current study.

It was surprising to note that in this study, the carbohydrate and olive oil groups had a lower serum triglyceride concentration than the milkfat and coconut oil groups. In a meta-analysis of 27 controlled dietary trials, Mensink and Katan (1992) predicted that replacement of carbohydrate with fat would cause a reduction in the serum triglyceride concentration in healthy human beings. It should be noted however, that this effect was predicted to occur under isocaloric conditions. Although the caloric density of the diets containing fat and carbohydrate were similar in the current study (13.1 mJ/kg versus 12.2 mJ/kg respectively), they were not isocaloric. Katan *et al* (1994) also predicted that in human beings, the replacement of saturated fat with monounsaturated fat would result in a slight increase in the serum triglyceride concentration. In the current study however, pigs fed the oleic acid-enriched diet (olive oil) had a significantly lower mean serum triglyceride concentration than those fed diets containing more saturated fat (hydrogenated coconut oil and milkfat).

An increased ratio of serum LDL-C to HDL-C has been shown in experimental and epidemiological studies to be associated with an increased risk for atherosclerosis (Norday & Goodnight, 1990; Luria *et al*, 1991; Stampfer *et al*, 1991; Anon, 1993). Rudel *et al* (1995) however, found that African green monkeys receiving monounsaturated and saturated fat diets had significantly more coronary artery atherosclerosis than those that received a diet enriched in polyunsaturated fatty acids, despite the monounsaturated fat fed group having the lowest LDL-C to HDL-C ratio. These workers speculated that the beneficial effect of the low LDL-C to HDL-C ratio in this group was offset by deleterious changes in LDL composition and particle size. In the current study, the LDL-C to HDL-C ratio paralleled the serum HDL-C concentration, with the exception that the fish oil-fed pigs had a higher ratio than the carbohydrate-fed group. This may indicate that in pigs, the fish oil-supplemented diet is the most atherogenic, although this does not take into account other determinants of atherogenicity, such as rheological factors, platelet reactivity or those factors outlined above.

In summary, fish oil behaved like carbohydrate with respect to its effect on serum lipids in pigs. Furthermore, based on the LDL-C to HDL-C ratio, this study has shown that fish oil-enriched diets may be more atherogenic than the diets supplemented with coconut oil, milkfat and olive oil in pigs. Unlike human beings, this study has shown that changes in the total cholesterol concentration in pigs are more likely to reflect changes in the serum HDL concentrations than in the serum LDL concentration. This may be due to differences in lipoprotein metabolism between the two species although there are other possible explanations. The pigs used in this study were in a rapid growth phase, which may prevent extrapolation to adult human beings. It is also noteworthy that the serum lipid concentrations of the pigs in this study were much lower than those found in middle-aged North Americans (Stein & Myers, 1994). This may reflect the relatively low fat content of the diets used in this study. It may be useful to repeat this study with a diet that more closely mimics the average western diet. In the meantime, caution is warranted when making inferences about human lipoprotein metabolism from porcine studies.

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Chapter 4. Evaluation of a balloon angioplasty model of arterial injury in pigs for the assessment of the thrombogenicity of dietary fats.

4.1. Introduction

The effects of drugs and diet on thrombotic potential have been evaluated using a wide range of laboratory techniques. These techniques broadly fall into three categories. Firstly, there are assays of platelet function, some of which have been described in Chapter 1. Secondly, there are assays for estimating the activity of components of the coagulation and fibrinolytic systems. Thirdly, the antithrombotic properties of the vessel wall have been evaluated by studying concentrations of eicosanoids, nitrovasodilators and ecto-nucleotidases (Marcus *et al*, 1995). Of these, the first two categories have been the focus of most attention.

Tracy describes four major types of assay used to evaluate the activity of the coagulation and fibrinolytic systems: clot-based and clot lysis-based assays and type 1, 2 and 3 assays (Tracy, 1997). Examples of clot-based and clot lysis-based screening assays included the activated partial thromboplastin time and euglobulin clot lysis time respectively. These assays are integrative and cover entire systems. They are easy to perform but they are insensitive and do not provide information on specific factors. Type 1 assays determine the circulating concentrations of individual zymogens or their inhibitors. These can be clot-based, immunological or chromogenic assays. Although reproducible, sensitive and easy to perform, these assays do not necessarily reflect enzyme activity *in vivo*. In contrast, type 2 assays are immunoassays that measure the concentration of molecules that form as a result of *in vivo* enzyme activity. Examples include assays for thrombin-antithrombin III complex, which estimates the concentration of thrombin that has formed *in vivo*, and fibrinopeptide A, which estimates the *in vivo* activity of thrombin (Tracy, 1997). Type 3 assays directly measure the concentration of active enzymes in circulation. They are usually chromogenic or immunological-based assays, or both. Examples include assays for activated protein C and activated factor VII (Tracy, 1997).

Despite the ever-increasing level of sophistication for evaluating markers of thrombosis/fibrinolysis and recent technological advances for assessing platelet and vascular reactivity, these assays cannot replace *in vivo* thrombus formation as the end-point for evaluating thrombotic potential. They do not take into account the complex interplay between blood components, the vessel wall (and its disease status) and rheological factors, all of which contribute to thrombus formation. At best, assays for markers of coagulation and fibrinolysis and *in vitro* methods for evaluating platelet and vascular reactivity help provide an explanation for why some diets are likely to be more pro-thrombotic than others.

In Chapter 1, a number of important *in vivo* sub-primate models of arterial thrombosis and thrombolysis were discussed. The large number of models and animal species that have been used to evaluate the thrombus end-point is testament to the fact that there is no perfect technique. In the pilot study presented in this chapter, a balloon angioplasty model of arterial injury in pigs for assessing the thrombogenicity of dietary lipids was evaluated.

4.2. Materials and methods

Experimental animals

Twenty-four entire male pigs, comprising eight sets of three littermates, were used. The pigs were all of the same genotype (Large White (LW) x Landrace first-cross dams and LW sires) and were obtained from the Pig Research Unit, Massey University. All procedures involving animals in this study were conducted with the approval of, and under the guidelines established by, the Massey University Animal Ethics Committee.

Experimental diets

There were three dietary treatments with eight pigs per treatment. The composition of the diets is shown in Table 4.1. Barley was the main source of digestible energy and lactic casein and soy protein isolate were the main sources of crude protein. Sunflower seed oil was added to all diets to ensure adequate concentrations of LA, an essential fatty acid. The experimental diets were stored at room temperature and were used within 10 days of preparation.

Apparent digestible energy, crude protein, lysine and macro-elements in the experimental diets were calculated using a least-cost diet linear programme (Moughan, 1982), which used a nutrient composition database compiled by staff at the Monogastric Research Centre, Massey University, Palmerston North. Adequacy was assured by comparison with the requirements for growing pigs (Braude *et al*, 1981). The experimental diets contained (g/kg): 239 crude protein, 14.2 lysine, 11.2 calcium, 7.9 phosphorus, 3.8 sodium and 3.6 potassium. The carbohydrate, milkfat and fish oil diets provided 12.2, 14.1 and 14.3 mJ/kg ADE respectively. The lipid content of the carbohydrate diet was approximately 2.5% (w/w) whereas the milkfat and fish oil diets contained approximately 12% (w/w). No attempt was made to ensure that each diet contained the same level of cholesterol or antioxidants.

Shortly after starting the study, it became apparent that the diet containing the concentrated marine fish oil was unpalatable and this group was removed from the study. The fatty acid compositions of the milkfat and carbohydrate diets are shown in Table 4.2.

Table 4.1 Ingredient composition of the experimental diets.

Ingredient	Dietary Treatment		
	Carbo[1]	Fish	Milkfat
	(g/kg, as fed)		
Barley	540.00	540.00	540.00
Lactic casein[2]	100.00	100.00	100.00
Soy protein isolate[3]	100.00	100.00	100.00
Wheat bran	100.00	100.00	100.00
Limestone	17.45	17.45	17.45
Calcium hydrogen phosphate[4]	17.43	17.43	17.43
Trisodium orthophosphate[5]	10.29	10.29	10.29
Sunflower oil[6]	10.00	10.00	10.00
Vitamin/trace element premix[7]	2.75	2.75	2.75
Salt	1.95	1.95	1.95
Endox[8]	0.13	0.13	0.13
Corn starch	100.00		
Concentrated marine fish oil[9]		100.00	
Anhydrous milkfat[10]			100.00

[1] Carbohydrate diet
[2] Spec 100, Tui Milk Products, Palmerston North, New Zealand
[3] Supro 590, Columbit (New Zealand) Ltd, Auckland, New Zealand
[4] Technik Products, Auckland, New Zealand
[5] Pacific Raw Materials, Auckland, New Zealand
[6] Davis Trading Co. Ltd, Palmerston North, New Zealand
[7] The vitamin and trace element premix was prepared for the study by NRM New Zealand Ltd, Auckland, New Zealand. It supplied in the finished diet (per kg): 8 000 IU vitamin A, 1 500 IU vitamin D_3, 100 mg vitamin E, 1 mg vitamin K, 10 mg pantothenic acid, 3 mg riboflavin, 15 mg niacin, 1 mg pyridoxine, 1 mg thiamine, 15 : g vitamin B_{12}, 50 : g biotin, 2 mg folic acid, 200 mg choline, 100 mg vitamin C, 0.5 mg cobalt, 1 mg iodine, 10 mg copper, 80 mg iron, 60 mg manganese, 100 mg zinc and 0.2 mg selenium, 125 mg ethoxyquin, 8 mg sodium fluoride, 195 mg inositol, 20 mg para-aminobenzoic acid.
[8] Technik products, Auckland, New Zealand
[9] Scotia Pharmaceuticals Ltd, Carlisle, Scotland. The concentrated marine fish oil contains 30% v/v eicosapentaenoic acid (20:5, n-3) and 20% docosahexaenoic acid (22:6, n-3) with a total long chain omega-3 content of 60%.
[10] New Zealand Dairy Board, Wellington, New Zealand

Table 4.2 Fatty acid composition of the carbohydrate and milkfat diets.

Fatty acid	Dietary Treatment	
	Carbo[1]	Milkfat
	(g/kg, total fatty acids)	
12:0	0.1	2.6
13:0	-[2]	0.1
14:0	0.5	10.1
15:0	0.1	1.2
16:0	14.7	30.6
17:0	trace[3]	0.6
18:0	3.0	12.2
19:0	0.1	0.7
20:0	0.1	0.2
22:0	0.5	-
Total saturated	19.0	58.3
14:1	-	1.0
15:1	-	0.3
16:1	0.1	1.1
17:1	-	0.1
18:1 n-9	19.4	21.6
18:2 n-6	56.6	13.7
18:3 n-3	3.5	1.5
20:1	0.4	-
20:2	0.6	0.7
20:3	-	0.4
Total unsaturated	80.6	40.4

[1] Carbohydrate diet
[2] Not detected
[3] <0.1 g/kg

Experimental procedure

At 3 to 4 weeks of age, pigs from each litter were randomly assigned to one of the three dietary groups. From eight days of age to approximately five weeks of age, the pigs were fed a commercial pre-weaner ration and from five weeks of age to the start of the trial, the pigs were fed a commercial weaner ration *ad libitum*.

From birth to four weeks of age, the piglets were housed with their dam in standard farrowing crates. From 4 to 7½ weeks of age the pigs were housed on flat decks in a commercial weaner house. During the trial, the pigs were housed in a test house at the Pig Research Centre, Massey University, Palmerston North. The test house has four concrete pens either side of a central walkway. Each pen has a central race (3.3 x 1.5 m) that is accessible to a sleeping enclosure (3.3 x 1.4 m) and eight feeding stalls (0.4 x 1.4 m), which allowed for individual feeding of the pigs. Heating was provided by overhead infrared lamps in each of the sleeping enclosures.

Each set of siblings was randomly assigned to one pen in the test house. They were introduced to the test house three days before the start of the trial to allow sufficient time for them to become accustomed to the new environment.

The test diet was fed for 12 weeks. The quantity of food offered each day, in kilograms, was calculated by multiplying the metabolic bodyweight ($kg^{0.75}$) by 10%. Quantities were readjusted weekly. The pigs were fed half their daily allowance at approximately 0830 hours and half at approximately 1600 hours. If food was refused following the morning meal, it was offered again with the afternoon meal. The quantity of food remaining at the end of the day was recorded. Water was also continuously available from drinking nozzles situated in the races.

Fatty acid composition of platelet phospholipids

Forty-five millilitres of blood was drawn into a 50-mL syringe (Monoject, St Louis, USA) containing 5 mL of 0.11 mmol/L trisodium citrate (ratio of 1 part trisodium citrate to 9 parts blood) from each pig at the beginning of the study and after 1, 2 4, 6, 8 10 and 12 weeks on the diets. Immediately after collection, the syringe was gently inverted several times to ensure thorough mixing of the blood with the trisodium citrate. The blood was then transferred to conical flasks (Falcon 2070, Becton Dickinson, NJ, USA). Within 90 min of collection, the sample was centrifuged at 200 × g for 10 min at ambient temperature (3360 rotor, Omnifuge 2.0 RS, Heraeus Sepatech GmbH, Osterode, Germany) to prepare PRP. The PRP was centrifuged at 500 × g for 2 min to separate platelets from any remaining red blood cells. The purified PRP was then centrifuged at 2000 × g for 10 min at ambient temperature. The platelet pellet was washed three times in 20 mmol/L tris(hydroxymethyl)aminomethane (tris) buffer at pH 7.4 containing 138 mmol/L NaCl and 1 mmol/L sodium EDTA (Abeywardena *et al*, 1987), resuspended in 1.0 mL of 0.1 M KCl and transferred to 15 mL tubes. Chloroform/methanol (8.0 ml; 2:1, v/v) was added and the lipid within the sample was extracted (Folch *et al*, 1957). The lipid extract was then stored at –87°C until the platelet phospholipid fatty acid composition was determined.

At the time of the analysis, samples from each pig were pooled according to their treatment group and the time of blood collection. Thin layer chromatography was used to check for the presence of phospholipids in the lipid extracts and the purity of the lipid extracts. The solvent system used was hexane/diethyl ether/acetic acid (85/15/1, v/v/v) (Body & Newman, 1989). Triglycerides in the lipid extracts were transesterified by incubation with 1 mL of 14% BF_3 in methanol for 60 min at 100°C (Morrison & Smith, 1964). After cooling, water (2.0 mL) and hexane (2.0 mL) were added and the fatty acid methyl esters (FAMEs) and cholesterol were extracted into hexane. Thin layer chromatography was used to ensure that transesterification was successful. Fatty acid methyl esters were separated from cholesterol by silicic acid column chromatography using a glass column (140 mm × 10 mm ID) of BioSilA 100-200 mesh (3 g), activated at 80°C for 4 hours (Body & Shortland, 1974). The sample was loaded in 0.5 mL of chloroform and the FAMEs were eluted with the first 10 mL of chloroform that passed through the column. Fatty acid methyl esters were analysed by gas-liquid chromatography in a Hewlett-Packard Gas Chromatograph (Model 5890A, Palo Alto, USA) using a stainless steel column (25 mm × 0.22 mm ID) packed with BPX70 and helium carrier gas, programmed over 140-210°C at 2%/min.

Fatty acid composition of the experimental diets

Samples of the diet (4.0 g) were taken from two batches of each diet made on different dates and stored at --87°C until analysed at the end of the study. Each sample of diet was divided into 2-gm portions and total lipid was extracted using chloroform/methanol (40 mL; 2/1, v/v) and 0.1 M KCl (8 mL) (Folch *et al*, 1957).

Triglycerides in the lipid extracts of the diets were then transesterified by incubation with 14% BF_3 in methanol (2.0 mL) and benzene (0.5 mL) at 100°C for 90 min (Morrison & Smith, 1964). After cooling, water (4.0 mL) and hexane (4.0 mL) were added and the FAMEs extracted into hexane and analysed by gas-liquid chromatography as for the platelet phospholipids. The fatty acid composition of both samples were similar and the average values are shown in Table 4.2.

Preparation of radiolabelled platelets

Reagents

The anticoagulant, acid-citrate-dextrose (ACD), was prepared by dissolving 4.0 g of anhydrous citric acid, 12.78 g of trisodium citrate dihydrate and 6.0 g dextrose in 500 ml of distilled water. The pH was ca.5.0 and osmolarity was ca.300 mOsm/L (Dewanjee *et al*, 1981). Acid-citrate-dextrose saline was prepared by mixing 36 mL of ACD solution with 250 mL of isotonic saline and the pH was adjusted to 6.5 by titration with 1.0 M NaOH (Dewanjee *et al*, 1981). Sodium pertechnetate was produced no more than 12 hours prior to platelet radiolabelling in a 99-Molybdenum/99m-Technetium (^{99m}Tc) generator (Techne Lite, The Du Pont Merck Pharmaceutical Co., Massachusetts, USA).

Method

Autologous platelets labelled with ^{99m}Tc exametazine were prepared based on the methods of Danpure and Osman (1988) and Becker *et al* (1987,1988). On the morning of the last day of the study, the pigs were transported to a pen in the large animal hospital of the Institute of Veterinary, Animal and Biomedical Sciences, Massey University. They were anaesthetised and blood was collected using techniques described in Chapter 3. Forty-three millilitres of blood was collected into 7 mL of acid-citrate-dextrose.

Within half an hour of collection, each blood sample was centrifuged at 180 × g for 10 min at ambient temperature (3360 rotor, Omnifuge 2.0 RS, Heraeus Sepatech

GmbH, Osterode, Germany) to prepare PRP. The supernatant was transferred to another tube and centrifuged at 1000 × g at ambient temperature for 10 min to separate the platelets from the PPP. The PPP was subsequently used to resuspend the radiolabelled platelets. The platelet pellet was resuspended in 5 mL of ACD-saline and 200 MBq of the radiolabel, ^{99m}Tc exametazine, was added. The ^{99m}Tc exametazime was prepared by adding approximately 1.0 GBq of sodium pertechnetate to a single-dose vial containing 0.5 mg exametazime (Ceretec, Amersham International plc, Buckinghamshire, United Kingdom). This suspension was mixed frequently and incubated at ambient temperature for 60 min to ensure maximum labelling efficiency. To remove any unbound ^{99m}Tc exametazine, the suspension was spun at 1000 × g for 10 min. The platelet pellet was resuspended in 5.5 mL of the PPP and spun at 100 × g for 5 min to remove any remaining red blood cells or platelet aggregates. Five millilitres of the supernatant was aspirated into a syringe ready for injection into the donor pig.

Angioplasty

The balloon angioplasty model used in this study was based on the description of Steele *et al* (1985) who developed a carotid artery balloon angioplasty model in pigs to evaluate the pathophysiological response of arteries to mechanical injury. This model has subsequently been used to evaluate the efficacy of various antithrombotic strategies (Heras *et al*, 1989, 1990, 1992; Lam *et al*, 1991).

In the current study, angioplasty was performed in both the left and right external iliac arteries simultaneously in each pig. One artery was randomly assigned to receive an angioplasty catheter with a 10-mm balloon while the contralateral artery was distended to 12 mm. The procedure for performing angioplasty was as follows.

Anaesthesia was induced in the pigs with three-quarters of the dose of the anaesthetic agents given in the morning to harvest blood for radiolabelling platelets. The pigs were then intubated and maintained on halothane and 100%

oxygen using a circle system with vapouriser out of circuit (Fluotec 3, Cyprane, Keighley, England). Respiration was spontaneous. The radiolabelled autologous platelets were then injected into a marginal ear vein.

The pig was tied to a stainless steel table in dorsal recumbency with the hind limbs abducted. A groove between the sartorius and gracilis muscles can be palpated over the medial aspect of the distal femur. At this site, a 10-cm skin incision was made to the level of the patella. The underlying subcutaneous fascia was dissected exposing the aforementioned muscles. The loose connective tissue between the sartorius and gracilis muscles was blunt dissected and the insertion of gracilis was dissected in a cranial to caudal direction along approximately two-thirds of its length. The gracilis muscle was then reflected caudally exposing the fascia containing the sciatic nerve and the femoral artery and vein. This fascia was carefully dissected and the origin of the saphenous artery was identified. Once exposed, ca.1 mL of 1% lignocaine hydrochloride (Lopaine, Ethical Agents Ltd, Wiri, New Zealand) was dripped over the exposed vessels to reduce the risk of vasospasm. Approximately 5 mm proximal to the bifurcation of the saphenous artery, a 19-gauge ultra-thin-wall percutaneous entry needle (William A. Cook Australia Pty Ltd, Queensland, Australia) was inserted into the lumen of the femoral artery. A 0.035-inch wire guide (Cook, Indiana, USA) was introduced through the lumen of the entry needle and advanced ca. 30 cm. The entry needle was then removed and a balloon angioplasty catheter (Accent, William A. Cook Australia Pty Ltd, Queensland, Australia) was threaded over the wire guide. The catheter was 5 french and 40 cm long and the balloon was 3 cm long and was either 10 or 12 mm in diameter. Two balloon sizes were chosen in an effort to create deep and superficial arterial injuries. Deep injury was defined by the presence of a tear through the internal elastic lamina and into the media. Superficial injury was defined by the presence of thrombus in an artery that had not sustained a tear through the internal elastic lamina. Fifteen centimetres of the catheter was inserted into the vessel in order to situate the balloon in a straight segment of the external iliac artery between the deep circumflex iliac artery cranially and the deep femoral artery caudally. The wire guide was then retracted so its distal extremity was positioned within the angioplasty catheter. This procedure was repeated on the contralateral side with the angioplasty catheter of the other balloon size. On five consecutive occasions, the balloons of both catheters were inflated simultaneously for 30 s with normal saline to a pressure of 6 atmospheres with a Leveen inflator (Medi-Tech, Boston Scientific Corporation, Boston, USA) and then completely deflated for 30 s. The catheters were withdrawn immediately afterwards and gauze swabs were applied with firm digital pressure to the entry sites to assist haemostasis.

Harvesting the vessels

Forty-five minutes after angioplasty, a midline laporotomy was performed. The abdominal viscera were reflected to the right and the abdominal aorta caudal to the renal arteries was identified. Blunt dissection of the peritoneum on either side of the aorta facilitated placement of a 30-cm long length of surgical tape over the aorta dorsally. Ten centimetres caudal to this site a second length of surgical tape was passed dorsally over the aorta in an identical fashion.

Approximately 58 min after the angioplasty, the segment of aorta suspended between the two pieces of surgical tape was pulled ventrally and a 2-cm transverse incision was made in the vessel. The common branch of a Y-shaped perfusion device, made from polyethylene tubing, was inserted into the abdominal aorta and tied in place with the surgical tape. The side branches of the perfusion device were connected to the spigots of two, 10-L containers, which were located 1 m above the level of the table upon which the pig was lying. One container contained isotonic saline and the other contained 10% neutral buffered formalin. The pigs were initially perfused by gravity with ca. 3 L of 0.9% saline, to which 0.5 mL/kg of a 1% w/v solution of Evans blue dye (BDH Laboratory Supplies, Poole, England) was added. Evans blue is a diazo dye can be used to delineate areas of increased endothelial permeability (Stehbens, 1978) The pigs were then perfused with ca.3 L of 10% neutral buffered formalin. The caudal vena cava was incised prior to perfusion to allow the free-flow of fluid through the hind limbs. Blanching and the impartment of a blue hue to the hind limbs indicated successful perfusion of the saline solution. Straightening of the hind limbs indicated successful perfusion with formalin.

