LIPIDS AND LIPOPROTEINS IN NORMAL AND COMPLICATED PREGNANCIES

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ABSTRACT

It is clear that there are substantial changes in lipids and lipoproteins in normal pregnancy with up to four-fold rise in triglycerides and up to a 50% increase in cholesterol. It is generally held that such changes exist to meet the demands of the feto-placental unit in terms of cellular proliferation and development and also to support lactation. In some situations the mechanisms regulating this physiologic hyperlipidaemia may malfunction. In women with pre-eclampsia, plasma free fatty acids and triglyceride concentrations climb substantially above those observed in normal pregnancy and do so well in advance of the appearance of clinical manifestations of the disorder. Lipoprotein classes are not homogeneous entities but include subclasses of differing function and metabolic potential. For example, particles such as low density lipoprotein itself can be broken down into distinct subpopulations with small, dense LDL (LDL-III) exhibiting strong atherogenic potential, in particular, promoting the development of foam cells and stimulating endothelial dysfunction. In the non-pregnant situation, plasma triglyceride is the major factor promoting the synthesis of LDL-III. However, this relationship is not linear, rather recent cross-sectional studies imply a threshold effect: synthesis of LDL-III proceeding only once triglyceride concentrations go beyond a 'threshold' value. To the best of our knowledge no longitudinal studies have been designed to examine this phenomenon in individual subjects. The physiological changes in plasma triglyceride which accompany pregnancy provide such an opportunity. In addition, at the time this thesis was initiated there was no information on the concentrations of very low density (VLDL) and LDL subfractions in pre-eclampsia. The primary objectives of the thesis were: to establish the lipoprotein subfraction changes occurring during the physiological hyperlipidaemia of normal pregnancies and the pathological hyperlipidaemia of pre-eclampsia. In addition, the consequences of the altered lipoprotein metabolism in pre-eclampsia for the promotion of the characteristic endothelial dysfunction present in this disorder was examined, as well as the relationship between lipid changes and haemostatic factors during normal gestation. Finally, lipid and lipoprotein concentrations in pregnancies complicated by IUGR, a condition in which the placental appearance is similar to that seen in pre-eclampsia but where the maternal systemic problems of hypertension and proteinuria are absent, were documented.

Plasma lipids and lipoprotein subfractions were quantified in 10 normal pregnant women at 5 weekly intervals from 10 to 35 weeks of gestation, together with circulating hepatic lipase and serum oestradiol concentrations. Concentrations of $VLDL_1$, $VLDL_2$ and IDL increased in parallel as plasma triglyceride increased with advancing gestation, and LDL mass increased by 70%. In addition, in six of the 10 women studied, the LDL subfraction pattern was modified towards a smaller denser

pattern in a manner indicative of a "threshold" transition. The concept of a threshold was of critical importance to our understanding of this important aspect of lipoprotein physiology. This "threshold" transition, if it occurred, did so at varying gestational ages and triglyceride concentrations for different women.

Circulating concentrations of coagulation and fibrinolytic markers were measured and related to changes in plasma lipids and oestradiol. Strong and similar individual correlations were observed between increases in FVII, PAI, t-PA and D-dimer (but not vWF) and increases in both oestradiol and triglyceride. Associations between increments in plasma cholesterol and haemostatic factors (except for FVII), were somewhat weaker. These data suggest that oestradiol-induced hypertriglyceridaemia may be a cause of elevations in plasma Factor VII activity, PAI and t-PA, and fibrin turnover (D-dimer) during normal pregnancy, but is poorly related to the increase in vWF antigen.

Next, plasma concentrations of VLDL, and LDL subfractions and pre-heparin hepatic lipase activity were compared in women with pre-eclampsia and healthy, age, gestational age and weight-matched controls. Women with pre-eclampsia exhibited higher median plasma triglyceride,$VLDL_1$ and $VLDL_2$ concentrations, whereas total plasma cholesterol, IDL and total LDL concentrations were the same in cases and controls. Furthermore, women with pre-eclampsia demonstrated markedly elevated median plasma concentrations of small, dense LDL, LDL-III. Consistent with studies in non-pregnant populations, the concentration of small, dense LDL correlated positively with plasma triglyceride concentration. Since, both $VLDL_1$ and small, dense LDL-III are damaging to the endothelium, elevated concentrations of these lipoprotein species may contribute to the expression of pre-eclampsia.