Immediately after perfusion, the external iliac arteries on both sides were exposed by careful dissection and the damaged segments of both arteries were identified. At the site of angioplasty, the artery was dilated and coloured blue due to staining by the Evans blue dye. The artery was then cut ca. 2 cm either side of the stained segment and removed from the pig. The adventitia was removed and the lumen gently flushed with 0.9% saline in order to remove any remaining blood. Removing the adventitia helped to identify the limits of the damaged segment. The length of artery that had not been damaged was then trimmed off and the damaged segment was stored in a 10-mL draw sterile blood collection tube (Vacutainer, Becton Dickinson, New Jersey, USA) containing 10% buffered neutral formalin. A piece of carotid artery was also harvested to serve as an undamaged control. Control segments were trimmed so that their weight was the same as the weight of damaged arterial segments.

Quantitation of thrombus size

Radiolabel technique

A venous blood sample (6 mL) was drawn from a large bore abdominal vein at the time of the laporotomy and 3 mL was transferred to a blood collection tube containing EDTA (Vacutainer, Becton Dickinson, New Jersey, USA). The remaining 3 mL was transferred to a blood collection tube containing no anticoagulant (Vacutainer, Becton Dickinson, New Jersey, USA). The packed cell volume (PCV) and the platelet count were determined within 15 min of blood collection using an automated haematology analyser (Cobas Minos Vet, ABX Hematologie, Montpellier, France). EDTA-anticoagulated whole blood (1.0 mL) was transferred to a 10-mL draw sterile blood collection tube (Vacutainer, Becton Dickinson, New Jersey, USA). The tube containing the clotted blood was centrifuged at 2 250 × g for 10 min (11105 rotor, Centra -3C centrifuge, International Equipment Co, Massachusetts, USA) and 1 mL of serum was transferred to a second 10-mL draw sterile blood collection tube containing no anticoagulant (Vacutainer, Becton Dickinson, New Jersey, USA).

The next day, the segments of external iliac artery, the corresponding control segments and the 1-mL samples of whole blood and serum were taken to the Department of Nuclear Medicine, Palmerston North Hospital, Palmerston North, and analysed for gamma radioactivity in a well counter (mACE, EG & G Ortec, Oak Ridge, USA). Counting was always performed for 300 sec in an operating window of 108.99 - 184.93 kiloelectron volts (keV). 99m-technetium disintegrates with the emission of gamma radiation, 85.5% of which is at 141 keV, and its half-life is 6.02 hours (Anon, 1996). Background radiation was also counted from an empty 10-mL draw blood collection tube under the same conditions. Due to the short half-life of ^{99m}Tc and the fact that the samples could not be analysed simultaneously, all counts were decay-corrected to the time that the first sample was counted (Equation 4.1) (Parker *et al*, 1984).

$A_t = A_0 \cdot e^{-\lambda t}$ .... **Equation 4.1**

where A_t is the gamma radioactivity at time 't',
A_0 is the gamma-radioactivity at time 0,
λ is the transformation constant, $\log_e 2$ divided by the half-life of the isotope,
and t is the elapsed time.

Initially, a correction for unbound ^{99m}Tc in serum was made (Equation 4.2). The application of Equation 4.2 assumed that there was no unbound ^{99m}Tc in the packed red blood cell fraction and that ^{99m}Tc was not bound to blood cells other than platelets. Equation 4.2 provides an estimate of the counts per minute (cpm) associated with platelets per mL of whole blood.

$cpm_{wb} - (1 - PCV) \cdot cpm_s$ .... **Equation 4.2**

where cpm_{wb} is cpm per mL of whole blood,
and cpm_s is cpm per mL of serum.

The number of platelets per cpm can be determined from Equation 4.2 and from the automated platelet count (Equation 4.3).

$PLT \div [cpm_{wb} - (1 - PCV) \cdot cpm_s]$ .... **Equation 4.3**

where PLT is the platelet count per mL of whole blood.

Multiplying Equation 4.3 by the cpm of the arterial segment yields a platelet count per arterial segment (Equation 4.4).

$cpm_{art} \cdot PLT \div [cpm_{wb} - (1 - PCV) \cdot cpm_s]$ .... **Equation 4.4**

where cpm_{art} is the cpm per arterial segment.

Background radiation is then subtracted from all counts to yield the background-corrected number of platelets per arterial segment (Equation 4.5).

$PLT \cdot (cpm_{art} - cpm_{bgrnd}) \div [(cpm_{wb} - cpm_{bgrnd}) - (1 - PCV) \cdot (cpm_s - cpm_{bgrnd})$

$\Rightarrow PLT \cdot (cpm_{art} - cpm_{bgrnd}) \div [cpm_{wb} - cpm_s + PCV \cdot (cpm_s - cpm_{bgrnd})]$ .... **Equation 4.5**

where cpm_{bgrnd} is the background cpm in a blank tube.

Equation 4.5 was applied to the balloon-damaged arterial segment and the appropriate control segment of carotid artery. In the final calculation, the platelet count associated with the control segment was subtracted from the count associated with the balloon damaged arterial segment (Equation 4.6).

$(\text{Equation } 4.5)_{\text{Balloon-damaged segment}} - (\text{Equation } 4.5)_{\text{Control segment}}$ .... **Equation 4.6**

Morphometric technique

In the second method of quantitating thrombus size, the cross-sectional area of the thrombus was measured at 0.5-mm intervals. In order to correctly identify the terminal ends of the arterial segments, they were stained with one drop of Gill's haematoxylin. The segments were then cut in half transversally and following routine routine histological processing overnight, each half-segment was embedded in paraffin wax (Paraplast, melting point 56°C, Oxford Labware, St Louis, USA) in the following manner. Each half-segment was cut in half again and the terminal ends of each half were placed end-down in a 14-mm-deep mould. The quarter with the stained terminal end was placed in the middle of the mould whilst the other quarter was placed closest to the labelled end of the block. Next, 4-µm-thick sections were cut from the block on a rotary microtome until the first piece of artery could be identified in the section. This section was floated off in a water bath and mounted onto a microscope slide. Twenty 25-µm sections were then cut from the block and discarded and another 4-µm section was mounted on the microscope slide. These steps were repeated until no more artery was visible in the block. Following drying overnight at 60°C, the sections were stained by a modified Verhoeff's method and counterstained by a modified Martius scarlet blue trichrome method (see Appendix for staining method). With this method, elastin stained black, fibrin stained bright red, muscle and cytoplasm stained pink and erythrocytes stained bright yellow.

The sections obtained from each arterial segment were then examined by light microscopy for the presence of deep injury. This appeared histologically as a break in the normally continuous black-staining elastin fibres of the internal elastic lamina. The number of segments displaying deep injury divided by two provided an estimate of the total length of the tear in millimetres.

The cross-sectional area of the thrombus in each section was determined with the aid of image analysing software (Sigma Scan, version 1.10, Jandel Scientific, San Rafael, USA) interfaced with a binocular light microscope. Using 40x magnification, the outline of the thrombus was traced with a light pen, the pointer of which was visible when looking through the microscope. The image analysing software was calibrated at the beginning of the study using a slide micrometer and this calibration was used for all subsequent measurements. The total cross-sectional area of thrombus in each arterial segment was then determined.

Statistical analysis

Analysis of variance was used to compare the effect of dietary treatment on weight gain during the course of the study. The litter was included as a block within the statistical model. The analysis was performed using The SAS System for Windows, version 6.12 (SAS Institute Inc, Cary, USA).

The experiment was a split-plot (or nested) design with litters as blocks and pigs-within-litters as the main plots, within which the diet effect is estimated. Arteries-within-pigs form the sub-plot level from which the effect of balloon size is estimated. Despite the use of a large balloon, only five arteries could be seen with tears. Tear length was unsuitable for use as a covariate in ANOVA as a plot of the covariate against the variates did not show a linear relationship. Furthermore, there were insufficient torn arteries to assess whether the thrombus size associated with torn arteries varied with diet. Consequently, data from torn arteries were not included in further analysis of thrombus size.

The data were unbalanced because the torn arteries were not included in the analysis. Furthermore, one pig died 20 min after angioplasty and angioplasty was not successfully performed in two arteries. The data were therefore examined using the residual maximum likelihood (REML) method (Patterson & Thompson, 1971), which enables relatively unbiased estimates of treatment effects to be made for unbalanced and nested data. With this method, treatment effects were assessed using a chi-squared (Wald) test (Anon, 1993). The REML analysis was performed using Genstat 5 statistical software (Genstat 5 Committee, Oxford, England). Prior to analysis, a logarithmic transformation of the data was used to stabilize the variance.

Regression analysis was used to evaluate the level of agreement between the radiolabel method and the morphometric methods of evaluating thrombus size. Regression was performed on all data and on untorn arteries only. The regression analysis was performed using Excel 97 (Microsoft Corporation, Redmond, USA).

A P-value of <0.05 was considered statistically significant for all analyses.

4.3. Results

There was no significant difference in weight gain between the two groups of pigs over the duration of the study (P = 0.5). The mean weight gain (± SD) of the pigs receiving the milkfat and the carbohydrate diets over the course of the study were 64.1 ± 6.6 kg and 61.3 ± 10.1 kg respectively.

Fatty acid composition of platelet phospholipids

Although it was not possible to statistically compare the phospholipid fatty acid composition of the platelets between the two groups due to the pooling of samples, the fatty acid compositions were similar. Approximately 40% of the platelet phospholipid fatty acid composition consisted of saturated fatty acids. The most common saturated fatty acids within the platelet phospholipids of both dietary groups were 16:0 and 18:0, which accounted for ca.13% and ca.23% of the total phospholipid fatty acids respectively. Arachidonic acid, oleic acid and LA were the most common unsaturated fatty acids within the platelet phospholipids accounting for ca.28%, ca.17% and ca.8% of the total phospholipid fatty acids respectively. The concentrations of long-chain n-3 fatty acids and AA in the platelet phospholipids of both dietary treatments are shown in Figures 4.1 and 4.2 respectively. The effect of diet on platelet phospholipid concentrations of 5, 8, 11-eicosatrienoic acid (20:3 n-9) is shown in Figure 4.3.

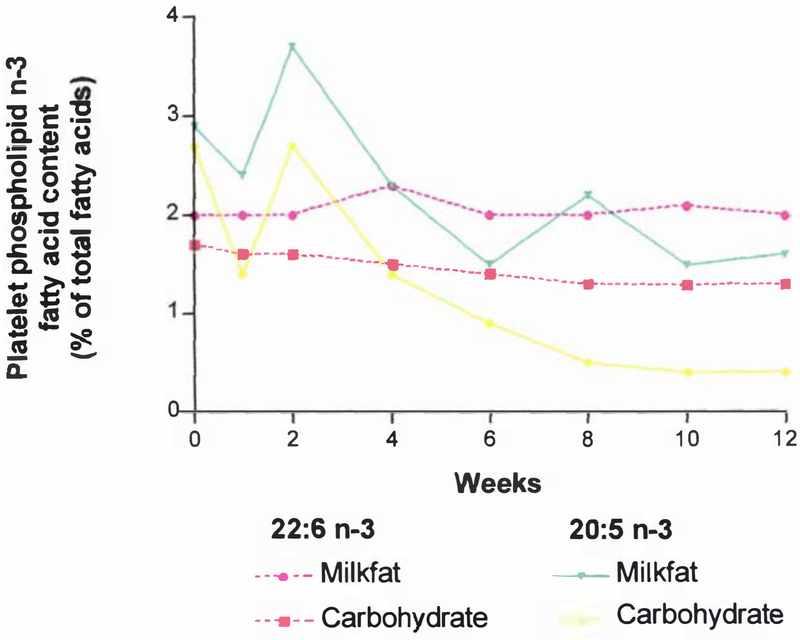

Figure 4.1 The effect of the milkfat and carbohydrate diets on the quantities of docosahexaenoic acid (22:6 n-3) and eicosapentaenoic acid (20:5 n-3) within platelet phospholipids, expressed as a percentage of the total fatty acid content within platelet phospholipids.

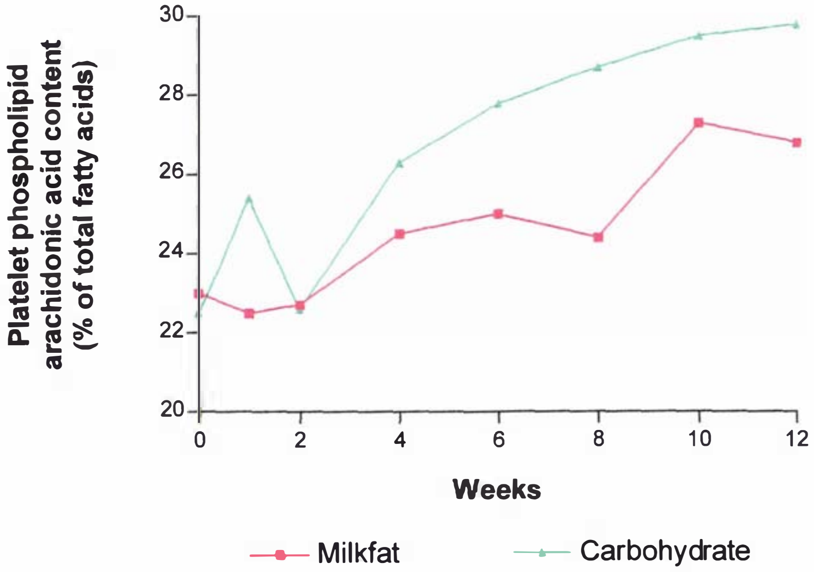

Figure 4.2 The effect of the milkfat and carbohydrate diets on the quantity of arachidonic acid within platelet phospholipids, expressed as a percentage of the total fatty acid content within platelet phospholipids.

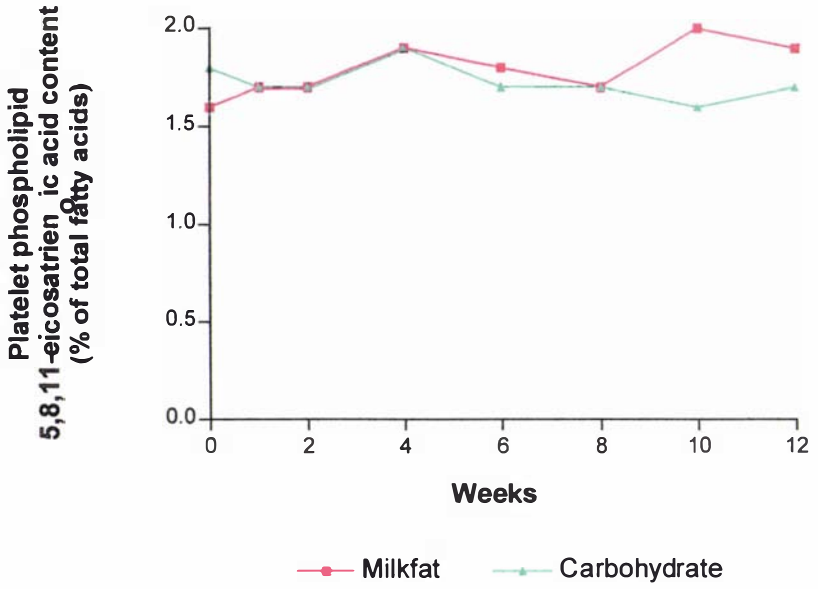

Figure 4.3 The effect of the milkfat and carbohydrate diets on the quantity of 5,8,11-eicosatrienoic acid within platelet phospholipids, expressed as a percentage of the total fatty acid content within platelet phospholipids.

Balloon angioplasty

The thrombi that formed at the site of arterial damage created by balloon angioplasty were platelet-rich (Figures 4.4 – 4.7). Five external iliac arteries, from a total of 32 that were subjected to angioplasty, sustained tears. Three pigs (one from the carbohydrate group and two from the milkfat group) sustained tears in one artery only and in one pig (from the carbohydrate group), both external iliac arteries were torn. Four torn arteries resulted from the use of the larger, 12-mm diameter, angioplasty balloon.

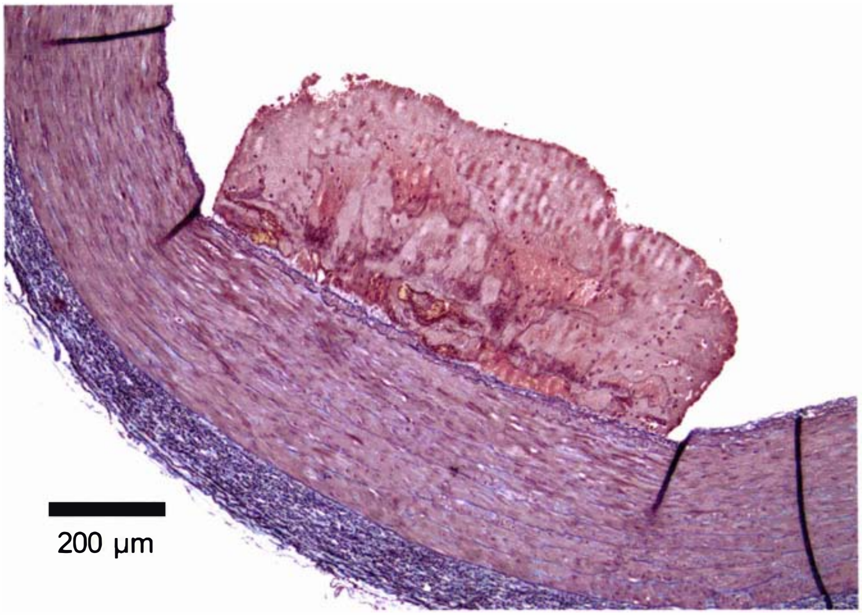

Figure 4.4 Photomicrograph of a thrombus that has formed within the external iliac artery at a site of damage created by a balloon angioplasty catheter. In this section, there is no evidence of deep injury. Stained using a modified Verhoeff's method and counterstained using a modified Martius blue trichrome method.

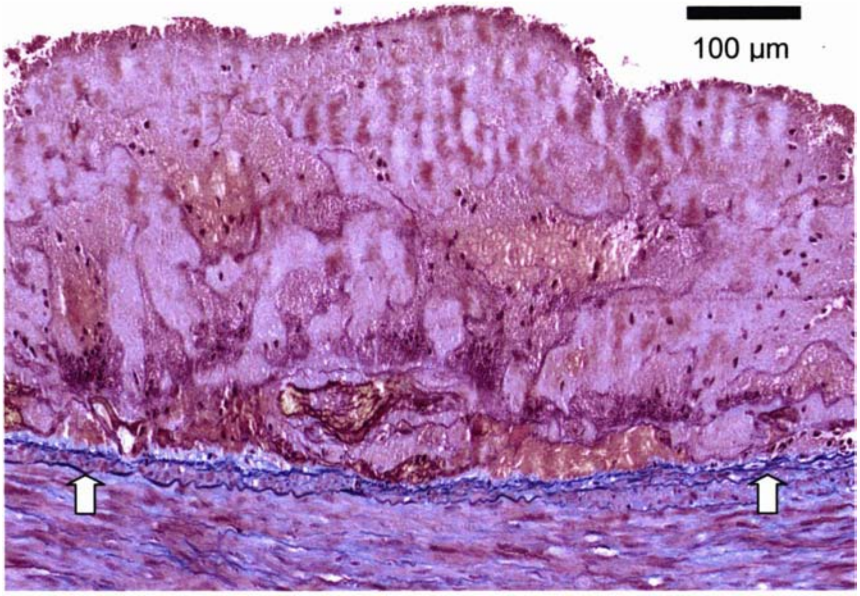

Figure 4.5 Higher magnification of Figure 4.4. Note the intact internal elastic lamina (arrows). The thrombus consists largely of platelets (mauve) but there are some fibrin-rich (red) areas. The nuclei of many leucocytes (black) can be seen within the thrombus. Stained using a modified Verhoeff's method and counterstained using a modified Martius blue trichrome method.

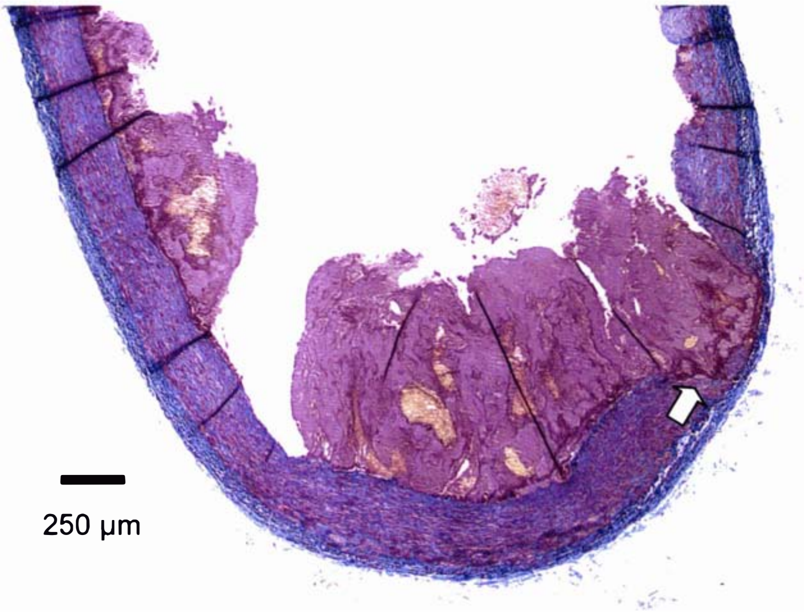

Figure 4.6 Photomicrograph of a thrombus that has formed within the external iliac artery at a site of damage created by a balloon angioplasty catheter. Unlike the artery shown in Figures 4.4 and 4.5, a tear can be seen extending into the media, indicated by the arrow. In this section, lacunae of erythrocytes (yellow) can be seen in the thrombus. Stained using a modified Verhoeff's method and counterstained using a modified Martius blue trichrome method.

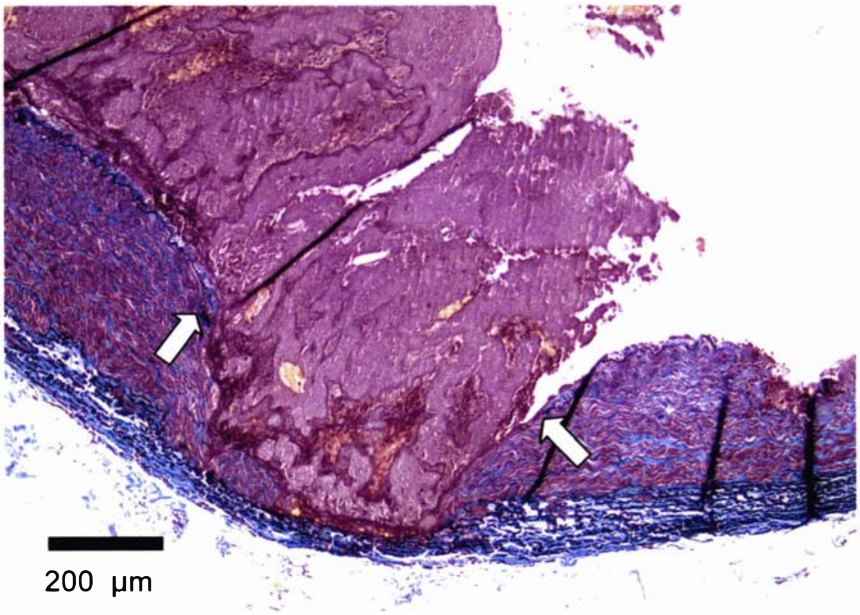

Figure 4.7 Higher magnification of Figure 4.6. The location of the torn ends of the internal elastic lamina are shown with arrows. Stained using a modified Verhoeff's method and counterstained using a modified Martius blue trichrome method.

Three-quarters of the radiolabel was found in the packed cell fraction (median, 75.4%; lower quartile, 65.2%; upper quartile 76.7%) one hour after angioplasty. The associations between the length of the tear and the size of the thrombus, determined by both methods, are shown in Figures 4.8 and 4.9. The raw data, excluding the torn arteries, are shown in Figure 4.10.

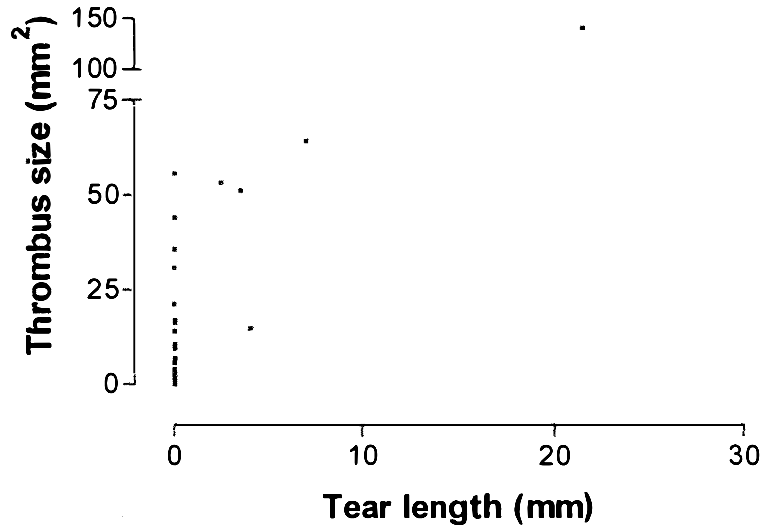

Figure 4.8 Thrombus size, evaluated morphometrically, versus arterial tear length.

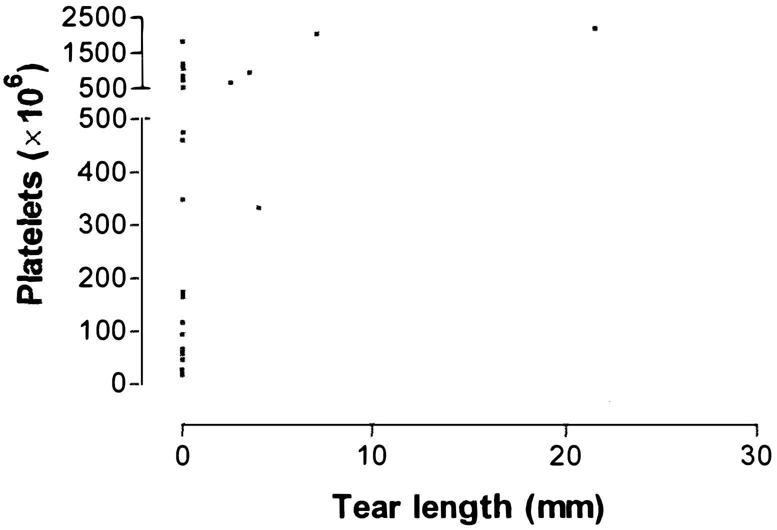

Figure 4.9 Number of platelets incorporated into arterial thrombus versus arterial tear length.

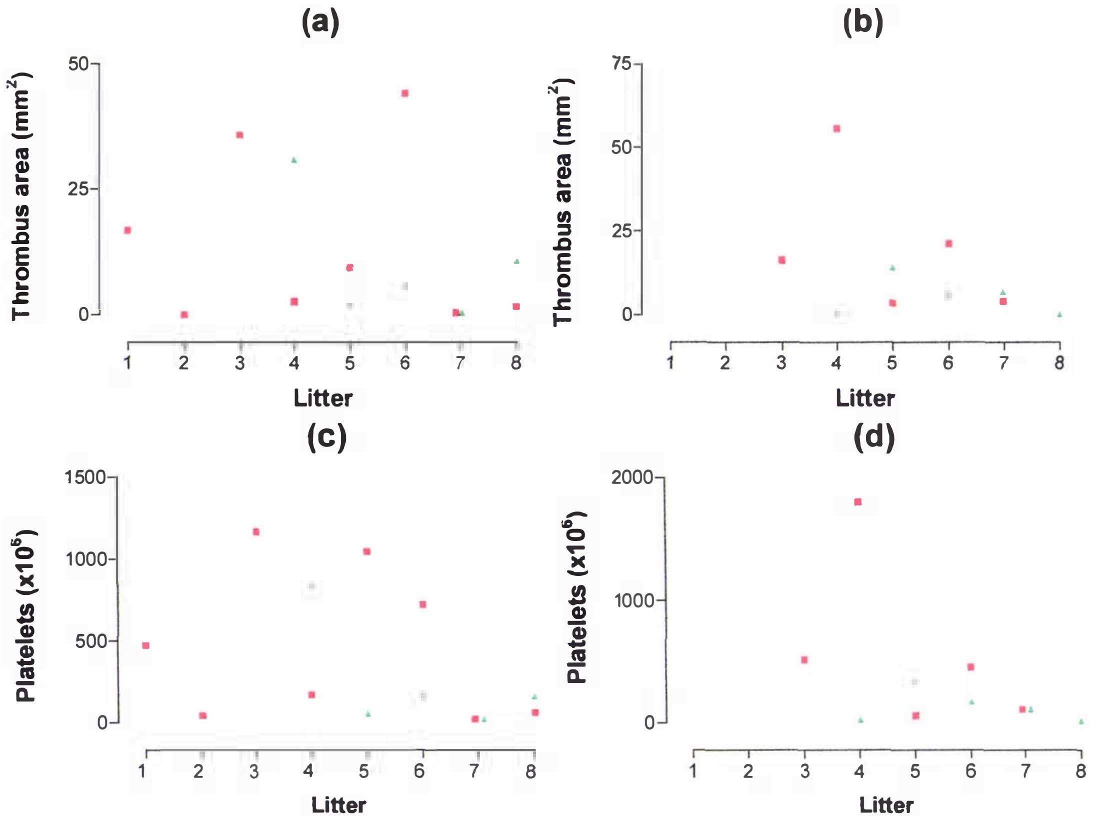

Figure 4.10 The raw data, excluding the arteries that tore, are shown for each litter. Graphs (a) and (b) show thrombus size, determined morphometrically, created by the 10-mm and 12-mm diameter angioplasty balloons respectively. Graphs (c) and (d) show the thrombus size, determined by the radiolabelled platelet method, created by the 10-mm and 12-mm diameter angioplasty balloons respectively. ■ Milkfat diet. ▲ Carbohydrate diet.

There is some evidence ($p = 0.1$) to suggest that the milkfat diet may have had a greater thrombotic tendency than the carbohydrate diet when thrombus size was quantitated by the radiolabelled platelet method (Table 4.3). There was no significant difference in the size of the thrombus between the diets when evaluated by the morphometric method. The size of the angioplasty balloon did not affect the size of the thrombus in the arteries that did not tear (Table 4.4).

Table 4.3 The effect of feeding milkfat or a carbohydrate diet for 12 weeks in growing pigs on predicted mean thrombus size in the external iliac artery following balloon angioplasty. Thrombus size was determined by a radiolabelled platelet method and by a morphometric method. Only arteries that did not tear following angioplasty are presented.

Variable	Dietary Treatment		SED[1]	DF[2]	p-value
	Milkfat (n=13)	Carbo[3] (n=10)			
Platelet number ($\times 10^6$)[4]	5.53 (252.1)	4.59 (98.5)	0.56	17	0.10
Thrombus size (mm^2)[4]	2.18 (8.85)	1.31 (3.71)	0.62	19	0.18

[1] Approximate standard error of differences between means.
[2] Residual degrees of freedom.
[3] Carbohydrate diet.
[4] Values are means of log-transformed data. Back-transformed means are shown in parentheses.

Table 4.4 The effect of the diameter of the angioplasty balloon on the predicted mean thrombus size in the external iliac artery of growing pigs fed a milkfat or a carbohydrate diet for 12 weeks. Thrombus size was determined by a radiolabelled platelet method and by a morphometric method. Only arteries that did not tear following angioplasty are presented.

Variable	Balloon Size		SED[1]	DF[2]	p-value
	10-mm (n=13)	12-mm (n=10)			
Platelet number ($\times 10^6$)[3]	5.11 (165.2)	5.02 (151.2)	0.56	17	0.92
Thrombus size (mm^2)[3]	1.82 (6.17)	1.67 (5.31)	0.62	19	0.79

[1] Approximate standard error of differences between means.
[2] Residual degrees of freedom.
[3] Values are means of log-transformed data. Back-transformed means are shown in parentheses.

There was a good correlation between the two methods of evaluating thrombus size when all data were evaluated ($r^2=0.77$, $p<0.0001$) (Figure 4.11). A similar was also obtained when the torn arteries were excluded ($r^2=0.79$, $p<0.0001$) (Figure 4.12).

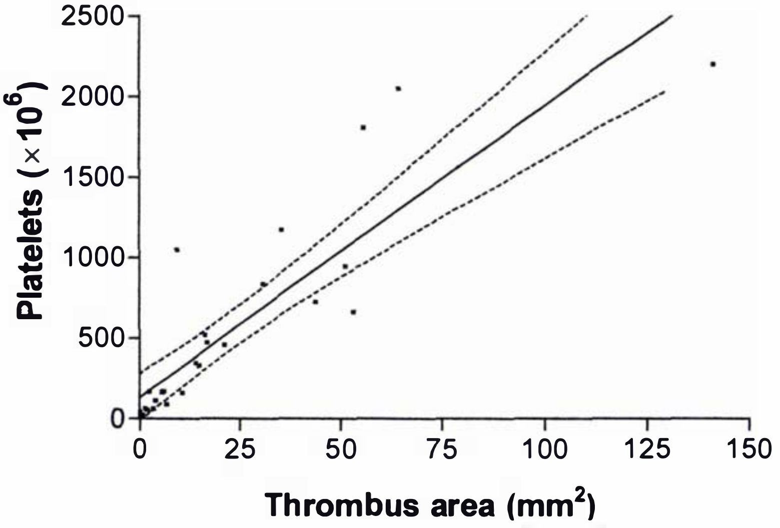

Figure 4.11 Number of platelets within the thrombus versus the thrombus size as evaluated morphometrically. All data are shown. The 95% confidence limits are illustrated as dashed lines.

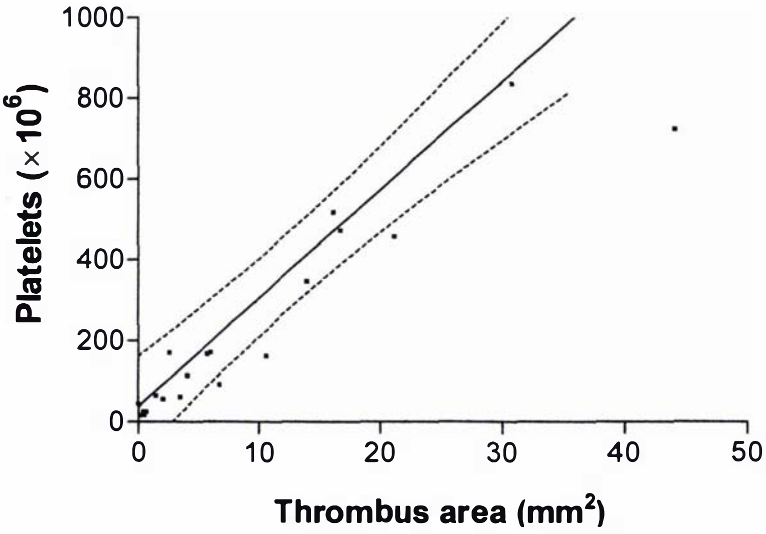

Figure 4.12 Number of platelets within the thrombus versus the thrombus size as evaluated morphometrically. Only arteries that did not sustain tears during balloon angioplasty are shown. The 95% confidence limits are illustrated as dashed lines.

4.4. Discussion

Despite the small number of observations in this study (8 pigs per treatment) and the exclusion of a number of observations due to the presence of deep tears, there was some evidence ($p = 0.1$) to suggest that the pigs fed the milkfat diet may have a greater thrombotic tendency than those fed the carbohydrate diet when thrombus size was evaluated by the radiolabelled platelet method. Given the low power of this pilot study, evaluation of a larger number of animals would be required before the null hypothesis (no differences between treatment groups) could be accepted.

Although there was no 'gold standard' technique for evaluating thrombus size in this study, the good correlation between the radiolabelled platelet and morphometric methods served to cross-validate their use (Figures 4.11 and 4.12). It should be noted, however, that these methods provide slightly different estimates of thrombus size. As can be seen in Figures 4.4 to 4.7, the thrombi that formed were predominantly platelet-rich, although many thrombi had fibrin-rich and erythrocyte-rich areas. The morphometric method takes into account the contribution of fibrin and erythrocytes to overall thrombus size whereas the radiolabelled platelet method only estimates the contribution of platelets to thrombus size. However, the radiolabelled platelet method has potential advantages over the morphometric method. Whereas the morphometric method 'sampled' the area of the thrombus at intervals of 0.5 mm along its entire length with the sum of these areas providing an estimate of thrombus size, the radiolabelled platelet method estimated the number of platelets in the entire thrombus. Furthermore, there is an element of subjectivity inherent with the morphometric method, which is not present in the radiolabelled platelet method. Using the morphometric method, it was not possible to precisely trace the outline of the thrombus and the interface of the thrombus with the artery was occasionally difficult to recognise, particularly when a deep tear was present. These features of the morphometric method may have resulted in its poorer ability to discriminate between the dietary treatments ($P = 0.18$, morphometric method; $P = 0.10$, radiolabelled platelet method).

There was a trend to suggest that the milkfat diet tended to be more thrombogenic than the carbohydrate diet, despite the similarity between the platelet phospholipid fatty acid composition of the two treatment groups (Figures 4.1 – 4.3). The pigs receiving the milkfat diet tended to have less arachidonic acid and more eicosapentaenoic acid and docosahexaenoic acid in their platelet phospholipids than the pigs receiving the carbohydrate diet. As discussed in detail in Chapter 1, increasing the ratio of EPA to AA within platelet phospholipids usually results in antiaggregatory and vasodilatory effects due to the formation of series 3 eicosanoids at the expense of series 2 eicosanoids. These findings suggest that if the milkfat diet is more thrombogenic than the carbohydrate diet, it is unlikely to be mediated by the platelet phospholipid ratio of eicosapentaenoic acid to arachidonic acid.

In human beings (Renaud *et al*, 1986b) and rats (McGregor *et al*, 1980), the concentration of 5, 8, 11-eicosatrienoic acid (20:3 n-9) within platelet phospholipids has been shown to correlate strongly with the clotting activity of platelets and thrombin-induced platelet aggregation. This fatty acid is derived principally from dietary stearic acid (18:0) by desaturation and elongation (Sprecher, 1983) and is metabolised by the lipoxygenase pathway to a pro-aggregatory, hydroxylated fatty acid (Lagarde *et al*, 1983, 1985). However, in the current study, the 20:3 n-9 content of the platelet membranes of the two dietary treatments was very similar throughout the duration of the study, despite the milkfat diet containing approximately fourteen times[a] more saturated fat and nineteen times[a] more stearic acid than the carbohydrate diet. This suggests that if the milkfat diet is relatively more thrombogenic than the carbohydrate diet, it is unlikely to be mediated by the platelet phospholipid concentration of 5, 8, 11-eicosatrienoic acid.

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[a] The approximate content of fatty acids within each diet can be calculated by multiplying the total lipid content of the diet (see Results) with the dietary fatty acid composition data shown in Table 4.2. For example, the total saturated fat content of the carbohydrate diet is approximately 2.5 g total fat/100 g diet and the stearic acid content within the carbohydrate diet is approximately 0.3 g /100 g total fatty acids. 2.5 × 0.3 = 0.75 g/100 g diet. This is only approximate because the estimate of the total fat within the diet includes small quantities of other lipids such as phytosterols and cholesterol. It also includes glycerol from triglycerides.

Although the possible prothrombotic tendency of the milkfat is unable to be explained on the basis of the platelet phospholipid fatty acid composition, other factors, not related to eicosanoid production, may potentially contribute to such an effect. Thrombin has been shown to play a pivotal role in the development of arterial thrombosis. Previous studies have shown that pre-treating pigs with heparin results in a dose-dependent reduction in the quantity of platelets and fibrin deposited on segments of carotid arteries that had sustained deep injury following balloon angioplasty (Heras *et al*, 1989, 1990). Heparin is a glycosaminoglycan that indirectly inhibits thrombin activity by increasing the activity of antithrombin III 2000- to 4000-fold (Olson & Bjork, 1994). Macroscopic mural thrombi could be totally abolished by pre-treatment with hirudin, a specific thrombin inhibitor (Heras *et al*, 1989, 1990). Using the same model of arterial injury, the anti-thrombotic effects of hirudin, the cyclooxygenase inhibitor, aspirin, and the thromboxane inhibitor, dipyridamole, were compared (Lam *et al*, 1991). Pre-treatment with low-dose aspirin (1 mg/kg) alone and a combination of high-dose aspirin (20 mg/kg) and dipyridamole significantly reduced platelet deposition but these treatments were not as effective as hirudin. High-dose aspirin alone and dipyridamole alone were ineffective at inhibiting platelet deposition on the subendothelium. Furthermore, low molecular weight (fractionated) heparin, which maintains its ability to inhibit factor Xa but has less efficacy at inhibiting thrombin than unfractionated heparin, was less effective at preventing arterial thrombosis than unfractionated heparin at doses that produce similar anti-factor Xa activity (Heras *et al*, 1992). In the current study, it is therefore possible that differences in thrombin generation may explain the tendency for thrombi to be larger in the group receiving milkfat. The effect of diet on thrombin generation *in vivo* is investigated in Chapter 6 by determining the thrombin-antithrombin III complex (TAT) concentration. In that study, despite there being significant differences in the TAT concentration between other dietary treatments, there was no significant difference between the carbohydrate and milkfat groups at an inclusion rate of 4% w/w.

The level of consumption of long-chain omega-3 fatty acids derived from marine fish oil is inversely related to platelet aggregability in human beings (Siess *et al*, 1980; Renaud *et al*, 1986a; Kwon *et al*, 1991). Furthermore, there is a low incidence of coronary heart disease in some populations consuming large quantities of fish (Bang & Dyerberg, 1980; Kromann & Green, 1980) . On this basis, it was decided that the inclusion of a group of pigs consuming a diet enriched with long-chain omega-3 fatty acids would serve as a 'negative control' for the evaluation of the balloon angioplasty model in pigs. An earlier study had shown that another brand of marine fish oil (MAXEPA®, Seven Seas Ltd, Hull, England) was palatable when included in the diet at 10% w/w (KG Thompson, KAC James, personal communication). The marine fish oil chosen for the current study (supplied by Scotia Pharmaceuticals Ltd, Carlisle, Scotland) was used in preference to MAXEPA® because it was a more concentrated source of EPA and DHA. The group of pigs receiving the diet containing concentrated marine fish oil was removed from the study after 6 weeks because the oil was unpalatable and feed intake was severely affected. A subsequent palatability trial showed that 4% w/w of high EPA/DHA oil was the maximum dietary inclusion rate that was palatable for pigs.

Thrombus has been reported to develop only in the arteries of pigs that have sustained tears following balloon angioplasty (Steele *et al*, 1985; Lam *et al*, 1991). In contrast, previous experience at Massey University has shown that following balloon angioplasty of the femoral arteries of pigs, thrombi will develop in the absence of a tear through the internal elastic lamina (KG Thompson, personal communication). On the basis of these conflicting results, one objective of this study was to create approximately equal numbers of deep and superficial injuries by using catheters with two different sized balloons in the left and right external iliac arteries of each pig. An 8-mm diameter balloon had previously resulted in tears in 9 out of 91 arteries in pigs with a mean weight of 72 kg (KG Thompson, personal communication). In order to create a greater number of tears, and to compensate for the anticipated heavier final weight of the pigs used in this study, catheters with 10-mm and 12-mm diameter balloons were used.

Thrombus was observed in histological sections of arterial segments that did not sustain tears (Figures 4.4 and 4.5), which concurs with the findings of KG Thompson (personal communication). Serial sections of the entire length of the damaged segments of arteries were taken at 0.5-mm intervals for histology so it is unlikely that a tear would have been missed. Unlike the study of Steele *et al* (1985), the pigs in the current study did not receive heparin at the time of angioplasty. Since heparin reduces the activity of thrombin (Olson & Bjork, 1994), this drug may have prevented the development of thrombi following superficial injury in the study of Steele *et al* (1985). In another study, thrombi were not observed in arteries that sustained superficial injury in pigs that received 0.9% saline rather than heparin but only two or three cross-sections of damaged artery were examined by light microscopy (Lam *et al*, 1991). It is therefore possible that microscopic thrombi were inadvertently missed because of the small number of sections examined.

Despite the intention to create an equal number of deep and superficial injuries, only 5 of 30 (16.7%) arteries sustained tears. Of those arteries that sustained tears, 4 of 5 were 7 mm or less and one tear was 21.5 mm long. Although the small number of tears precluded statistical analysis, the arteries with the longest and the second longest tears had the largest and second largest thrombi. The difficulty in controlling the length of tear, and the apparent impact that tear length had on thombus size, suggests that creating deep injury may be a difficult variable to control. Furthermore, large thrombi that form at the site of deep injury may mask subtle dietary effects. On this basis, creating superficial injury rather than deep injury may be more desirable in refining the *in vivo* mdoel for thrombogenicity studies. In the clinical setting however, fissuring of atheromata (Stary type IV lesion (Stary, 1989)), rather than deendothelialisation, is probably required in order for the acute coronary syndromes to develop (Fuster, 1994). With this in mind, deep injury may more closely model the pathophysiology of atherothrombosis.

There are a number of shortcomings with the model described in this chapter. The size of the thrombus is determined at only one point in time, one hour after angioplasty. As discussed in Chapter 1, thrombosis and embolisation are dynamic events. Evaluating thrombus size at one predetermined point in time may not provide the best end point for determining the thrombogenicity of diets. Using the Badimon perfusion chamber, Lasilla *et al* (1990) showed that in individual preparations of artery with the media exposed, embolisation could usually be detected at the point of maximum platelet deposition (the apex of an 80% stenosis) 30 minutes or more after exposure to blood. These results suggested that a larger thrombus was more likely to dislodge. Despite this, Lasilla *et al* (1990) showed that over the 50-minute study period, the average rate of platelet accumulation was always greater than the average rate of platelet loss over a wide range of rheological conditions (0% to 80% stenosis). The effect of degree of arterial injury (superficial or deep) on the extent of thromboembolism is unknown. It is tempting to speculate that a thrombus overlying superficial injury is not as well anchored as thrombus overlying deep injury and for any given thrombus size or set of rheological conditions, maybe more prone to embolism. The influence of thromboembolism on the results of the current study is unknown but scintigraphic techniques, or the use of flow probes distal to the site of arterial injury, could be used in future to estimate its impact on final thrombus size.