Fourth, we have related lipid concentrations to endothelial behaviour in myometrial vessels bathed in plasma from normal and pre-eclamptic pregnancies. A significant correlation was observed between endothelial behaviour and apo AI concentrations, whereas no associations were seen with other lipid parameters measured. These results were consistent with data from studies in non-pregnant individuals where HDL cholesterol has been positively and strongly related to endothelial function. We postulated that this relationship may reflect a direct effect of HDL particles since they are antioxidative and cardioprotective. Alternatively, rather than a direct influence of Apo AI on endothelial behaviour, some other factor(s) dictating Apo AI metabolism and thus Apo AI concentrations may relate to endothelial behaviour. In particular, the efficiency of triglyceride catabolism which in the non-pregnant situation, is a major determinant of Apo AI and HDL-cholesterol concentrations, may be involved.

Next, a mechanistic scheme for the generation of the hyperlipidaemia in pre-eclampsia was devised. It was suggested that increased plasma cytokine (IL-1, TNF-α) concentrations resulting from activation of macrophages/neutrophils either directly or indirectly through endothelial activation, or the placenta itself, may enhance the peripheral lipolysis already activated in normal pregnancy by HPL. This, in association with cytokine mediated *de novo* hepatic fatty acid synthesis, would then result in an increased flux of free fatty acids to the liver. These are channelled predominantly into hepatic triglyceride synthesis and lead to an increased secretion (over and above that of normal pregnancy) of large, triglyceride-rich VLDL particles, which are also removed less efficiently than normal. Accumulation of free triglyceride occurs in the hepatocyte when there is saturation of this pathway. This triglyceride accumulation is sufficient to explain the fatty changes in the liver seen in pre-eclampsia and AFLP, which as previously discussed may be a variant of the pre-eclampsia process. Furthermore, it was suggested that increased concentrations of triglyceride-rich lipoproteins in the circulation may contribute both directly and, through the generation of small, dense LDL, indirectly to endothelial dysfunction and therefore expression of pre-eclampsia in the mother, despite potentially benefiting fetal nutrition.

Finally, we examined lipid changes in pregnancies complicated by IUGR. The results of this study demonstrated that LDL levels which normally increase by around 70% in uncomplicated pregnancies, fail to rise appropriately in pregnancies complicated by IUGR. The mechanisms for this observation are unclear but clearly, given the importance of LDL particles in delivering cholesterol to the placenta for hormonal synthesis and for transport to the fetus, such low levels may contribute to the pathogenesis of growth restriction. In addition, these data suggest that LDL-cholesterol measurements may be useful in identifying mothers with IUGR pregnancies.

In conclusion, the results of our novel studies have given deeper insight into the unique features associated with the hyperlipidaemia of normal pregnancy, particularly with reference to lipoprotein subfraction concentrations. Some of this information is clearly relevant to our understanding of the factors which govern generation of small, dense LDL in the non-pregnant population, and atherosclerosis. Furthermore, data presented in this thesis suggest that in some situations (pre-eclampsia and IUGR) mechanisms regulating this physiologic hyperlipidaemia may malfunction, with potential ramifications for the clinical expression or diagnosis of these conditions.

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PUBLICATIONS AND PRESENTATIONS ENSUING FROM WORK PERFORMED AS PART OF THESIS

1. PUBLICATIONS

1. **Sattar N,** Greer IA, Loudon J, Lindsay G, McConnell M, Shepherd J, Packard CJ. Lipoprotein subfraction changes in normal pregnancy: threshold effect of plasma triglyceride on appearance of small, dense low density lipoprotein. *J Clin End Metab* 1997; **82**: 2483-2491.