Another disadvantage with this model is its expense. The most significant cost is associated with purchasing, housing and feeding the pigs for 12 weeks. Since the pigs are killed at the end of the study, there is no cost-recovery. Considerable cost is also associated with radiolabelling platelets, anaesthesia, the angioplasty procedure and preparing serial sections of artery.

Some costs associated with this model could be reduced by using a smaller breed of pig and/or feeding the test diets for a shorter period of time. In New Zealand, the Kune Kune breed may be a suitable experimental model as it is smaller than the commercial Landrace, Large White and Duroc breeds[b]. However, it may be more difficult to control for genotype as this breed is more heterogenous than

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[b] Information obtained from: www.geocities.com/Heartland/Valley/5330/whatpig.html#phys

commercial breeds[b]. Similarly, it may not be possible to obtain enough littermates for a large study to statistically block for litter. Unlike other breeds of pig, the Kune Kune is a grazer rather than a forager and they do not thrive on commercial pig rations[b]. More work would be required to investigate the suitability of this breed as a model for cardiovascular research.

The length of time required to feed the test diets in order to document different dietary effects on the thrombus end-point with this model is unknown. A conservative approach was taken for this study with the selection of a 12-week feeding period but it is accepted that a shorter period may have been satisfactory. In terms of the platelet phospholipid fatty acid composition, the arachidonic acid content continued to trend upwards during the 12 weeks of the study. On the other hand, there was very little change in the platelet phospholipid content of eicosapentaenoic acid in the group receiving the carbohydrate diet from approximately 8 weeks. Although the group that received the concentrated marine fish oil was removed after six weeks on the diet, the platelet phospholipid fatty acid composition of this group was determined over this period. The platelet phospholipid content of eicosapentaenoic acid, arachidonic acid and docosahexaenoic acid for this group are presented in the Appendix. This quite clearly demonstrates that most of the changes induced by the marine fish oil supplement on the platelet phospholipid composition of these fatty acids occurred in the first two weeks on the diet. The length of the feeding period required before changes in the activities of the coagulation cascade, the fibrinolytic system, and the activity of the constitutive inhibitors of coagulation could be observed remains to be determined.

This model does not take into account two important features of ischaemic heart disease in human beings. Firstly, the damaged segments of artery were healthy rather than diseased and secondly, the external iliac arteries rather than the coronary arteries were used. As previously discussed, fissuring of atheromata (Stary type IV lesion (Stary, 1989)) is probably required in order for the acute coronary syndromes to develop (Fuster, 1994). Using the Badimon perfusion chamber, Fernandez-Ortiz *et al* (1994) showed that the lipid core of an atheroma is the most thrombogenic component of the vessel wall. Consequently, it would be ideal to investigate the effect of dietary lipids on the degree of thrombosis at sites of atherosclerosis. Accelerated atherosclerosis can be induced in pigs by combining angioplasty-induced endothelial cell denudation with the feeding of an atherogenic diet containing large quantities of saturated fat and cholesterol (Lee & Lee, 1975). If the objective is to examine the influence of dietary lipids on thrombosis however, the dietary manipulation required of this model renders it unsuitable. Spontaneous atherosclerosis also occurs at an accelerated rate in pigs with inherited hypercholesterolaemia LDL cholesterol (IHLC), which closely resembles familial defective apolipoprotein B-100 in human beings. By one year of age, the IHLC mutant pig develops focal lesions in the major coronary, iliac and femoral arteries, comprising macrophage-derived foam cells and smooth muscle cells (Prescott *et al*, 1991). By two years of age, stenotic lesions with fibrous caps and necrotic cores, cholesterol clefts, calcium deposits and neovascularisation deep within the lesion are common in the major coronary vessels (Prescott *et al*, 1991). Peripheral vascular lesions were more smooth muscle cell-rich and fibrotic. In three-year-old pigs, neovascularisation is observed throughout the intimal lesions and haemorrhage and rupture are common (Prescott *et al*, 1991). In these pigs, the pathophysiological events leading to atherosclerosis and the morphology of the lesions are akin to human atherosclerosis (Overturf & Loose-Mitchell, 1992). Consequently, the use of these pigs may represent an improvement on the model used in this study.

The external iliac arteries, rather than the coronary arteries, were used because sophisticated imaging equipment was not required, which in turn makes the model more cost-effective and accessible. In a recent study, larger thrombi, with more consistency in thrombus size between pigs, were obtained in coronary arteries subjected to deep injury than in carotid arteries (JJ Badimon, personal communication). This may have been due to the ability to induce a more consistent degree of injury. Smaller within-group variances are more likely to result in significant differences between groups.

In summary, this model, using radiolabelled platelets for estimating thrombus size, shows promise as a method of evaluating the effects of dietary lipids on thrombosis. Although statistical significance between dietary treatments was not reached, there was a trend for the pigs receiving milkfat to have greater thrombus size than the pigs receiving the carbohydrate diet. The use of a larger number of animals per treatment group and the satisfactory inclusion of a fish oil group may have resulted in differences. This study has clearly shown that deep injury is not required in order for thrombosis to occur, at least within the external iliac arteries of non-heparinised pigs. In fact, creating superficial injury may be advantageous as it appears that the length of a tear may be difficult to control and tears are likely to be accompanied by greater thrombus size. The influence of tearing may potentially mask any dietary effects. The model could also be made more relevant to human atherothrombosis research by using coronary arteries instead of the external iliac arteries and by using the IHLC mutant pig. It should be noted however, that creating deep tears at the site of atheroma would need to be achieved if the IHLC mutant pig was to be used to its fullest advantage. The possible advantages derived from these approaches would need to be weighed against their practicality and expense.

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Chapter 5. The effect of a milkfat diet and a low-fat diet on coagulation screening tests, fibrinogen concentrations and factor VII activity in pigs.

5.1. Introduction

In Chapter 4, a balloon angioplasty model of arterial injury was evaluated in pigs as a means for assessing the thrombogenicity of dietary fats. Despite the small numbers of observations, there was a trend for the pigs ingesting the milkfat diet to have larger thrombi at the site of arterial injury than the carbohydrate group. In order to determine whether the increase in thrombus size might be to increased activity of the coagulation cascade, the activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TT) were determined in this study to evaluate the overall activity of enzymes of the intrinsic, extrinsic and common coagulation pathways.

The APTT principally evaluates the activity of factors VIII, IX, XI and XII, prekallikrein and high molecular weight kininogen (HMWK) (Spaethe, 1984). The APTT will also detect severe functional deficiencies in factors II, V, X or fibrinogen (Basu *et al*, 1972). In this test, a platelet factor 3 substitute (partial thromboplastin) and a contact activator (ellagic acid, kaolin or celite) are incubated with a sample of citrated plasma from the test animal. The contact activator provides a negatively charged surface, which activates factors XII and XI in the presence of kallikrein and HMWK. The partial thromboplastin provides a suitable surface for activation of factors IX, X and II. Following a set incubation period, calcium ions are added in excess to activate factors IX, X and II. The intrinsic and common clotting cascades are activated and a fibrin clot is formed.

The PT is used primarily to evaluate the activity of enzymes of the extrinsic and common coagulation pathways (Spaethe, 1984). The PT is therefore dependent of the plasma concentrations and activities of activated factors II, V, VII and X. A sample of citrated plasma from the test animal is incubated with a tissue factor substitute (plain thromboplastin) and calcium ions in excess. The plain thromboplastin provides a suitable surface for activation of factors VII, X and II, whilst the calcium ions serve as a cofactor. The extrinsic and common clotting cascades are activated and a fibrin clot is formed.

The TT is used to evaluate the concentration and function of fibrinogen in plasma and to detect the presence of fibrin(ogen) split products (FSP) and to determine the efficacy of heparin and clot lysis therapy (Spaethe, 1984). Thrombin cleaves fibrinopeptides A and B from fibrinogen. The net result is the formation of fibrin monomers, which accumulate to form soluble fibrin polymers. Increased plasma concentrations of FSP or heparin, as occurs in clot lysis therapy and heparin therapy respectively, results in an increased thrombin time.

As well as the screening tests outlined above, clot-based assays for fibrinogen and factor VII are evaluated in this study. In 1986, the results of the Northwick Park Heart Study indicated that plasma factor VII activity and fibrinogen concentrations were strongly associated with increased risk of first-time major episodes of ischaemic heart disease (Meade *et al*, 1986a). In the same year, an international study compared groups of men of similar age in communities at very low (Gambia), high (England and Czechoslovakia) and very high (Scotland and Finland) risk of ischaemic heart disease (Meade *et al*, 1986b). A strong and positive relationship between factor VII activity and ischaemic heart disease was consistently found, although there was a less consistent relationship with fibrinogen concentrations, particularly in high-risk communities. These early epidemiological studies prompted further studies investigating the role of markers of haemostasis and fibrinolysis on ischaemic heart disease. A meta-analysis of six large, prospective epidemiological studies provides testament to the now widely accepted view that the plasma fibrinogen concentration is a risk factor for cardiovascular disease (Ernst & Resch, 1993). Despite this association, the link between diet and plasma fibrinogen concentrations is tenuous.

Since 1986 there has been strong, but by no means unanimous, support for the thesis that increased factor VII activity is a risk factor for cardiovascular disease. In a recent study, 3000 middle-aged men clinically free of cardiovascular disease were ranked according to a risk score for fatal coronary heart disease (Miller *et al*, 1996). The risk score took into account well-established risk factors such as smoking habit, systolic blood pressure, serum cholesterol concentration and obesity. Plasma factor VII coagulant activity, factor VII antigen concentration and activated factor VII concentration were positively and significantly associated with risk for coronary heart disease. Conversely, another study failed to show significant differences in factor VII coagulant activity between men who had a previous myocardial infarct and men who had stable angina, suggesting no role for increased factor VII activity in acute coronary syndromes (Biasucci *et al*, 1996). Merlini *et al* found no differences in activated factor VII concentrations between patients presenting to hospital with acute myocardial infarction, unstable angina, stable angina and age- and sex-matched healthy individuals (Merlini *et al*, 1995).

In contrast to the tenuous association between plasma fibrinogen concentrations and dietary fat intake, there is a large body of evidence to suggest that dietary fat induces changes in factor VII activity. In healthy individuals, diets high in fat are associated with significantly higher concentrations of factor VII coagulant activity than low-fat diets (Mitropoulos *et al*, 1994). Whereas factor VII coagulant activity is positively associated with plasma concentrations of saturated fatty acids, it is negatively associated with both plasma concentrations of LA and the ratio of polyunsaturated to saturated fatty acids (Folsom *et al*, 1994). Tholstrup and co-workers (1994a,b) have shown that a diet enriched with myristic acid (14:0) results in greater factor VII coagulant activity than a diet enriched with palmitic acid (16:0) and that diets enriched in saturated fatty acids with 12 to 16 carbon atoms result in greater factor VII coagulant activity than diets enriched in stearic acid (18:0). A recent study has indicated a gender difference: in women, but not in men, diets enriched with lauric or myristic acid increased factor VII activity when compared with a diet enriched in oleic acid (Temme *et al*, 1999). As well as the effects of specific fatty acids upon factor VII activity, there is evidence to suggest that the intake of sufficient quantities of fat to induce postprandial lipaemia can increase factor VII coagulant activity and the concentration of factor VII in circulation in the activated form (Miller *et al*, 1986; Sanders *et al*, 1996).

5.2. Materials and methods

Experimental animals, diets and procedure

The experimental animals, diets and procedures were as described in Chapter 4.

Blood sampling and handling

Blood samples of the pigs were obtained from the right brachiocephalic or jugular vein following an overnight fast. Samples were taken between 0800 and 1000 hours at the beginning of the study and 1, 2, 4, 6, 8, 10 and 12 weeks later.

Nine millilitres of blood was drawn into a 10-mL syringe (Monoject, St Louis, USA) containing 1 mL of 0.11 mmol/L trisodium citrate. The citrated blood was transferred to a labelled polystyrene conical tube (Falcon 2095, Becton Dickinson, NJ, USA).

Within one hour of collection, the sample was centrifuged at 2 250 x g for 10 min (11105 rotor, Centra -3C centrifuge, International Equipment Co, Needham

Heights, Ma., USA) and the supernatant (plasma) was harvested. Approximately 2 mL of plasma was used to determine the PT, TT, APTT, factor VII concentration and fibrinogen concentration.

Clotting time and clotting factor assays

Assays for APTT, PT, TT, fibrinogen, and Factor VII were determined turbidometrically using a dual-channel coagulometer (Coatron Junior, TECO Medical Instruments GmbH, Ergoldsbach, Germany) within four hours of blood collection. Duplicate determinations were performed and the mean result was recorded. Assays were re-run when duplicate clotting times differed by more than 1 s. Control limits (mean ± 2 standard deviations) were determined using control plasma (Dade Ci-Trol Coagulation Control, Level 1, Baxter Diagnostics Inc, Illinois, USA). On each day that the assays were performed, control samples were run at the beginning and at the end of a daily run and whenever a new bottle of reagent was reconstituted.

Activated Partial Thromboplastin Time

A 0.1-mL sample of citrated plasma was incubated with 0.1 mL of activated partial thromboplastin reagent (Dade Actin FS activated PTT reagent, Baxter Diagnostics Inc, Illinois, USA) in a reaction cuvette for 3 min at 37°C. A soybean phospholipid is used in this reagent as the platelet factor 3 substitute and ellagic acid for contact activation of factors XI and XII (Spaethe, 1984). With the coagulometer in the PTT mode, the cuvette was placed in the reaction chamber and 0.1 mL of 0.02 M calcium chloride (Baxter Diagnostics Inc, Illinois, USA), which had been prewarmed to 37°C, was added to initiate clot formation.

Prothrombin Time

A 0.1-mL aliquot of citrated plasma was prewarmed to 37°C for 2 min in a reaction cuvette. The cuvette was then transferred to the reaction chamber and 0.2 mL of a solution containing acetone-dehydrated rabbit brain thromboplastin and calcium ions at a concentration of 10 mmol/L (Dade Thromboplastin IS, Baxter Diagnostics Inc, Illinois, USA) was added forcibly to the cuvette. The reaction time was recorded with the coagulometer in the PT mode.

Thrombin Time

Citrated plasma was diluted 1:2 with Owren's veronal buffer (Baxter Diagnostics Inc, Illinois, USA). A 0.2-mL aliquot of the diluted sample was incubated in a reaction cuvette at 37°C for 3 min. Bovine thrombin (0.1 mL; Dade Data-Fi thrombin reagent, 20 NIH units/mL, Baxter Diagnostics Inc, Illinois, USA) was added to the sample and the clotting time was recorded with the coagulometer in the TT mode.

Fibrinogen

Plasma fibrinogen concentrations were determined using the principle developed by Clauss (1957). Citrated plasma was diluted 1:10 with Owren's veronal buffer (Baxter Diagnostics Inc, Illinois, USA). A 0.1-mL aliquot of the diluted sample was incubated in a reaction cuvette at 37°C for 3 min. To initiate clot formation, 0.1 mL of reconstituted bovine thrombin (Dade Data-Fi thrombin reagent, 100 NIH units/mL, Baxter Diagnostics Inc, Illinois, USA) was added to the sample. The thrombin reagent was reconstituted with a 1 mg/mL light kaolin solution (Fort Richard Laboratories Ltd, Auckland, New Zealand), in accordance with the method recommended by the manufacturer of the coagulometer. The same batch of thrombin reagent was used for all fibrinogen determinations. The plasma fibrinogen concentration was determined by comparing the clotting time with a 5-point standard curve, which had been made using a human fibrinogen standard (Dade Data-Fi Fibrinogen Calibration Reference, Baxter Diagnostics Inc, Illinois, USA).

Factor VII

Citrated plasma was diluted 1:10 with Owren's veronal buffer (Baxter Diagnostics Inc, Illinois, USA). A 0.1-mL aliquot of the diluted sample was mixed with 0.1 mL of immunoabsorbed factor VII deficient human plasma (Baxter Diagnostics Inc, Illinois, USA) and incubated in a reaction cuvette at 37°C for 2 min. The cuvette was then transferred to the reaction chamber of the coagulometer and 0.2 mL of a solution containing acetone-dehydrated rabbit brain thromboplastin and calcium ions at a concentration of 10 mmol/L (Dade Thromboplastin IS,) was added forcibly to the cuvette. The reaction time was recorded with the coagulometer in the PT mode. The factor VII activity of the sample was evaluated by comparing the reaction time with a response curve obtained by plotting the dilution of a calibrated reference plasma (Factor Assay Reference Plasma, Baxter Diagnostics Inc, Illinois, USA) on the abscissa and the clotting time on the ordinate of two-cycle log-log paper.

Statistics

One-way analysis of variance (ANOVA), with the litter included as a blocking factor, was used to compare the effect of dietary treatment on weight gain. Variables were compared using repeated measures ANOVA. Each pen of pigs was included in the statistical model as a blocking factor. Since both the univariate tests for within-subject (time) effects and the interactions involving these effects required a type H covariance structure, a sphericity test was applied to determine whether the data satisfied this condition. If the data did not satisfy the assumption of type H covariance, Huyhn and Feldt's estimator was applied to adjust the numerator and denominator degrees of freedom. All statistical analyses were conducted using The SAS System for Windows, version 6.10. A p-value of less than 0.05 was considered significant for all comparisons.

5.3 Results

There was no significant difference in weight gain between the two groups of pigs during the study ($p = 0.5$). The mean weight gain (±SD) of the pigs receiving the milkfat and the carbohydrate diets were 64.1±6.6 kg and 61.3±10.1 kg respectively.

The APTT data are shown in Figure 5.1. There was a significant blocking effect ($p = 0.03$). There was some evidence to suggest that the pigs fed the milkfat diet to have a longer APTT than the pigs fed the carbohydrate diet ($p = 0.07$). The activated partial thromboplastin time decreased in a linear fashion over time ($p = 0.0001$).

The PT data are shown in Figure 5.2. There was no significant blocking effect ($p = 0.25$) or effect of diet ($p = 0.17$). The PT decreased in a linear fashion with time ($p = 0.0001$).

The TT data are shown in Figure 5.3. There was no significant blocking effect ($p = 0.12$) or effect of diet ($p = 0.14$). Thrombin times changed in a quadratic fashion over time ($p = 0.003$).

The fibrinogen data are shown in Figure 5.4. There was no significant blocking effect ($p = 0.95$) or effect of diet ($p = 0.62$). Fibrinogen times changed in a quadratic fashion over time ($p = 0.04$).

The factor VII data are shown in Figure 5.5. There were no significant blocking effect ($p = 0.72$) or effect of diet ($p = 0.28$). Factor VII activities increased in a linear fashion with time ($p = 0.01$).

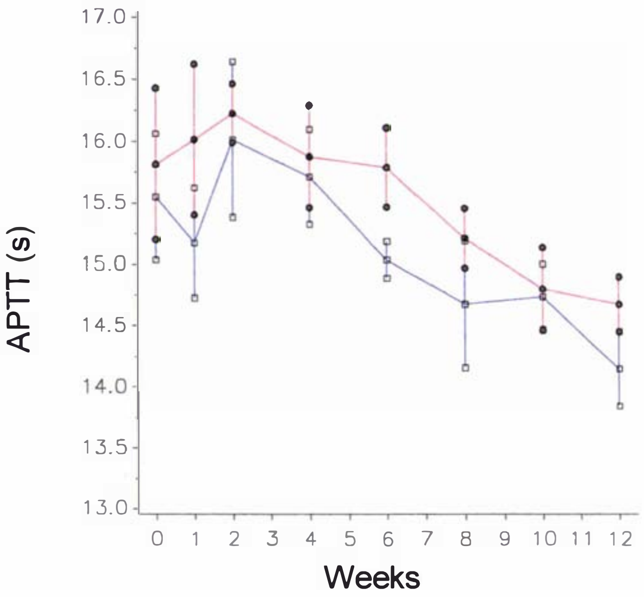

Figure 5.1 The effect of feeding a carbohydrate diet (blue line, open squares) and a milkfat diet (red line, filled circles) on the activated partial thromboplastin time in growing pigs over a 12-week period. Error bars indicate one standard error.

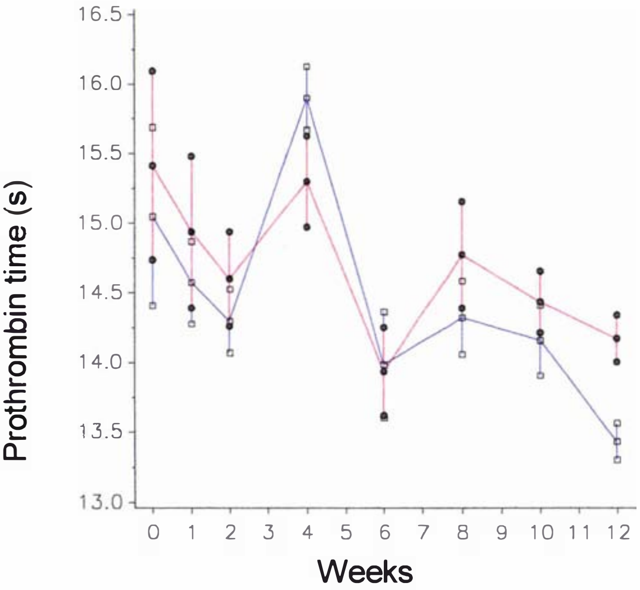

Figure 5.2 The effect of feeding a carbohydrate diet (blue line, open squares) and a milkfat diet (red line, filled circles) on the prothrombin time in growing pigs over a 12-week period. Error bars indicate one standard error.

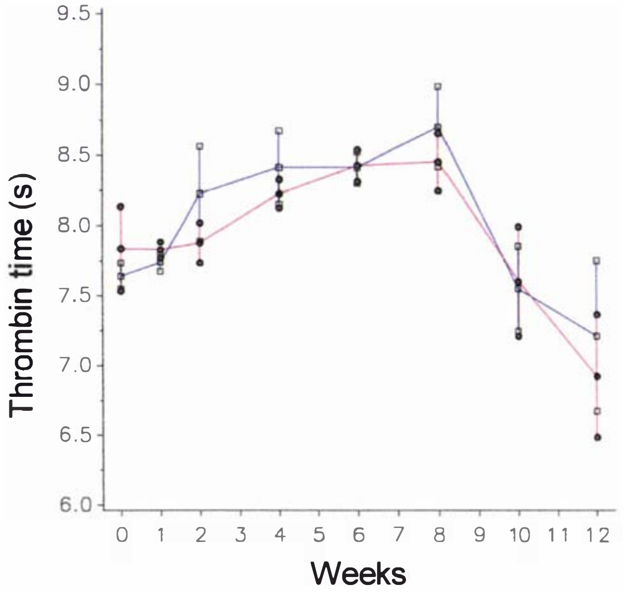

Figure 5.3 The effect of feeding a carbohydrate diet (blue line, open squares) and a milkfat diet (red line, filled circles) on the thrombin time in growing pigs over a 12-week period. Error bars indicate one standard error.

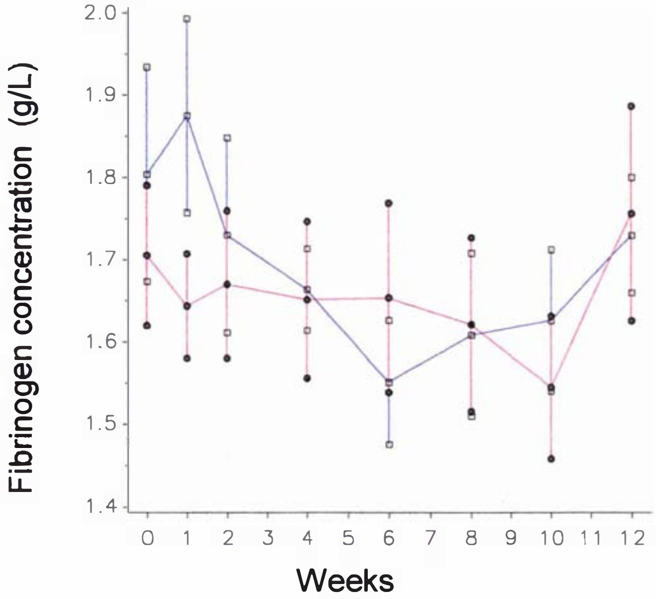

Figure 5.4 The effect of feeding a carbohydrate diet (blue line, open squares) and a milkfat diet (red line, filled circles) on plasma fibrinogen concentrations in growing pigs over a 12-week period. Error bars indicate one standard error.

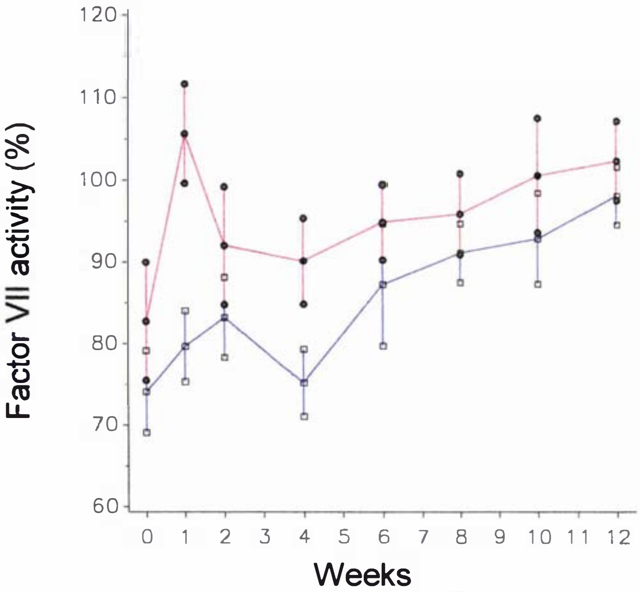

Figure 5.5 The effect of feeding a carbohydrate diet (blue line, open squares) and a milkfat diet (red line, filled circles) on factor VII activities in growing pigs over a 12-week period. Error bars indicate one standard error.

5.4 Discussion

In this study, there was some evidence to suggest that the group receiving the milkfat diet had a longer APTT than the group receiving the carbohydrate diet (p = 0.07). This suggests that the milkfat diet may induce less activity within the intrinsic clotting cascade and/or the common clotting cascade then the carbohydrate diet. Given that there were no significant differences between the two groups in both the TT, which evaluates the activity of the common clotting cascade, and the fibrinogen concentration, any difference in the APTT was most likely due to altered activity of one or more components of the intrinsic clotting cascade.

Contact activation is an *in vitro* event that triggers clot formation through the intrinsic clotting cascade. Its relevance to *in vivo* coagulation is controversial because isolated deficiencies of factors essential to contact activation such as factor XII, prekallikrein and high molecular weight kininogen, do not induce bleeding in human beings (Roncales & Sancho, 2000). Consequently, the importance of a difference in the APTT between the two groups in this study is uncertain. Furthermore, since the APTT is a screening assay that evaluates the activity of a number of clotting factors, it is not possible to determine which part of the intrinsic clotting cascade has been specifically affected by diet. In human beings, immunological techniques have been developed to evaluate the plasma concentrations of activated factor XII, which serves as a means of evaluating the degree of contact activation, and activated factor XI bound to its natural inhibitors (α1-antitrypsin, protein C inhibitor, antithrombin III and C1 inhibitor), which evaluates the concentration of activated factor XI in circulation (Roncales & Sancho, 2000). To the author's knowledge, these assays have not been validated for use on porcine plasma samples.

In Chapter 4, there was some evidence ($p = 0.1$) to suggest that the milkfat diet induced a larger thrombus in the external iliac arteries of pigs than did the carbohydrate diet. In the current study however, the milkfat diet tended to result in a longer rather than a shorter APTT. It would appear therefore that any possible change in activity within the intrinsic clotting cascade does not explain the greater thrombotic tendency of the milkfat diet.

The APTT, PT and TT are generally affected by gross alterations in clotting factor activity (Spaethe, 1984) and although there were no significant effects of diet on the TT and PT in this study, it is possible that there were still some significant differences in the activity of individual clotting factors. Factor VII activities and plasma fibrinogen concentrations were evaluated because of their well-documented association with ischaemic heart disease. Although this study was of low statistical power, no trends were detected in the concentrations of these parameters over time. It is therefore unlikely that the use of a larger number of animals would have resulted in rejection of the null hypothesis (no differences between treatment groups).

The APTT and PT decreased, and factor VII activity increased, in a linear fashion over the 12-week period in both treatment groups. A possible explanation for this observation is that the activity of the intrinsic and extrinsic clotting cascades increases with age in growing pigs. It is difficult to provide a physiological explanation for the quadratic relationship of the TT and fibrinogen concentration with time. It should be noted that the changes in the clotting times and fibrinogen concentrations over the 12-week period of the study were very small and it is possible that the inherent variability of the semi-automated clot-based techniques used in this study may have accounted for these observations, despite the use of controls.

In summary, a possible mechanism for the tendency for the milkfat diet to be more thrombotic than the carbohydrate diet, as discussed in Chapter 4, was not identified using the tests employed in this study. Other markers of thrombosis, such as thrombin-antithrombin III concentrations or activated factor VII concentrations, may be more sensitive indicators of activity of the coagulation cascade than the tests employed here.

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Chapter 6. The effect of dietary lipids on blood concentrations of markers of thrombosis, platelet indices, white and red blood cell counts and platelet reactivity in pigs.

6.1. Introduction

Despite early evidence from postmortem and angiographic studies suggesting that thrombosis plays a role in ischaemic heart disease, it was not until 1991, when the results of the Caerphilly Collaborative Heart Disease Study became available, that an association between platelet reactivity and ischaemic heart disease was established (Elwood *et al*, 1991). In that study, there was more than a two-fold increase in the odds of a past myocardial infarction in men whose ADP-induced platelet aggregation was in the highest 20%. This increase in risk was independent of confounding variables such as smoking status, lipoprotein cholesterol concentrations, blood pressure and body mass index.

Components of the coagulation and fibrinolytic systems, including fibrinogen, factor VII, factor VIII, tissue plasminogen activator, plasminogen activator inhibitor and plasminogen are also associated with coronary heart disease (Pearson *et al*, 1997). In fact, it has been suggested that a 'hypercoagulable state' may exist in people most at risk of developing coronary heart disease (Fuster *et al*, 1992).

Of the above factors, the plasma fibrinogen concentration has been most consistently linked to coronary heart disease. A recent meta-analysis of six, large prospective epidemiological studies has highlighted the importance of plasma fibrinogen concentration as a risk factor for cardiovascular disease

(Ernst & Resch, 1993) but the effect of diet on plasma fibrinogen concentration is inconclusive.

A number of haematological variables have also been positively associated with coronary heart disease. These include mean platelet volume (Cameron *et al*, 1983; Martin *et al*, 1983; Kishk *et al*, 1985; Novo *et al*, 1994; Pizulli *et al*, 1998), plateletcrit (Cameron *et al,* 1983; Martin *et al,* 1983) and white blood cell count (Grimm *et al*, 1985; Lowe *et al*, 1985; Burr *et al*, 1992; Weijenberg *et al*, 1996; Cooper *et al*, 1999; Krumholz *et al*, 1999; Held *et al*, 2000). Large platelets aggregate more readily, have a higher capacity to produce thromboxane, serotonin and β-thromboglobulin and express more glycoprotein receptors than small platelets (Thompson *et al*, 1984; van der Loo & Martin, 1999). Temporal associations have been made between platelet size and acute coronary events (Cameron *et al*, 1983; Martin *et al*, 1983; Kishk *et al*, 1985; Novo *et al*, 1994; Pizulli *et al*, 1998). Platelet volume has been shown to be increased for up to seven weeks following myocardial infarction suggesting that platelet size is chronically increased and may occur prior to infarction (Cameron *et al*, 1983; Martin *et al*, 1983). Furthermore, platelet volume, measured after myocardial infarction, has been shown to be a risk factor for further ischaemic episodes and death (Martin *et al*, 1991). There is also evidence to suggest that the plateletcrit may be an important determinant of ischaemic heart disease risk. In a study of patients presenting to a coronary care unit within 12 hours of developing acute chest pain, multiple regression analysis showed that the platelet mass was a highly significant determinant of the bleeding time (Milner & Martin, 1985). The bleeding time was significantly shorter in patients found to have myocardial infarction than patients with chest pain but no infarction. The importance of platelet mass to haemostasis is also highlighted in thrombocytopenic patients where the plateletcrit is a better predictor of bleeding tendency than the platelet count (Mohr *et al*, 1986).

The total leucocyte count has repeatedly been shown to be a risk factor for coronary death and non-fatal coronary events (Grimm *et al*, 1985; Lowe *et al*, 1985; Burr *et al*, 1992; Weijenberg *et al*, 1996; Cooper *et al*, 1999; Krumholz *et al*, 1999; Held *et al*, 2000). In many of these studies, cigarette smoking was shown to be strongly associated with the leucocyte count but despite this, it remained an independent risk factor following multivariate analysis. A meta-analysis of seven long-term prospective studies performed prior to 1998 showed that subjects with a single baseline leucocyte count in the upper tertile, compared with the lower tertile, had a relative risk of coronary heart disease of 1.4 (95% CI, 1.3 - 1.5) (Danesh *et al*, 1998). The total leucocyte count has also been shown to be positively associated with the severity of established coronary artery disease (Kostis *et al*, 1984; Amaro *et al*, 1993; Miche *et al*, 1995; Bovill *et al*, 1996) and to established risk factors for cardiovascular disease (Bovill *et al*, 1996). Although an association between the total leucocyte count and coronary heart disease has been demonstrated, causality has not been established. Strong associations exist between total leucocyte count and several variables, including platelet count, heart rate, triglyceride concentration, fasting blood glucose concentration, diastolic blood pressure and total cholesterol concentration (Capuano *et al*, 1995; Freedman *et al*, 1996). Furthermore, counts are higher in American whites versus American blacks, smokers versus non-smokers and men who consider themselves in good health versus those who consider themselves in poor health (Freedman *et al*, 1996). With respect to cardiovascular risk, unravelling the contribution of the total leucocyte count from the contribution of these other variables will be difficult. It is possible that an elevated total leucocyte count is a marker for a chronic inflammatory/infectious state. A number of acute phase reactants (albumin, fibrinogen and c-reactive protein), which increase during inflammation, were shown to be independent risk factors for coronary heart disease (Danesh *et al*, 1998). Furthermore, there appears to be an exacerbation of the inflammatory process during the acute stage of myocardial infarction and during coronary artery reperfusion (Mehta *et al*, 1998). There is also evidence to suggest that infection with *Helicobacter pylori* and *Chlamydia pneumoniae* is associated with coronary heart disease (Patel *et al*, 1995). The association occurred despite adjustment for a large number of confounders, including most of the known risk factors for coronary heart disease.

More recently, markers of thrombin generation (fragment F1 + 2 (F1 + 2) and thrombin-antithrombin III complex (TAT)) and thrombin activity (fibrinopeptide A (FPA)) have been positively associated with ischaemic heart disease. Thrombin plays a pivotal role in thrombosis. Not only is it responsible for cleaving fibrinogen to yield fibrin, it is also a potent platelet agonist (Badimon *et al*, 1994). Markers of thrombin formation and activity are elevated in patients with previous episodes of ischaemic heart disease (Merlini *et al*, 1994; Martinez-Sales *et al*, 1998). Furthermore, they are positively associated with known risk factors for ischaemic heart disease (Lowe *et al*, 1991; Miller *et al*, 1996), have been shown to assist in predicting which patients are likely to die following acute myocardial infarction and have been shown to be independent risk factors for death from acute coronary events (Agewell *et al*, 1998). Despite these associations, to the author's knowledge only one study has investigated the effect of diet on markers of thrombin formation (Temme *et al*, 1999).

The objective of the present study was to evaluate the effect of dietary lipids on blood concentrations of fibrinogen and markers of thrombin generation, platelet indices, white and red blood cell counts and platelet reactivity in pigs. Where appropriate, the results have been compared with the current state of knowledge on the effects of dietary lipids on these variables in human beings.

6.2. Materials and Methods

Experimental animals, diets and procedure

The experimental design, including animals, diets and procedure are described in Chapter 3.

Analytical methods

Thrombin-antithrombin III complex concentration

Assay method

On the first and last day of the study, plasma thrombin-antithrombin III complex (TAT) concentrations were assayed in duplicate using a quantitative sandwich enzyme immunoassay designed for the determination of TAT concentrations in human plasma (Enzygnost TAT, Behring, Behringwerke AG, Marburg, Germany) according to the manufacturer's instructions. Blood was collected into syringes containing 3.8% trisodium citrate at a ratio of 9 parts blood to 1 part citrate. Plasma was harvested within two hours of collection following centrifugation at 1500 × g for 10 min and transferred to 1.8-mL cryo tubes (A/S Nunc, Rosklide, Denmark). Samples were stored at -87°C until assayed.

An overview of the method is as follows. In the first incubation step, TAT present within the sample binds to rabbit anti-human thrombin antibodies, which is attached to the surface of plastic wells. Unbound constituents are removed by washing and in a second incubation, free antithrombin III determinants are bound to an excess of peroxidase-conjugated rabbit anti-human antithrombin III antibodies, which are added to the well. Following a second wash, hydrogen peroxide and chromogen are added and after 30 min the peroxidase-dependent reaction is stopped with dilute sulphuric acid. The resultant colour change is then determined by reading each sample against distilled water at 492 nm.

Validation of the assay for use on porcine samples

Prior to the study, this assay was validated for use on porcine samples using two pigs. Anaesthesia was induced as previously described (Chapter 3) and maintained by halothane and oxygen. The cranial superficial epigastric vein was cannulated with a 3.2-cm, 20 gauge catheter (Wingless Quik-Cath, Baxter, Deerfield, IL, USA) and normal saline was continuously infused for 30 min at a rate of 200 µL/kg/hr with the aid of a syringe pump (Ivac Syringe Pump model 2000, Ivac Corporation, Hampshire, United Kingdom). This was immediately followed by an infusion of reconstituted thromboplastin (Dade Thromboplastin IS, Baxter Diagnostics Inc, Illinois, USA) at 200 µL/kg/hr for 60 min in order to induce a 'hypercoagulable state'. Saline was then infused at the same rate for a further 120 min. Blood was taken directly from the cranial vena cava into 4.5-mL draw vacutainers containing 0.5 mL of 3.2% buffered citrate at the following intervals: prior to the saline infusion (-30 min), immediately prior to thromboplastin infusion (0 min) and then 15, 30, 45, 60, 90, 120 and 180 minutes later.

Baseline plasma TAT concentrations, measured at –30 and 0 minutes, ranged between 7 and 18 µg/L (Figure 6.1). Thrombin-antithrombin III complex concentrations increased during the infusion of thomboplastin and peaked at concentrations of approximately 47 µg/L. The TAT concentrations returned to baseline one hour after discontinuing the thromboplastin infusion. During the study, all TAT concentrations were within the measurement range (2 - 60 µg/L) indicated by the manufacturer of the assay (Anon, 1994).

These results suggested that the assay was capable of detecting an increasing concentration of thrombin generation in response to an intravenous infusion of thromboplastin in pigs and support the observations of Ravanat *et al* (1995), who found that the immunoreactivity of porcine plasma samples was equivalent to that of human beings.

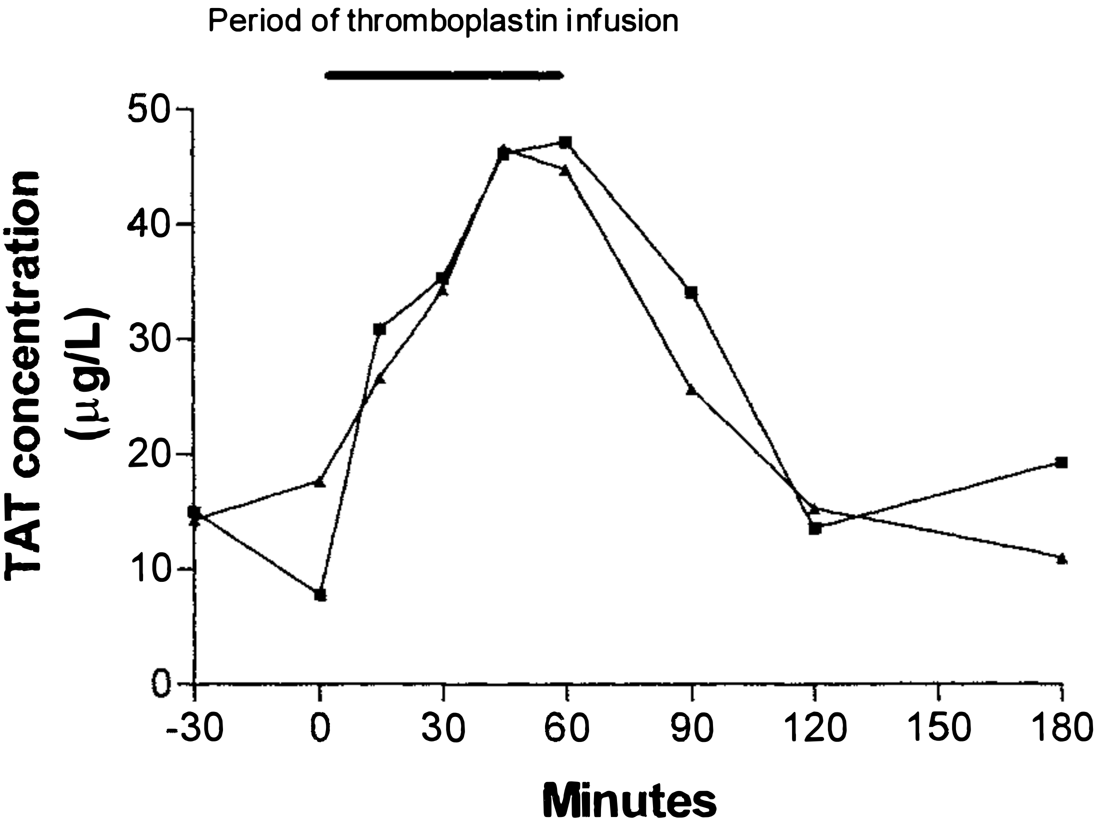

Figure 6.1 Changes in plasma TAT concentrations in two pigs in response to intravenous infusion of thromboplastin.

Plasma fibrinogen concentrations

Blood was collected into syringes containing 3.8% trisodium citrate at a ratio of 9 parts blood to 1 part citrate. Blood was centrifuged at 1500 × g for 10 min within two hours of collection and the plasma harvested and transferred to 1.8-mL cryo tubes (A/S Nunc, Rosklide, Denmark). Samples were stored at –87°C until they were analysed as a batch approximately one month later.

Plasma fibrinogen concentrations were determined in a dual-channel, semi-automated coagulometer (Coatron Junior, TECO Medical Instruments GmbH, Ergoldsbach, Germany) using the principle developed by Clauss (1957). Citrated plasma was diluted 1:10 with Owren's veronal buffer (Baxter Diagnostics Inc, Illinois, USA). A 0.1-mL aliquot of the diluted sample was incubated in a reaction cuvette at 37°C for 3 min before the addition of 0.1 mL of bovine thrombin (Dade Data-Fi thrombin reagent, 100 NIH units/mL, Baxter Diagnostics Inc, Illinois, USA), which initiated clot formation. The thrombin reagent had been reconstituted with a 1 mg/mL light kaolin solution (Fort Richard Laboratories Ltd, Auckland, New Zealand) in accordance with the method recommended by the manufacturer of the coagulometer. The same batch of thrombin reagent was used for all fibrinogen determinations. The plasma fibrinogen concentration was determined by comparing the clotting time with a 5-point standard curve, which had been made using a human fibrinogen standard (Dade Data-Fi Fibrinogen Calibration Reference, Baxter Diagnostics Inc, Illinois, USA). Fibrinogen concentrations were determined in duplicate on each sample. The assay was repeated if duplicate determinations disagreed by more than 10%. Control limits (mean ± 2 standard deviations) were determined using control plasma (Dade Ci-Trol Coagulation Control, Level 1, Baxter Diagnostics Inc, Illinois, USA). During the analysis, controls were run at the beginning of the study, after every 10 samples, at the end of the study and whenever a new bottle of thrombin reagent was used. No control value outside the control limits was encountered during the study.

Haematology

An automated veterinary haematology analyser (Cobas Minos Vet, ABX Hematologie, Montpellier, France), which used an impedance method, was used to determine the red blood cell count, white blood cell count, platelet count, mean platelet volume (MPV) and plateletcrit (PCT) in whole blood containing ethylenediaminetetraacetic acid (EDTA, final concentration 1.8 mg/mL). Analysis was carried out within one hour of blood collection. Any abnormalities in the analysis of the samples, such as platelet clumping and the presence of red blood cell fragments, were indicated by the analyser with flags. Flagged parameters were excluded from the analysis.

Platelet aggregation

Preparation of platelet-rich and platelet-poor plasma

Citrated blood was transferred to 12-mL plastic tubes and centrifuged at 200 × g for 10 min at ambient temperature (3360 rotor, Omnifuge 2.0 RS, Heraeus Sepatech, Kalkberg, Germany). The supernatant, platelet-rich plasma (PRP), was removed and the remaining contents of the tube were centrifuged at 5000 × g for 15 min. The supernatant, platelet poor plasma (PPP) was harvested. Any remaining red blood cells in the PRP were removed by centrifugation at 500 × g for 1 min.

Standardisation of platelet-rich plasma

The platelet count in the PRP was standardised using the following procedure. The platelet count was determined in whole blood containing the anticoagulant, EDTA, using the automated haematology analyser. The platelet count was divided by the ratio of the volume of PRP harvested to the volume of the original whole blood sample to give an estimate of the platelet count in the PRP (PLT_{PRP}). The quantity, in millilitres, of PRP (Q_{PRP}) required to yield 4 mL of standardised (360×10^9/L) PRP was calculated as follows:

$Q_{PRP} = 360 \div PLT_{PRP} \times 4$ .... **Equation 6.1**

The quantity of PPP (Q_{PPP}), in millilitres, required was calculated as follows:

$Q_{PPP} = 4 - Q_{PRP}$ .... **Equation 6.2**

The required volumes of PRP and PPP were then added together and gently mixed.