2. **Sattar N,** Bendomir A, Berry C, Shepherd J, Greer IA, Packard CJ. Lipoprotein subfractions in pre-eclampsia: pathogenic parallels to atherosclerosis. *Obstet Gynecol* 1997; **89**: 403-408.

3. **Sattar N**, Gaw A, Packard CJ, Greer IA. Potential pathogenic roles of aberrant lipoprotein and fatty acid metabolism in pre-eclampsia. *Br J Obs Gyn* 1996; **103**: 614-620.

4. **Sattar N**, Greer IA. Apo AI and Birthweight [letter]. *Lancet* 1998; **351**: 835.

2. Oral Presentations to Learned Societies.

1. **Sattar N**, Rumley A, Greer IA, Packard CJ Shepherd J, Lowe G· Fibrinolytic markers in pregnancy: relationship to perturbations in lipoprotein levels. British Association of Haemostasis and Thrombosis, St Andrews, March 1995.

2. **Sattar N**, Lindsay G, Greer IA, Packard CJ, Shepherd J. Lipoprotein Subfractions Changes in Normal Pregnancy. Association Of Clinical Biochemists, Glasgow, May 1995.

3. **Sattar N**, Lindsay G, Greer IA, Packard CJ, Shepherd J. Lipoprotein Subfractions Changes in Normal Pregnancy. European Atherosclerosis Society, Utrecht, Netherlands, June 1995.

4. **Sattar N**, Greer IA, Glen A, Gaw A. The value of non-fasting lipids in predicting pregnancy complications. International Society for the Study of Hypertension in Pregnancy. Oxford, September 1997.

5. **Sattar N**, Greer IA, Rumley A, Packard CJP, Lowe G· A longitudinal study of the relationship between lipid, haemostatic and hormonal changes during normal human pregnancy. International Society for the Study of Hypertension in Pregnancy. Oxford, September 1997.

6. **Sattar N**, Packard CJ, Greer IA. Lipids and lipoproteins in normal pregnancy and pre-eclampsia. Toxtalks meeting, San Fransisco, July 1997.

POSTER PRESENTATIONS

1. **Sattar N**, Bendomir A, Berry C, Shepherd J, Greer IA, Packard CJ. Lipoprotein subfractions in pre-eclampsia: pathogenic parallels to atherosclerosis. European Atherosclerosis Society meeting Florence 1996.

2. **Sattar N**, Bendomir A, Berry C, Shepherd J, Greer IA, Packard CJ. Lipoprotein subfractions in pre-eclampsia: pathogenic parallels to atherosclerosis. Scottish Society for Experimental Medicine, Glasgow 1997.

3. R Hayman, **N Sattar**, A Warren, I Greer, I Johnson, P Baker. A relationship between Myometrial resistance artery behaviour and circulating lipid composition? Supplement to journal of the Society for Gynaecological Investigation Vol 5, Number 1 (supplement) Jan/ Feb 1998 T445 p147A

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AUTHORS DECLARATION

The work presented in this thesis was performed solely by the author, except where the assistance of others is acknowledged.

N Sattar, April 1998

DEDICATION

This thesis is dedicated to my parents
for their gentle encouragement and strong support throughout the years.
I endeavour to live up to the standards they have set in their lives.

CHAPTER I

INTRODUCTION

1. PLASMA LIPIDS AND LIPOPROTEINS

1.1 Lipids

Lipids and fat soluble compounds are critical for normal metabolism in biological systems. Three specific lipid compounds, cholesterol, triglyceride, and phospholipids are found widespread throughout the body. Cholesterol occurs in both polar (free cholesterol), and non-polar (cholesteryl ester) forms. Cholesterol serves as a structural component of cell membranes and for the synthesis of steroid hormones and bile acids. Phospholipids, amphoteric molecules having a polar head and a non polar tail, are structural components of cell membranes. Phospholipids also function in intracellular signalling, solubilisation of non polar substances in the intestine, and stabilisation of alveoli in the lung. Triglycerides, which are non polar substances, are used primarily as a source of energy and fatty acids. Certain fatty acids derived from triglycerides are also involved in cell signalling. These three lipid components can be synthesised *de novo* or provided in the diet.