Platelet agonist

Native equine collagen fibrils (Collagenreagent Horm, Nycomed Arzneimittel GmbH, Munchen, Germany) were used as the platelet agonist at four different final concentrations. The collagen is supplied in a vial containing 1 mg of collagen fibrils per mL of isotonic glucose (pH 2.7). A fresh stock solution of collagen (96 μg/mL) was made every second day using SKF Horm buffer (Nycomed Arzneimittel GmbH, Munchen, Germany). Every day, the stock solution was diluted further with SKF Horm buffer to make working collagen solutions at concentrations of 12, 24, 36 and 48 μg/mL. When added to the sample, the final concentrations of collagen fibrils per mL of standardised PRP were 2, 4, 6 and 8 μg respectively.

Performing Aggregometry

Platelet aggregation in PRP was measured by the turbidometric method of Born (1962) using a 4-channel aggregometer (Monitor IV, Helena Laboratories, Beaumont, Tx, USA). The aggregometer was pre-warmed for 20 min prior to use. A light intensity check was performed each day before use to ensure that all four channels were functioning correctly. Samples (250 μL) of standardised PRP were transferred to four reaction cuvettes (each containing a magnetic stirring rod) and incubated at 37°C for 2 min in the heating block. The cuvettes were then transferred to the channels of the aggregometer and 50 μL of each of the four concentrations of working collagen solution were added to the cuvettes using an electronic pipette. The test was initiated automatically following depression of the electronic pipette. At the conclusion of the 6-min aggregation time, the percent aggregation versus time curve was printed out. Each test was performed in duplicate.

Filtragometry

Filters and filter housing

The technique of filtragometry was reviewed in Section 4 of Chapter 1. In previous filtragometry studies (Hornstra & ten Hoor, 1975; Larsson *et al*, 1990; Soderback *et al*, 1991; Broijersen *et al*, 1993), nickel filters with a pore size of 20 μm, a diameter of 6 mm and a free surface (total surface area of the pores divided by the total surface area, expressed as a percentage) of 50% were used. Due to the expense of these filters, a monofilament polyester sifting and filter fabric (Polymon, No. PES-20/13, Schweizerische Seidengazefabrik AG, Zurich) was initially assessed for suitability. This material was available with a 20-μm mesh opening but unlike the nickel filters, the fabric had a free surface of only 13%. Furthermore, due to the weave of the fabric and its flexible nature, the pore size was nominal rather than absolute. A smaller free surface meant that fewer pores needed to be blocked for any given change in pressure (Hornstra & ten Hoor, 1975). This may improve the sensitivity of the technique, particularly when the number of platelet aggregates is small. On the other hand, the nominal rating of the fabric was potentially disadvantageous with some pores being slightly greater or less than 20 μm. This could potentially contribute to the variability of the technique.

A carpenter's punch was used to make 13-mm diameter discs of the filter fabric suitable for use in Swinnex 13 filter holders (Millipore Corporation, Massachusetts, USA). Pre-filters were fashioned in a similar manner from synthetic, waterproof paper (Yupo, Oji-Yuka Paper Company Ltd, Tokyo, Japan). These were cut into O-shaped rings, which reduced the effective area of the filter fabric to a diameter of 4 mm. This is comparable to previous studies (Hornstra & ten Hoor, 1975; Larsson *et al*, 1990; Soderback *et al*, 1991; Broijersen *et al*, 1993).

Since the total surface area of the filter, the free surface of the filter and the draw rate of the pump were known, the flow rate per pore could be compared with that described previously (Table 6.1). Despite reducing the effective filter diameter of the fabric to 4 mm, the flow rate per unit area was half that created in the previous studies.

Scanning electron microscopy of the filters showed marked platelet adhesion to the monofilament polyester (Figure 6.2). It was apparent that the physical properties of the monofilament polyester aided cell adhesion and spreading. The adhesion of cells to a non-physiological surface is believed to be a four-step process involving the adsorption of plasma proteins, cellular contact with the material, the formation of bonds of attachment and the spreading of platelets on the material (Baier & Meyer, 1984). For this reason, and because the pore size was nominal only, this material was not used in subsequent studies.

Table 6.1 A comparison between the flow rate across the filter in previous studies (Hornstra & ten Hoor, 1975; Larsson *et al*, 1990; Soderback *et al*, 1991; Broijersen *et al*, 1993) with the flow rate across the filter in the current study.

Parameter	Previous studies	Current study	
		Fabric filter	Nickel filter
Filter diameter (mm)	2.3	4	2.3
Flow rate (mL/min)	2.0	1.8	1.8
Filter area (mm^2)	4.15	12.57	4.15
Free surface (%)	50	13	50
Pore area (mm^2)	2.08	1.63	2.08
Flow rate/pore area (mL/min/mm^2)	0.96	0.49	0.87

Nickel filters, as used in previous studies, were subsequently used. Hornstra and ten Hoor (1975) reported that nickel filters did not support platelet adhesion and aggregation and consequently, obstruction of the filter is likely to be due to the formation of platelet clumps that form *in vivo*. The construction of the filter housing was not described in any of the previous studies so the Swinnex filter holders, used in the initial investigation of the polyester fabric, were adapted for use. These reusable filter holders comprise of four parts: the housing, which is in two halves that screw together, a gasket, and a polypropylene filter support, which the filter sits upon. One of the housing halves has a female luer lock inlet while the other half has a male luer slip outlet. Both halves of the filter housing were modified to take two stainless steel inserts. The filter support was not used. The stainless steel insert made for the male part of the housing was made with a 25-μm central depression (the thickness of the filter). The insert made for the female half had a flat face and when the filter housing was screwed together, the two faces of the inserts were flush and provided a tight seal. Occasionally however, the filter would become displaced within the depression so the stainless steel inserts were modified. The depression for the filter was deepened to 1 mm and the insert made for the female part of the housing was made with a flange that would tighten down onto the outside of the filter when the housing was screwed together.

Despite the use of the nickel filters and an experimental design similar to that used in previous studies, platelets could be seen adhering to the nickel filters (Figure 6.3). Developing a filter made of material sufficiently inert to prevent platelet adhesion was cost-prohibitive so for pragmatic reasons, the nickel filters continued to be used. It was recognised that the filtragometry technique described here would not only determine the degree of platelet aggregation occurring prior to filtragometry, as proposed by Hornstra and ten Hoor (1975), but it would determine the degree of platelet aggregation occurring during filtragometry.

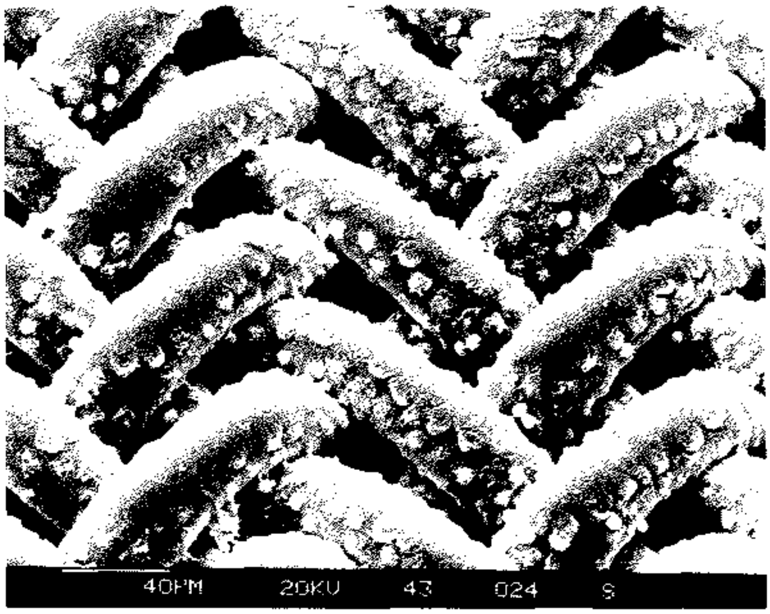

Figure 6.2 Scanning electron micrograph of the monofilament polyester filter with 20-µm pores evaluated for suitability for filtragometry. Platelets can be seen adhering to the filter. For this reason, and because the pore size was nominal rather than absolute, this material was not evaluated further.

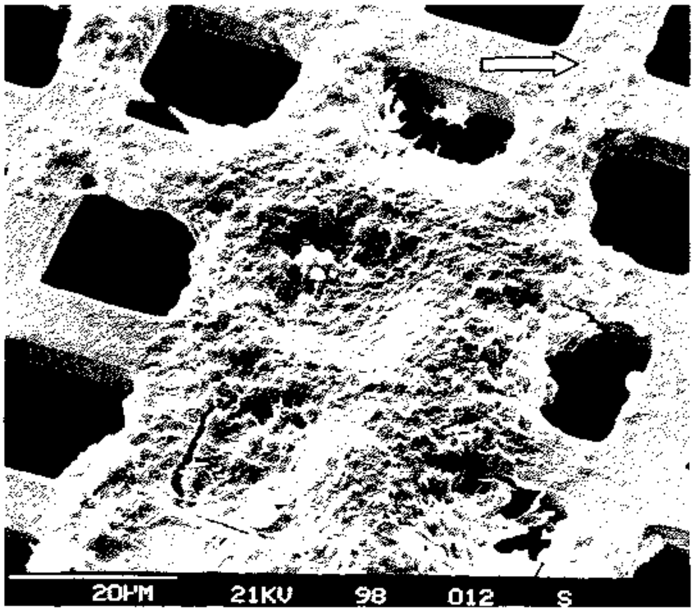

Figure 6.3 Scanning electron micrograph of a nickel filter with 20-µm pores following filtragometry. The arrow indicates an area of platelets that have adhered to the nickel filter.

Evaluation of a suitable anticoagulant

Homstra and ten Hoor, in their inaugural description of filtragometry, evaluated the use of three anticoagulants (Homstra & ten Hoor, 1975). The anticoagulants included sodium citrate, at a final concentration in blood of 0.0034 mg/mL; hirudin, at a final concentration of 0.1 mg/mL; and heparin, at a final concentration of 5 IU/mL. The time taken for the pressure to reach 5 mmHg (t_A) was significantly longer (287 s versus 131 s) when citrate was used as the anticoagulant. Scanning electron micrography of the filters showed that the pores were occluded by platelet aggregates in which red blood cells were trapped. Fibrin was not observed. For this reason, it was initially decided that citrate would not be used and that heparin would be investigated as an anticoagulant in this study at final concentrations of 5 IU/mL and 10 IU/mL. Unlike the study of Homstra and ten Hoor (1975) however, large quantities of fibrin could be seen on the filters at both heparin concentrations (Figure 6.4). Consequently, the use of trisodium citrate, at a final concentration of 0.0038 mg/mL in blood, was evaluated. Fibrin strands were not evident in scanning electron micrographs when this anticoagulant was used (Figure 6.5) although more sophisticated techniques, such as immunogold labelling (Poujol *et al*, 1998), may have revealed the presence of smaller quantitites of fibrin. Trisodium citrate was subsequently used as the anticoagulant for this study.

Low-molecular weight heparin (Fragmin, Kabi, Stockholm, Sweden) has been used by other groups of researchers at a final concentration in blood of 10 IU/mL (Soderback *et al*, 1991; Broijersen *et al*, 1993) but due to its expense, it was not considered for use in this study.

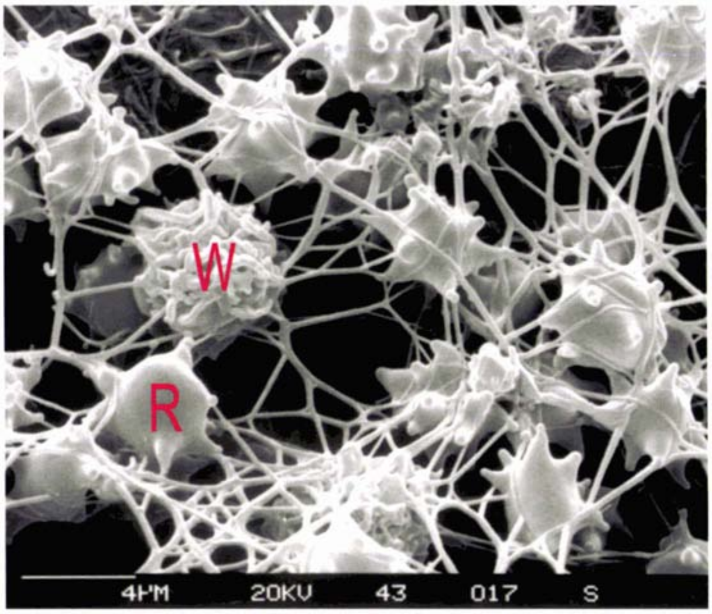

Figure 6.4 Scanning electron micrograph of a nickel filter following filtragometry using heparin at a concentration of 10 IU per mL of whole blood. Note the presence of crenated red blood cells (R) and white blood cells (W) entangled in strands of fibrin.

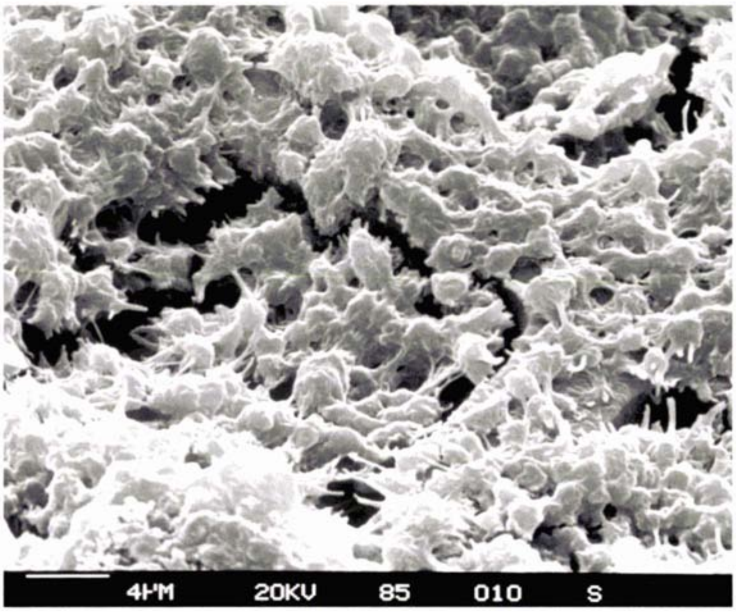

Figure 6.5 Scanning electron micrograph of a nickel filter following filtragometry using trisodium citrate at a final concentration in blood of 0.0034 mg per mL of whole blood. Note the sheet of platelets obstructing a pore in the filter and the absence of fibrin.

Description of the filtragometry system

A diagram showing the components of the filtragometry system used in this study is shown in Figure 6.6. The system was primed with a 3.8% (w/v) trisodium citrate solution at the start of each study. Whole blood was drawn from a 10-mL tube by means of a Harvard Apparatus infusion/withdrawal pump (unknown model number, Harvard Apparatus Ltd, Massachusetts, USA) at a rate of 1.6 mL/min, which was as close to the draw rate used in previous studies (1.8–2.0 mL/min) as this pump allowed (Hornstra & ten Hoor, 1975; Soderback *et al*, 1991). The addition of a pulsatile blood pump (model 1405, Harvard Apparatus Ltd, Kentucky, USA) to the filtragometry system, prevented the sedimentation of red blood cells. This was set to withdraw and redeliver 0.6 mL of blood to and from the tube each second. Pressure tubing, specifically designed as a component in pressure monitoring assemblies (PT60M, Viggo-Spectramed, Singapore), was used to connect componentry. The blood tube, the filter and the interconnecting tubing were immersed in a water bath at 37^{O}C. All solutions used in the filtragometry system were made with double-distilled water and then filtered to remove particles larger than 0.22 µm (model 25952-1L, Corning disposable polystyrene filtration system, New York, USA). All components of the filtragometry system were siliconised prior to use (SurfaSil, Pierce, Illinois, USA).

The pressure difference was measured with a pressure transducer (Statham P23XL, Gulton-Statham, New York, USA) on either side of the filter. The pressure transducers interfaced with a preamplifier (NeuroLog NL900-424, Digitimer Ltd, Hertfordshire, UK). The analogue signal was converted to a digital signal (MacLab model 8S, ADInstruments, Sydney, Australia) and the data were recorded by a PowerMac 7100/80 (Apple Computer Inc., California, USA) running MacLab Chart, version 3.4.2 (ADInstruments, Sydney, Australia).

A small pressure difference across the filter was noted when the priming solution (citrate) was drawn across the filter. This baseline pressure difference was subtracted from the pressure difference associated with drawing blood across the filter to give a net change in pressure (ΔP).

The end-point found to be the most repeatable by Hornstra and ten Hoor (1975) was the time taken to reach 5 mmHg (T_a), which corresponds to a 25% blockage of the filter. However, in the filtragometry system described for the current study, a sudden loss of pressure was noted in some tracings when ΔP exceeded approximately 5 mmHg. Occasionally, a small gas bubble could be seen in the tubing distal to the filter at the same time as the drop in pressure. Although there were no leaks evident in the system, gas contained within blood may have come out of solution, resulting in trace artefact. It is also possible that on some occasions, dislodgement of a platelet aggregate from the filter may have accounted for the drop in pressure. In this study, the tracings appeared to be most repeatable over the first two to four minutes of exposure of blood to the filter. Consequently, the end-point used in this study was the ΔP after two minutes of exposure of the filter to the blood ($\Delta P_{2\ min}$).

Scanning Electron Microscopy

As part of the evaluation process, many filters were examined by scanning electron microscopy after blood had passed over them to ensure that platelet aggregates were obstructing the pores. The filters were prepared for scanning electron microscopy in the following manner. Phosphate-buffered saline (PBS) was drawn over the filter at the end of the study to remove extraneous blood components. The filters were then placed in PBS containing 2% glutaraldehyde overnight. The following morning, the filters were processed by gently mixing them for 10 min on a mechanical mixer in the following solutions (in order): PBS (twice) and acetone at concentrations of 25%, 50%, 75% and 95% v/v. The filters were then mounted with double-sided tape onto aluminium studs and coated in gold using a Balzers SCD050 sputter coater (BAL-TEC AG, Liechtenstein). Specimens were examined using a Cambridge 250, mark 3 scanning electron microscope (Cambridge, England) at an acceleration voltage of 20 kV.

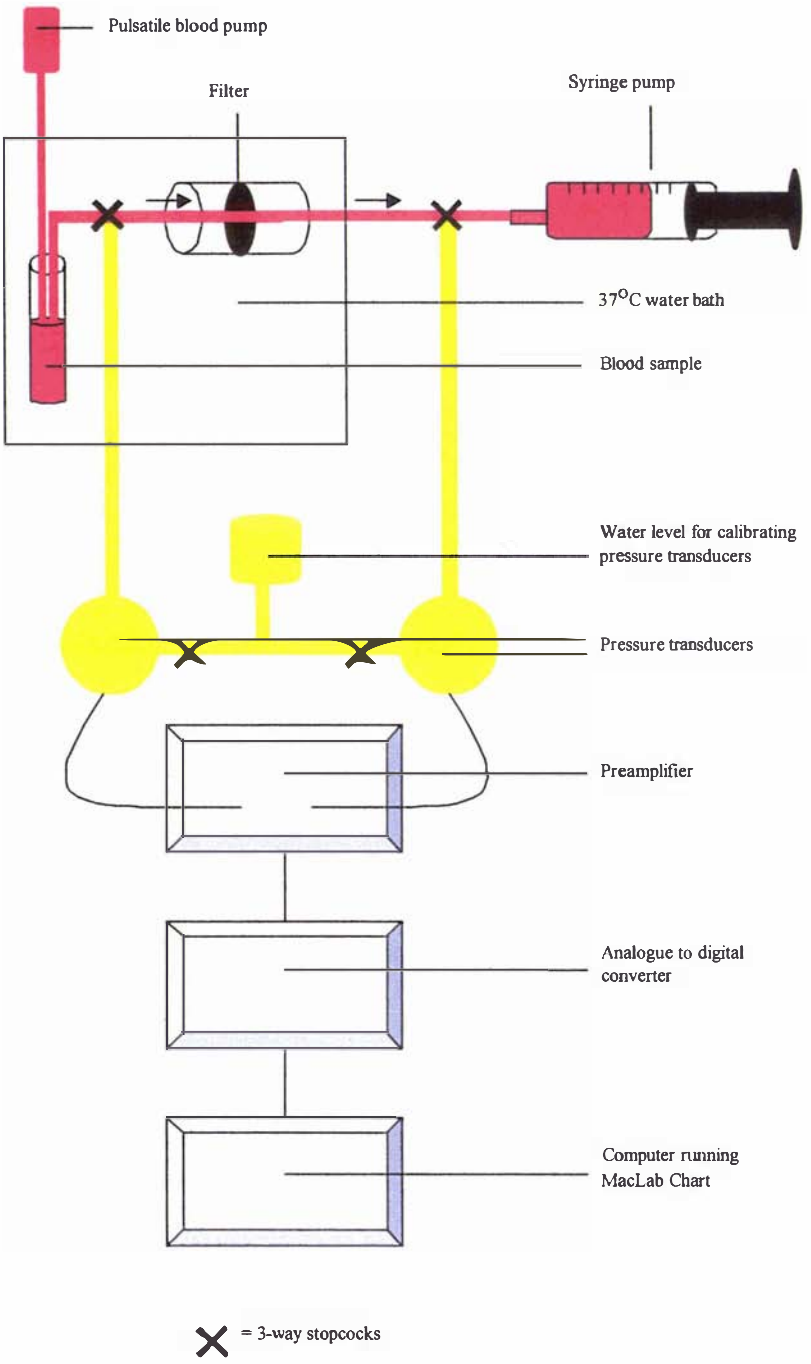

Figure 6.6 Diagram of the filtragometry system used in this study.

Statistical analysis

Analysis of variance was used to compare the effect of diet on the concentration of each of the haematological variables using the concentration at day 1 as a covariate. The statistical model considered the effect of blocking for each pen of pigs.

The aggregometry response to collagen was considerably less than that previously reported in our laboratory (KAC James, unpublished data). The method used to standardise the PRP was the only notable difference in methodology. Previously, the platelet count was estimated manually using a haemocytometer, whereas in this study, a calculation was used to standardise the PRP. In a subsequent study, involving a group of pigs of the same age (n=16), the method for determining the platelet count of the PRP used in the present study was compared with an automated platelet count of the PRP. The results of this subsequent study are reported in the Appendix to Chapter 6. This additional study showed that the method used here overestimated the platelet count within the PRP, resulting in its overdilution with PPP. The calculation used in this study was based on an erroneous assumption that the PRP would contain almost all the platelets within the blood sample.

Despite this error, the aggregometer did not at any stage during the study indicate that the absorbances of the standardised PRP samples were too low for aggregometry to be performed. This indicated that although the standardised PRP samples had a lower platelet count than anticipated, the results could still be evaluated. At the 2 µg/mL collagen concentration, only one sample (from the group receiving milkfat) exceeded 10% aggregation. The large number of 'non-responders' at the other concentrations of agonist precluded the evaluation of standard end-points, such as the percent maximum aggregation or the maximum slope of the aggregation trace (Kinlough-Rathbone *et al*, 1983). Consequently, the data were evaluated binomially (ie. 'responders' vs 'non-responders'). A response was defined as maximum platelet aggregation >10%.

A chi-square analysis of the aggregometry data was performed to determine whether there was any interaction between dietary group and collagen concentration. For each diet and collagen concentration, ANOVA was performed on the proportion of samples in each group to exhibit more than 10% aggregation. A plot of the residuals versus the fitted values showed no apparent trends, thus validating the use of ANOVA in this case.

For the filtragometry data, a residual plot indicated increasing variance with group means, even after the data were log transformed. Consequently, the data were analysed using Friedman's test. This is a non-parametric analysis of ranks that allows for a blocking variable. Missing values were estimated with treatment group medians.

All analyses were performed using Genstat 5 statistical software (Genstat 5 Committee, Oxford, England). A p-value of <0.05 was considered significant.

6.3. Results

There was no refusal of food by any pigs throughout the duration of the study. The weight gain was less in the pigs fed the carbohydrate diet than in pigs fed the coconut and milkfat diets (Table 3.2).

The effect of varying the lipid component of the diet on the mean concentrations of TAT and fibrinogen, platelet indices and red and white blood cell numbers is shown in Table 6.2. Compared with the olive oil and coconut oil groups, the mean log plasma TAT concentration was higher in the fish oil group. Compared with all other groups, the mean fibrinogen concentration was higher in the fish oil group. The mean platelet count was higher in the fish oil group than all other groups and the olive oil group had a lower platelet count than all other groups.

The mean MPV was higher in the olive oil group than all other groups. The group receiving the carbohydrate diet had a higher mean MPV than groups receiving either the milkfat or fish oil diets. The mean plateletcrit was highest in the fish oil group. The group receiving the carbohydrate diet had a higher mean plateletcrit than the groups receiving olive oil and milkfat. The mean WBC was higher in the carbohydrate and fish oil groups than the olive oil and coconut oil groups. There was no significant difference in the mean RBC count across treatment groups.

Table 6.2 Mean concentrations of markers of thrombosis and haematological variables in growing pigs fed diets containing fish oil, milkfat, olive oil, coconut oil or cornstarch at 4% w/w.

Variable	Units	Dietary Treatment					LSD[1]	DF[2]
		Carbo[3]	Fish	Milkfat	Olive	Coconut		
TAT[4]	µg/L	2.51^{ab} (12.30)	2.79^{b} (16.28)	2.14^{ab} (8.50)	1.93^{a} (6.89)	1.98^{a} (7.24)	0.75	25
Fibrinogen	mg/dL	187.0^{a}	208.0^{b}	174.3^{a}	174.5^{a}	177.9^{a}	9.3	19
Platelets	$\times 10^{9}$/L	503^{b}	649^{c}	479^{b}	413^{a}	486^{b}	61	19
MPV[5]	fL	7.71^{b}	7.33^{a}	7.37^{a}	8.14^{c}	7.40^{ab}	0.34	19
Plateletcrit	mL/L	3.93^{b}	4.79^{c}	3.49^{a}	3.37^{a}	3.66^{ab}	0.42	19
WBC count[6]	$\times 10^{9}$/L	20.6^{b}	20.8^{b}	19.8^{ab}	15.9^{a}	16.2^{a}	2.8	18
RBC count[7]	$\times 10^{12}$/L	6.9^{a}	6.7^{a}	6.6^{a}	6.9^{a}	6.6^{a}	0.5	19
Aggregation[8]		0.83^{a}	0.17^{d}	0.50^{b}	0.33^{c}	0.61^{b}	0.15	8

[1] Least significant differences of the means at the 5% level.
[2] Degrees of freedom.
[3] Carbohydrate diet.
[4] Thrombin-antithrombin III complex concentration. The data have been log transformed. Back-transformed data are presented in brackets.
[5] Mean platelet volume
[6] White blood cell count
[7] Red blood cell count
[8] Proportion of samples in each treatment group that demonstrated more than 10% aggregation.
Differing letter superscripts denote significant differences between treatment groups.

Chi-square analysis of the aggregometry data indicated that there was no interaction between the collagen concentration and diet (p=0.87; 12 df). Given the uniform unresponsiveness to collagen at the 2 µg/mL concentration, data from only the 4, 6 and 8 μg/mL collagen concentrations were used to investigate the effect of diet on platelet aggregation. As anticipated, there was a highly significant effect of collagen concentration on aggregation ($p < 0.01$, 8 df). Dietary effects are shown in Table 6.2. The extent of platelet aggregation, expressed as the proportion of samples in each group that demonstrated more than 10% aggregation, was as follows (from greatest to least): carbohydrate>coconut>milkfat>olive oil>fish oil.

The average pressures at 2 min from duplicate filtragometry runs are shown in Figure 6.7. There was a significant effect of diet ($p = 0.040$). When the analysis was re-run excluding the coconut oil group, there was no significant difference between the remaining treatment groups. This suggests that the group receiving coconut oil had the lowest median $\Delta P_{2\ \mathrm{min}}$ and there were no significant differences between the other dietary groups.

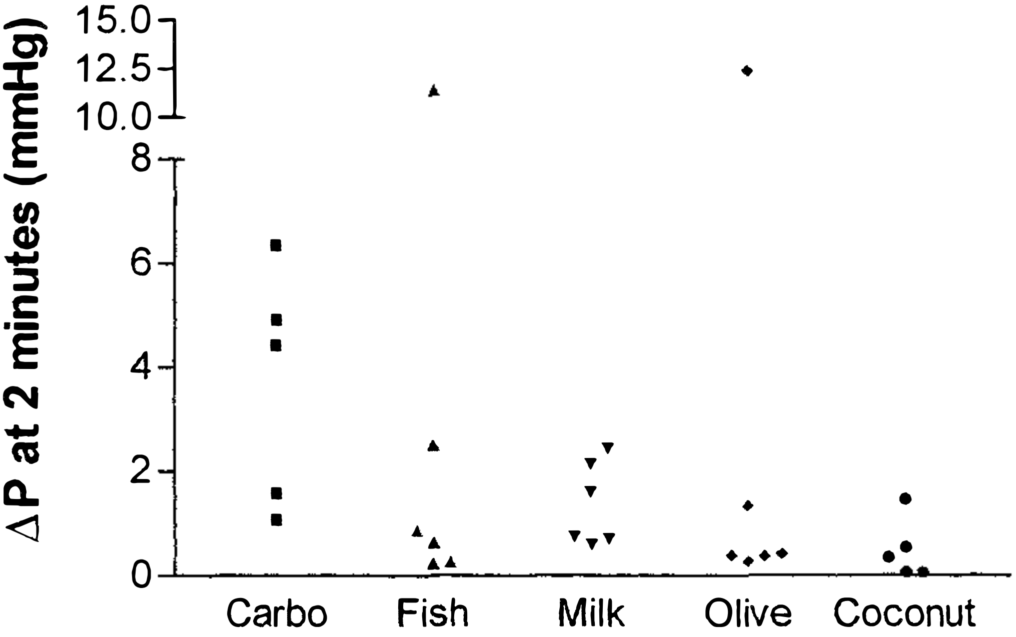

Figure 6.7 The change in pressure (in mmHg) that develops two minutes after drawing whole blood across a nickel filter is shown for each group of pigs who have been fed diets containing either 4% w/w cornstarch (carbohydrate), fish oil, milkfat, olive oil or coconut oil.

6.4. Discussion

In this study, the inclusion of fish oil in the diet of pigs had affected some variables in a way that could be interpreted as 'prothrombotic' compared to the other dietary treatments. The fish oil group had the highest concentrations of TAT, fibrinogen, platelets and white blood cells and the highest plateletcrit. In contrast, platelet function, as assessed by aggregometry, was lowest in the group receiving fish oil. Some populations that consume large amounts of n-3 fatty acids have remarkably low rates of acute myocardial infarction (Bang & Dyerberg, 1980; Kromann & Green, 1980). In a recent, critical review of all prospective cohort studies investigating the association between fish consumption and mortality associated with coronary heart disease, intakes of 40-60 g/d were associated with a reduction in risk in high-risk populations (as evaluated by coronary heart disease death rate) (Marckmann & Gronbæk, 1999). Conversely, fish intake was not associated with a reduction in death rate in low-risk populations. It is currently unclear whether this protective effect in high-risk populations is provided by long-chain n-3 polyunsaturated fatty acids or by some other component of fish. However, animal models of myocardial infarction have demonstrated reduced infarct size when the diet is supplemented with purified n-3 fatty acids (Otsuji *et al*, 1993; Zhu *et al*, 1994).

It is possible that the very high inclusion rate of n-3 fatty acids in the current study was responsible for some of the apparent 'prothrombotic' effects of fish oil. In the largest prospective cohort study examining the relationship between fish intake and coronary death performed to date (n = 44 895), the risk of coronary heart disease was slightly higher in the group which ate the most fish (120 g/d) compared to the group which ate the least (<10 g/d) (Ascherio *et al*, 1995). A smaller study from Finland also showed an association between increased consumption of non-fatty, freshwater fish and coronary heart disease mortality but a similar relationship existed between fish consumption and mercury intake (Salonen *et al*, 1995). The increased incidence of coronary heart disease mortality may have been due to mercury-induced lipid peroxidation rather than a direct effect of fish consumption (Salonen *et al*, 1995).

The pigs receiving olive oil had the lowest, or equal lowest, TAT, fibrinogen, plateletcrit and WBC concentrations. Platelet function, as assessed by aggregometry, was the second lowest. Compared to the other dietary lipids and using the profile of tests evaluated in this study, olive oil appears to be relatively 'anti-thrombotic'. This is noteworthy because the consumption of a Mediterranean-style diet, which is rich in olive oil, is apparently protective against coronary artery disease (Ferro-Luzzi & Branca, 1995; Majem-Serra *et al*, 1995; Carluccio *et al*, 1999). Mechanisms responsible for this protective effect may be associated with lower levels of lipid peroxidation associated with consumption of a Mediterranean-style diet (Mancini *et al*, 1995) and a direct inhibitory effect of oleic acid on endothelial activation (Carluccio *et al*, 1999). The current study indicates that olive oil may also inhibit the activity of the coagulation cascade and platelet aggregation.

Thrombin-antithrombin III concentration

A key finding in this study was that the inclusion of fish oil in the diet of pigs resulted in significantly higher plasma TAT concentrations than both the olive oil and coconut oil diets. Despite the key role of thrombin in thrombosis (Badimon *et al*, 1994), and its association with ischaemic heart disease (Lowe *et al*, 1991; Merlini *et al*, 1994; Miller *et al*, 1996; Agewell *et al*, 1998; Martinez-Sales *et al*, 1998), to the author's knowledge there has been only one other study that has investigated the effect of diet on markers of thrombin formation and/or activity. This was a randomised, crossover study in healthy men and women who consumed diets enriched in lauric, palmitic and oleic acid for 6 weeks (Temme *et al*, 1999). No significant difference in plasma F1 + 2 concentrations were observed between groups.

In the current study, the effect of dietary fats on plasma TAT concentrations in pigs was evaluated because unlike FPA and F1 + 2, there is good cross-reactivity between the commercial immunoassay, developed for use on human samples, and porcine plasma (Ravanat *et al*, 1995). Also, preliminary work described in this chapter, demonstrated the ability of this assay to detect thrombin in porcine plasma. The lack of difference in plasma TAT concentration between the pigs receiving the oleic acid-enriched diet (olive oil) and those receiving the lauric and palmitic acid-enriched diet (coconut oil) is in agreement with the findings of Temme *et al* (1999) in healthy human beings. The significance of the fish oil-induced elevation in the plasma TAT concentration, in terms of increased risk of thrombosis, cannot be determined from this study and a model of thrombosis, such as that described in Chapter 4, would be required to address this issue. Whether lower daily intakes of fish oil have similar effects on thrombin formation is also unknown.

Plasma fibrinogen concentration

The fish oil diet resulted in higher plasma concentrations of fibrinogen than the other diets. This finding is in contrast with a crossover study in men in which the effects of ingesting diets containing milkfat, partially hydrogenated fish oil and partially hydrogenated soybean oil for three weeks were compared (Almendingen *et al*, 1996). In that study, the milkfat diet resulted in a small but significantly higher median plasma fibrinogen concentration than the partially hydrogenated fish oil diet. According to a recent review by Knapp (1997), most studies have failed to document an effect of dietary n-3 fatty acid supplementation on plasma fibrinogen concentrations in human beings. Reports of increases in plasma fibrinogen concentration following supplementation with marine fish oils are uncommon. Schmidt *et al* (1992) supplemented the diet of 24 healthy volunteers with 4 g of n-3 polyunsaturated fatty acids daily for nine months and found a significant increase in the plasma fibrinogen concentration, but this study lacked a control group. Highly significant, high-amplitude seasonal variations (peak-trough difference of 28%)

in plasma fibrinogen concentrations have been documented in human beings (Maes *et al*, 1995). Although the results from the current study may reflect a species difference, it is possible that the very high intake of marine fish oils may have had an unexpected prothrombotic effect. Dietary vitamin E concentrations were not standardised across the treatment groups and variables other than the type of fat may have been responsible for this effect. In healthy human beings, vitamin E concentrations within the diet have previously been shown to result in no significant changes in plasma fibrinogen concentrations (de Maet *et al*, 1994).

In the current study, apart from the fish oil diet, there was no significant difference in plasma fibrinogen concentration between any of the other dietary groups. In human beings, the effect of dietary lipids on plasma fibrinogen concentration remains unclear. Marckmann and co-workers (1994) found no significant differences in plasma fibrinogen concentrations between a low saturated/monounsaturated, high fibre diet and an average Danish diet when served for two weeks. Similarly, there were no significant differences in the plasma fibrinogen concentrations between vegans, ovolacto vegetarians and individuals who ate moderate or high quantities of meat, despite the marked difference in the dietary fat intake between these groups (Li *et al*, 1999). No differences were observed in the plasma fibrinogen concentrations between healthy individuals who ate diets enriched with myristic or palmitic acid (Tholstrup *et al*, 1994) or between healthy individuals who ate diets enriched in lauric or palmitic acid (Temme *et al*, 1999). In contrast, some studies have shown an effect of dietary fat on plasma fibrinogen concentrations. A diet high in stearic acid (18:0) resulted in significantly higher plasma fibrinogen concentrations in human beings than a diet high in myristic (14:0) and lauric (12:0) acid (Bladbjerg *et al*, 1995). In a recent study, diets enriched with monounsaturated fatty acids (oleic acid) resulted in lower plasma fibrinogen concentrations than diets enriched with saturated fatty acids (palmitic or lauric) (Temme *et al*, 1999). The difference was significant at the 0.05 level but not at the 0.02 level, which was considered statistically significant by the authors. In the current study, there was no significant difference between the oleic acid-enriched diet (olive) and the palmitic and lauric acid-enriched diet (coconut).

Platelet indices

In the current study, there was a tendency for the platelet count to be inversely proportional to the MPV. The pigs fed the fish oil diet had the highest platelet count but the lowest MPV. The effect of fish oil supplementation in the current study in pigs contrasts to most studies in human beings, which have shown that the consumption of long chain n-3 polyunsaturated fatty acids reduces platelet counts and/or increases platelet volumes (Dyerberg & Bang, 1979; Goodnight *et al*, 1981; Hay *et al*, 1982; Houwelingen *et al*, 1987; Brown & Roberts, 1991; Nelson *et al*, 1991; Innis *et al*, 1993). In human beings, the fall in platelet count with fish oil supplementation coincides with increased platelet survival, suggesting that the fall in platelet count is due to a decrease in platelet production (Hay *et al*, 1982). Platelet kinetic studies would need to be performed in pigs to determine whether the high platelet counts observed in the current study are due to an increase in platelet survival and/or an increase in platelet production.

In the current study, the pigs fed the oleic acid-enriched diet (olive oil) had the highest MPV and the fewest platelets. The addition of canola oil, which is also high in oleic acid, to milk formula given to neonate piglets has previously been shown to result in similar changes in platelet indices (Innis *et al*, 1993; Innis & Dyer, 1999). Increasing the ratio of oleic acid, supplied principally as canola oil, to palmitic acid (16:0) resulted in a decrease in platelet number and an increase in platelet size (Innis *et al*, 1993). Similar effects on platelet size and number were noted when the formulae were made of canola oil rather than soybean oil, the latter oil being higher in LA and palmitic acid (Innis & Dyer, 1999).

It is apparent however, that not all dietary lipids rich in oleic acid have identical effects on platelet indices. When neonate piglets were given a formula containing canola oil blended with other oils to mimic the lipid composition of infant formula, the platelet count was lower and the platelet volume was higher than a blend with a similar lipid composition containing predominantly soybean oil (Innis & Dyer, 1999). Furthermore, a formula made with high oleic sunflower oil, and with an almost identical fatty acid profile to a 100% canola oil formula, did not lower platelet counts or raise mean platelet volumes (Innis & Dyer, 1999). These findings suggest that specific properties of canola oil, rather than its fatty acid profile per se, may influence platelet morphology and number. It has been suggested that the effect of canola oil could be due to the position of fatty acids on the glycerol backbone of triglycerides or due to the quantities and/or types of phytosterols within canola oil (Innis & Dyer, 1999). Given that in the current study, olive oil had similar effects on platelet indices, it may be possible that olive oil and canola oil share similar properties, besides their oleic acid content. One similarity is that triolein, a triglyceride with oleic acid at all three positions on the glycerol backbone, is the predominant fatty acid in both oils (Eskin *et al*, 1996; Firestone *et al*, 1996).

The relative quantities of saturated and unsaturated fat in the diet influences platelet size and number. Newborn piglets, receiving milk replacement formula containing 100% soybean oil or canola oil, had lower platelet counts and higher mean platelet volumes than piglets receiving these oils blended with palm oil and coconut oil, which are good sources of saturated fatty acids (Innis & Dyer, 1999). In a recent cross-sectional study, the thrombotic tendency of male subjects who were habitual meat-eaters (high meat consumers and moderate meat consumers) was compared with that of vegetarians (vegans and ovolacto vegetarians) (Li *et al*, 1999). The platelet count was lower in the vegans than the ovolacto vegetarians but the vegans had larger mean platelet volumes than the other groups. These groups represent a wide spectrum of dietary fat intakes (saturated fat and cholesterol intakes being higher in the meat eaters and polyunsaturated fat intake being higher in the vegetarians) although other nutritional differences, such as energy or protein intake, may also explain this effect.

White blood cell count

The coconut and olive oil diets resulted in significantly lower WBC counts than the fish oil and carbohydrate diets. Two observations, with large residuals, were excluded from the analysis. Had these outliers been included, they would have lowered the mean WBC count in the milkfat group but raised the coconut oil mean WBC count. This would have resulted in only the olive oil group having significantly lower WBC counts than the fish oil and carbohydrate groups.

It is difficult to provide an explanation for the effect of dietary lipids on the WBC counts observed in this study. Fish oil supplementation has been shown to attenuate the chemotactic response of neutrophils and their generation of leukotriene B_4 (Sperling *et al*, 1993). Reduction in leucocyte margination and migration may provide a possible explanation for the higher number of leucocytes observed in circulation in the pigs receiving the fish oil diet than those receiving the olive oil and coconut oil diets. Differential white blood cell counts were not performed in this study so it is not possible to determine which cell type (lymphocytes and/or neutrophils) contributed to the differences. It could be argued however, that given that the carbohydrate diet is effectively a 'basal diet' and that there were no differences in WBC count between the carbohydrate, fish oil and milkfat diets, the olive and coconut oil diets may possess properties that lower WBC counts rather than the fish oil diet elevating WBC counts.

With the exception of long chain n-3 fatty acids, very few studies have investigated the effects of dietary fat on leucocyte counts. Dietary supplementation with fish, fish oil or purified long-chain n-3 polyunsaturated fatty acids in healthy subjects has been shown to either decrease the total leucocyte count, predominantly through a reduction in granulocytes, or result in no change in leucocyte number. A fish-based diet, fed as the evening meal to 12 healthy men for 6 weeks, reduced the total leucocyte and neutrophil count compared to a control, non-fish diet (Brown & Roberts, 1991). Similarly, compared to a basal diet, dietary supplementation with 6 g/d DHA for 90 days in 11 healthy men resulted in a decrease in the circulating number of total leucocytes and granulocytes (Kelley *et al*, 1998). In contrast, no differences in white blood cell numbers were observed in a study that compared the effects of a low-fat high-fish diet with a low-fat low-fish diets and the typical American diet in 22 healthy subjects over 40 years of age (Meydani *et al*, 1993). However, the high-fish diet only provided 1.23 g/d EPA and DHA, which is less than the previous two studies. Similarly, there was no difference in the effect of feeding 100 g/d fish paste or meat paste to healthy male subjects for 6 weeks (Houwelingen *et al*, 1987). In this study the fish paste provided 1.7 g EPA and 3.0 g DHA.

Red blood cell count

There was no significant difference in the erythrocyte count between dietary groups. Since the mean lifespan of porcine erythrocytes is 62 days (Bush *et al*, 1955), only one-third of the erythrocytes would have been replaced during the course of the study. This may have precluded an effect of diet on the RBC count from being observed.

Evidence for the role of erythrocytes in haemostasis and thrombosis is provided by a number of observations, recently reviewed by Andrews and Low (1999). Firstly, a reduction in bleeding time can be achieved by transfusing red blood cells and conversely, polycythaemic states can predispose to thrombosis. Secondly, some inherited red blood cell disorders are associated with an increased risk of thrombosis despite the fact that the classic components of haemostasis are normal in these patients. Red blood cells may participate in haemostasis by increasing blood viscosity, acting as a source of platelet agonists, expressing adhesion receptors and possibly by providing a source of phosphatidylserine for contact activation. Apart from one study that has shown increased mortality due to cardiovascular disease in women with high haematocrits (Elwood *et al*, 1974), there is little evidence to suggest that the erythrocyte count contributes to coronary events. The baseline haematocrit had no predictive value for recurrent coronary events or all-cause mortality in the PARIS-1 study (Lowe *et al*, 1985). In the Diet and Reinfarction Trial (DART), the haemoglobin concentration, which is strongly associated with haematocrit, was associated with decreased odds of all-cause mortality in patients who had previously experienced myocardial infarction (Burr *et al*, 1992). Although the red blood cell count has been shown to be higher in patients with coronary artery disease than those without, this was explained by cigarette smoking in multivariate analysis (Kostis *et al*, 1984).

Aggregometry and filtragometry

The platelet aggregation results were consistent with those from previous studies in human beings, which have shown that the platelets of subjects who consume diets containing a high content of long-chain n-3 fatty acids are likely to demonstrate less aggregation than the platelets of subjects whose diets contain high concentrations of saturated fatty acids, such as coconut oil and milkfat (see Chapter 1 for review). Oleic acid, which is abundant in olive oil, inhibits transmembrane signal transduction by decreasing the synthesis of phosphatidylinositol 4,5-biphosphate (PIP_2) (Nunez *et al*, 1990). This results in reduced platelet concentrations of diacylglycerol, an important secondary messenger in the platelet activation pathway (Marcus, 1996). These observations may explain why the group of pigs receiving the olive oil diet had the second lowest level of platelet aggregation.

Platelet aggregation was highest in the group of pigs that received the carbohydrate diet. In the study reported in Chapter 4, the group of pigs receiving a milkfat diet tended to have less AA and more EPA and DHA in their platelet phospholipids than the pigs receiving a carbohydrate diet. As has been discussed in Chapter 1, an increasing ratio of EPA to AA within platelet phospholipids usually results in antiaggregatory and vasodilatory effects due to the formation of series 3 eicosanoids at the expense of series 2 eicosanoids. The platelet phospholipid fatty acid compositions of the diets were not determined in this study, but a low ratio of EPA to AA within the platelet phospholipids of the pigs receiving the carbohydrate diet may explain this observation.

Aggregometry requires a standardised number of platelets and it does not take into account the effect of diet on platelet numbers or other blood components. Hornstra and ten Hoor (1975), who originally described the technique of filtragometry, considered that the nickel filter trapped platelet clumps that had formed *in vivo* because the *in vitro* conditions of the filtragometer did not support platelet adhesion and aggregation. They suggested that this technique might be a sensitive method for detecting prothrombotic states. Consequently, filtragometry was selected as the method for evaluating platelet function in whole blood. In the current study however, porcine platelets could be seen adhering and spreading on the nickel filters (Figure 6.3). This suggests that using the methodology described in this chapter, filtragometry is unlikely to provide a determination of platelet aggregate formation *in vivo*. At best, it provides a measure of platelet reactivity *in vitro* and consequently, it offers no advantages over whole blood aggregometry, or the use of newer technology analysing platelet function in whole blood such as the Platelet Function Analyzer 100® (reviewed in Chapter 1). Furthermore, this technique is labour-intensive and time-consuming and the repeatability of the method was poor when the change in pressure across the filter exceeded approximately 5 mmHg.

The lower $\Delta P_{2\ \mathrm{min}}$ in the group that received coconut oil is not in agreement with the aggregometry data, which indicated that this group had the second highest degree of platelet aggregability. When the raw data are examined (Figure 6.7), it can be seen that compared to the other groups, there was less spread in the data in the coconut oil group. The apparent lower $\Delta P_{2\ \mathrm{min}}$ in the group receiving coconut oil may therefore reflect this tighter clustering of data rather than a biological effect on platelet reactivity.

6.5. References

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Chapter 7. General discussion

7.1. *Discussion*

A myriad of epidemiological studies have been undertaken to determine potential risk factors for ischaemic heart disease in human beings. Well-established risk factors include, but are not limited to, plasma factor VII activity, plasma fibrinogen concentration, serum LDL-C concentration and cigarette smoking (Fuster *et al*, 1992). Epidemiological studies cannot prove cause and effect and it is incumbent on scientists to test the associations proposed by epidemiologists using appropriate end-points. Ideally, hypothesis testing should be conducted using the target species, but this is obviously associated with practical and ethical difficulties when human disease states are being investigated. Therefore, other species must be used. In many respects, the baboon provides the ideal model for atherothrombosis research because its vascular anatomy, platelet structure, coagulation and fibrinolytic structures and response to drugs closely resemble those of human beings (Harker *et al*, 1991). Practical considerations, such as the expense of the baboon model and the availability of test animals (the baboon is not available for biomedical research in New Zealand), need to be considered. The domestic pig (*Sus domesticus*) has been used extensively as a model in atherothrombosis research because this species has many physiological similarities to human beings (Ratcliffe & Luginbühl, 1971; Luginbühl *et al*, 1974; Lee & Lee, 1975; Gerrity *et al*, 1979; Dodds, 1982; Weiner *et al*, 1985, 1986; Fuster *et al*, 1986; Griggs *et al*, 1986; Kim *et al*, 1986; Rapacz *et al*, 1986; Thomas *et al*, 1986, 1990; Beynen & West, 1987; Nichols *et al*, 1990; Luhman *et al*, 1992; Mersmann *et al*, 1992; Softeland *et al*, 1992; Harris,KB *et al*, 1993; Atar *et al*, 1994; Moon *et al*, 1994). With this in mind, a major objective of the research reported here was to determine the suitability of the pig as a model for determining the effect of dietary lipids on lipid metabolism. This was the subject of Chapters 2 and 3. Chapter 4 describes a pilot study that was undertaken to evaluate a balloon angioplasty model of arterial injury in pigs for assessing the thrombogenicity of dietary lipids. Chapters 5 and 6 focussed on identifying the effect of dietary lipids in pigs on potential indicators of thrombosis.

In order to make the pig a more practical model for lipoprotein research, the validation of simple methods for determining serum HDL-C, LDL-C and VLDL-C concentrations in pigs were evaluated in Chapter 2. Compared with the more cumbersome method of sequential ultracentrifugation, the use of simple methods would allow large numbers of samples to be processed quickly and inexpensively. The study presented in Chapter 2 showed that there was excellent agreement between the sequential ultracentrifugation method and the simple methods for determining serum LDL-C and HDL-C concentrations. These methods were subsequently used in the study presented in Chapter 3. There was poor agreement between the simple method for determining the VLDL-C concentration and sequential ultracentrifugation. This may have been due to technical difficulties associated with harvesting VLDL from the small samples obtained following sequential ultracentrifugation. Another study, with a similar experimental design but using much larger serum samples, should be performed to determine whether this is indeed the case. The work presented in Chapter 2 has been published in *Nutrition Research* and the paper is appended at the end of this thesis.

The study presented in Chapter 3 determined the effect of a spectrum of dietary lipids commonly eaten by human beings on the fasting serum lipid composition of pigs. The results were then compared with the results of similar studies performed in human beings. This study showed that dietary lipids that have a hypercholesterolaemic effect in pigs tend to raise serum HDL-C concentrations, but not LDL-C concentrations. It was hypothesised that the lack of dietary influence on the serum LDL-C concentration could be due to either a lack of specificity of the ACAT enzyme or a lower level of regulation of the porcine LDL receptor by hepatocellular free cholesterol concentration. It was also proposed that since porcine plasma lacks CETP activity, very little cholesteryl ester contained within the HDL fraction is transferred to the LDL fraction (Knipping *et al*, 1987) and that this may reduce the effect of dietary lipids on LDL cholesterol in pigs. Regardless of the mechanism, such results would not be expected if a similar study were performed in human beings (Zanni *et al*, 1987; Katan *et al*, 1984).

The effects of dietary lipids on the serum HDL-C concentrations in pigs were similar to those previously observed in human beings and animals. The carbohydrate and fish oil diets resulted in lower serum HDL-C concentrations than did the milkfat, hydrogenated coconut oil and olive oil diets. Substituting dietary carbohydrate with saturated fat has previously been shown to result in an increase in the serum concentration of HDL-C in man and some animal species (Fisher *et al*, 1983; Chong *et al*, 1987; Quig & Zilversmit, 1989; Carlson & Kottke, 1990; Katan *et al*, 1994). The activity of LCAT, a key determinant of the serum HDL-C concentration, is reduced when diets are enriched with fish oil (Thornburg *et al*, 1995). In rabbits, coconut oil has been reported to raise HDL-C concentrations more profoundly than other sources of saturated fat. Trilaurin appears to be the triglyceride within coconut oil responsible for this effect (Quig & Zilversmit, 1989; Carlson & Kottke, 1990). In the study presented in Chapter 3 however, there were no significant differences in the mean serum HDL-C concentration between the hydrogenated coconut oil group and either the milkfat or olive oil groups.

The effects of dietary fat on serum triglyceride concentrations in the pigs in the current study were dissimilar to those usually documented in healthy human beings. In a meta-analysis of 27 controlled dietary trials, Mensink and Katan (1992) predicted that replacement of dietary carbohydrate with fat would result in a reduction in the serum triglyceride concentration in man. In the study presented in Chapter 3 however, the pigs receiving the milkfat and coconut oil diets had higher serum triglyceride concentrations than those receiving the carbohydrate diet. Katan *et al* (1994) predicted that the replacement of dietary saturated fat with monounsaturated fat would result in a slight increase in the serum triglyceride concentration in human beings, but in the research presented here, the oleic acid-enriched diet (olive oil) resulted in a significantly lower mean serum triglyceride concentration than the diets containing more saturated fat (milkfat and coconut oil). The fish oil-fed pigs had significantly lower serum triglyceride concentrations than those receiving either milkfat or coconut oil, which is in agreement with previous studies in human beings (Herold & Kinsella, 1986; Harris,WS, 1989, Katan *et al*, 1994).

Taken together, the results of Chapter 3 would suggest that there are substantial differences in lipid metabolism between young domestic pigs and adult human beings. Caution is therefore warranted when extrapolating studies measuring porcine serum triglyceride and cholesterol concentrations to man. Whether these differences would have been observed if adult pigs were used remains to be determined. In the current research programme, young pigs from commercial breeds (crosses of Duroc, Large White or Landrace) were used because they are less expensive to purchase and feed and they are easier to handle than their adult counterparts. Since maturity is reached at a lighter bodyweight, the use of adult, smaller breeds of pig may have been advantageous. With the exception of the Kune Kune however, small breeds, such as the Yucatan mini-pig and the Vietnamese pot-bellied pig, are not widely available in New Zealand. Kune Kune pigs were considered, but unlike commercial pig breeds, these pigs graze and do not thrive well on commercial pig rations[a]. Furthermore, there is more genetic heterogeneity within the Kune Kune breed and litter sizes are smaller. The work presented in Chapter 3 is in press (Allan FJ, Thompson KJ, James KAC, Manktelow BW, Koolaard JP, Johnson RN, McNutt PV. Serum lipoprotein cholesterol and triglyceride concentrations in pigs fed diets containing fish oil, milkfat, olive oil and coconut oil. *Nutrition Research* 21, 785-796, 2001).

A balloon angioplasty model of arterial injury in pigs is described in Chapter 4. In that study, there was some evidence to suggest that the pigs fed the milkfat

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[a] Information obtained from www.geocities.com

diet may have a greater thrombotic tendency than those fed the carbohydrate diet when thrombus size was evaluated by the radiolabelled platelet method. This finding was interesting because there were only a small number of observations and the fish oil group, which was initially included as a 'negative control', had to be withdrawn from the study.

Unlike previous studies (Steele *et al*, 1985; Lam *et al*, 1991), the study presented in Chapter 4 showed that arterial thrombus will form in the absence of deep injury. Creating superficial injury, rather than deep injury, may be desirable because of the difficulty in controlling the length of the tear (4 of 5 arteries had tears < 7mm long and one had a tear 21.5 mm long). Furthermore, deep injury appeared to be associated with thrombus size (the arteries with the longest and second longest tears had the largest and second largest thrombi) and it is possible that the influence of tearing may mask subtle dietary effects.

There are four major shortcomings associated with the model described in Chapter 4. Firstly, arterial thrombosis and thromboembolism are dynamic events (Lassila *et al*, 1990) and this is not evaluated in this model. In future studies, scintigraphic techniques, or the use of flow probes, could be employed to determine the impact of thromboembolism on final thrombus size.

Secondly, the model is expensive. As has already been mentioned, the use of a smaller breed of pig may lower costs associated with housing and feeding. Feeding the test diets for a shorter period of time would have an obvious impact on cost reduction. The 'ideal' feeding period, which optimises the ability to detect a dietary effect with the shortest feeding period, will not be easy to determine because thrombosis is multifactorial, involving the interaction between platelets, other blood cells, the vessel wall and components of the coagulation and fibrinolytic systems.

Thirdly, healthy arteries, rather than atherosclerotic arteries, are subjected to damage in this model. The acute coronary syndromes typically develop as a consequence of fissuring of atheromata (Fuster, 1994), resulting in exposure of the lipid core, the most thrombogenic component of the arterial wall (Femández-ortiz *et al*, 1994). This aspect of the pathogenesis of ischaemic heart disease is not taken into account in the current model. In order to improve the pathophysiological relevance of the current model, thrombus size could be evaluated at sites of angioplasty-induced atheroma rupture. Inherited hypercholesterolaemia LDL-C (IHLC) pigs, which develop atheromata by 2 years of age, would represent the ideal porcine model for this purpose (Overturf & Loose-Mitchell, 1992). Compared to healthy arteries, thrombus is likely to be larger at the site of atheroma rupture (Fernández-ortiz *et al*, 1994) and this model would indicate whether dietary lipids have the ability to modulate thrombus size in the face of high-level thrombogenic stimulus. It should be recognised that with this modification of the model, deep injury is created and as previously discussed, tear length may be difficult to control. A further consideration is that this modification of the current model would be expensive because IHLC pigs would need to be at least 2-years-old prior to undertaking studies.

The fourth major disadvantage of the model described in Chapter 4 is that the external iliac arteries, rather than the coronary arteries, were used. Angioplasty of the coronary arteries would have been ideal because thrombus formation at this site is the cause of ischaemic heart disease. However, the coronary arteries were not used because of the unavailability of angiography. Larger thrombi, with more consistency in thrombus size between pigs, were obtained in coronary arteries subjected to deep injury as compared with those obtained in carotid arteries (JJ Badimon, personal communication). Smaller within-group variances are more likely to result in significant treatment effects.

Whether or not the trend for the pigs fed the milkfat diet to have a greater thrombotic tendency than those fed the carbohydrate diet was due to changes in the activity of the coagulation cascade was the subject of Chapter 5. The APTT, PT, TT and plasma fibrinogen and factor VII concentrations were evaluated. The only dietary effect noted was a trend for the APTT to be longer in the pigs receiving the milkfat diet than the carbohydrate diet, indicating that a greater propensity for fibrin formation in pigs receiving the carbohydrate diet than the milkfat diet. Clearly, this finding cannot explain the greater thrombotic tendency of the pigs consuming the milkfat diet.

The effects of a wider range of dietary lipids on an array of variables that may influence thrombus size were evaluated in the study presented in Chapter 6. This study underscored the difficulties associated with determining whether a 'pro-thrombotic' state exists on the basis of examining platelet function or markers of thrombosis. The fish oil group had the highest concentrations of plasma TAT and fibrinogen, the greatest numbers of circulating platelets and white blood cells and the highest plateletcrit. These findings would suggest that the fish oil diet is more likely to induce a 'pro-thrombotic' state than the other test diets. Conversely, compared with the other dietary treatments, platelet aggregation was least in the fish oil group, indicating that thrombus size *in vivo* may be less when fish oil is fed. The dilemma is that we do not know which of these variables has the greatest influence on eventual thrombus size. Hence, it is essential that a thrombus end-point be established in an appropriate model. Once the model is established, the effects of other dietary factors, such as proteins, antioxidants and dietary supplements can also be evaluated. Examining the effects of diet on markers of thrombosis is of secondary importance although such studies provide insights into mechanisms that may lead to thrombosis.

In summary, this research programme has shown that there are differences between human beings and growing pigs in the effects of dietary lipids on plasma lipid composition. Caution is therefore warranted when extrapolating results from similar studies in growing pigs to human beings. A balloon angioplasty model of thrombosis in pigs was established and shows promise as a means of determining the thrombogenicity of dietary fats. A number of potential improvements to the model have been suggested. The development of such a model could greatly enhance studies aimed at furthering our understanding of the effects of diet on thrombosis in human beings.

7.2 References

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Appendix

Chapter 4

The solutions required and the method used to stain the specimens for morphometric determination of thrombus size.

Solutions Required:

1 Verhoeff's haematoxylin
2 0.2% martius yellow in 95% ethanol with 2% phosphotungstic acid
3 1% crystal ponceau in 1% aqueous acetic acid
4 0.2% aniline blue in 1% aqueous acetic acid
5 1% aqueous phosphotungstic acid
6 Saturated alcoholic picric acid

Method:

1	Bring sections to 70% ethanol	
2	Mordant in saturated alcoholic picric acid	5 min
3	Running water	5 min
4	Rinse in 70% ethanol	2 dips
5	Verhoeffs haematoxylin	10 min
6	Rinse of excess stain in water	2 dips
7	Differentiate until grey/blue in 2% ferric chloride	3-5 s
8	2% sodium thiosulphate to remove iodine	10 s
9	Rinse in running water	2 min
10	Rinse in 70% ethanol	2 min
11	Cover with martius yellow	2 min
12	Rinse briefly in water	
13	Cover with crystal ponceau	5 min
14	Rinse briefly in water	
15	1% phosphotungstic acid	5 min
16	Drain and cover with aniline blue	5 min
17	Rinse briefly in water	
18	1% aqueous acetic acid	1 min
19	Dehydrate in 100% ethanol (2 changes)	
20	Clear and mount	

Results:

Elastin fibres and nuclei	black	Erythrocytes	yellow
Muscle and cytoplasm	pink	Collagen	blue
Fibrin	red		

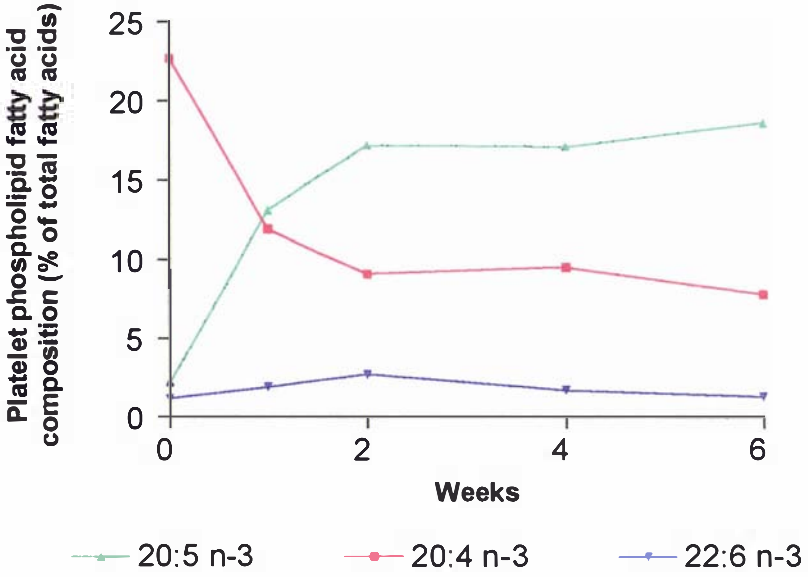

Figure A-1 The effect of feeding a diet containing 10% w/w concentrated marine fish oil to pigs for 6 weeks. Although the diet was not palatable, and the pigs did not eat their daily ration, it can be seen that most of the changes in the platelet phospholipid fatty acid content of eicosapentaenoic acid, arachidonic acid and docosahexaenoic acid was achieved in the first two weeks of feeding the diets.

Chapter 6

The platelet count of the PRP for aggregometry was standardised empirically using the method described in Chapter 6. The aggregometry response was lower than that which has been previously achieved in pigs of this age in our laboratory. In previous studies in our laboratory, platelet counts of the PRP had been performed using a haemoctyometer prior to standardising the PRP to 360 $\times 10^9$/L. In the current study, it was suspected that the empirical method overestimated the platelet count in the PRP resulting in its excessive dilution during standardisation. In a subsequent study in a group of pigs of the same age (n=16), the empirical method for determining the platelet count of the PRP used in Chapter 6 was compared to an automated platelet count of the PRP (Figure II). The regression equation was Y = 1.6x – 73.4 ($r^2 = 0.55$, p = 0.001). This demonstrates that the calculated platelet count, predicted by regression, overestimates the actual platelet count by approximately 60%. This subsequently led to the excessive dilution of the PRP and is likely to have accounted for the lower than anticipated aggregometry readings.

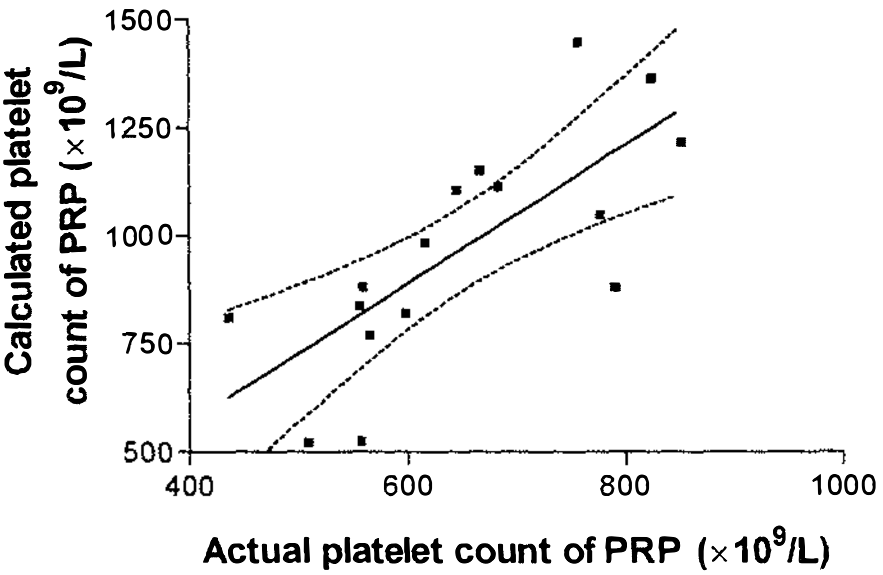

Figure A-2 Calculated platelet count of platelet-rich plasma, using the formula described in Chapter 6, versus an automated platelet count of platelet-rich plasma, using a haematology analyser (Cobas Minos Vet, ABX Hematologie, Montpellier, France) in a group of growing pigs (n=16).

Glossary

14:0	Myristic acid
16:0	Palmitic acid
18:0	Stearic acid
$\Delta P_{2\,\mathrm{min}}$	Change in pressure after two minutes
% en	Percentage energy supplied by a nutrient within a diet
AA	Arachidonic acid (20:4 n-6)
ADE	Apparent digestible energy
ADP	Adenosine diphosphate
APTT	Activated partial thromboplastin time
ATP	Adenosine triphosphate
apoB 100	Apolipoprotein B-100
CETP	Cholesteryl ester transfer protein
CFRs	Cyclic flow reductions
cpm	Counts per minute
DHA	Docosahexaenoic acid (22:6 n-3)
EDTA	Ethylenediaminetetra-acetate
EPA	Eicosapentaenoic acid (22:5 n-3)
F1 + 2	Fragment F1 + 2
FAMEs	Fatty acid methyl esters
FPA	Fibrinopeptide A
HDL	High density lipoprotein
HDL-C	High density lipoprotein cholesterol
IHLC	Inherited hypercholesterolaemia LDL cholesterol
IP_3	Inositol 1,4,5-trisphosphate
keV	Kiloelectron volts
LA	Linoleic acid (18:2 n-6)
ALA	α-linolenic acid (18:3 n-3)
LCAT	Lecithin:cholesterol acyl transferase
LDL	Low density lipoprotein
LDL-C	Low density lipoprotein cholesterol
OT	Obturation time
PCV	Packed cell volume
PFA-100®	Platelet Function Analyzer-100®
PGH_2	Prostaglandin H_2
PGI_2	Prostacyclin I_2
PGI_3	Prostacyclin I_3
PIP_2	Phosphatidylinositol 4,5-biphosphate
PLTP	Phospholipid transfer protein
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
PT	Prothrombin time
TAT	Thrombin-antithrombin III
TF	Tissue factor

TT	Thrombin time
TXA_2	Thromboxane A_2
TXA_3	Thromboxane A_3
TXB_2	Thromboxane B_2
VLDL	Very low density lipoprotein
VLDL-C	Very low density lipoprotein cholesterol