The difficulty of transporting lipids in blood, an aqueous medium, has been overcome by the formation of lipid/lipoprotein particles referred to as lipoproteins. Lipoproteins are spherical particles which contain the non polar lipids, cholesteryl ester and triglyceride, in their core and proteins and the more polar lipids, phospholipid and free cholesterol, on their surfaces. The polar portions of the latter are orientated towards the aqueous medium. The protein components, referred to as apolipoproteins (apo) or apoproteins, are found on the surface and extend into the core of the lipoprotein particle depending on the type of apolipoprotein. Currently six major subclasses of lipoproteins have been identified. These are: chylomicrons, very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL), high density lipoprotein (HDL) and lipoprotein little "a" [Lp(a)]. Each of these has a unique composition and content of protein and lipid which define their function.

The function and metabolic paths of the major lipoprotein subclasses are summarised as follows:

1.2 Chylomicrons

Chylomicrons are the largest of the lipoprotein particles. These particles have apolipoprotein apo B-48 as their structural protein and also contain apo-AI, A-IV, C's, and E. Chylomicrons are synthesised by the intestine following the ingestion of fat

and serve to transport dietary lipid from the intestine to various tissues in the body. Upon entering the circulation via the thoracic lymph duct the following scenario is envisioned to occur: The chylomicron particle binds to heparan sulfate proteoglycans lining the vascular epithelium where, in the presence of apo C-II, the triglyceride is lipolysed by lipoprotein lipase (LPL). This is likely a dynamic process, lipolysis of triglyceride within a single particle occurring at multiple sites. Another process which occurs involving chylomicron particles is the exchange of triglyceride for cholesteryl ester with HDL particles. The exchange is mediated by cholesteryl ester transfer protein (CETP). As the chylomicron loses lipid mass during lipolysis and becomes enriched in cholesteryl ester its affinity for the C apolipoproteins, which inhibit the binding of chylomicrons to hepatic chylomicron receptors is decreased. At the same time, apo E content of the chylomicron particle is increased which increases the affinity of the particle for the chylomicron remnant receptors. At some point, the ratio of apo C: apo E is low enough to promote avid binding of this particle to hepatic receptors, at which point the particle is removed from the circulation.

1.3 Very Low Density Lipoproteins

VLDL is the next largest lipoprotein particle. It is synthesised in the liver and has apoB-100 as its major structural protein. VLDL function primarily to carry lipid from the liver to different tissues throughout the body. The metabolism of VLDL is thought to be similar to that of chylomicrons, the particle undergoes LPL-mediated lipolysis and CETP-mediated exchange of triglyceride for cholesteryl ester with HDL. As with chylomicrons, VLDL lose apo C apoproteins and gain apo E during lipolysis. Unlike chylomicrons, lipolysed VLDL are thought to have a number of metabolic fates: 1. receptor mediated removal by the liver, 2. receptor - mediated removal by a proposed VLDL receptor found in skeletal muscle, 3. conversion to LDL via IDL.

VLDL can be separated into two major subfractions distinct in their metabolic properties and defined by their hydrated density - $VLDL_1$ (Sf 60-400), larger, triglyceride-rich particles, and $VLDL_2$ (Sf 20-60), smaller, relatively cholesterol - enriched species. Both can be synthesised directly by the liver, and in addition $VLDL_2$ are formed by the delipidation of $VLDL_1$. $VLDL_1$ are the preferred substrate of CETP and readily undergo neutral lipid exchange whilst $VLDL_2$ are overproduced in common hypercholesterolaemias.

1.4 Intermediate Density Lipoproteins

IDL are best characterised as being VLDL remnants, in transit between VLDL and LDL. These particles are rich in cholesteryl ester. In addition to the main structural protein, apo B-100, IDL has a relatively high concentration of apo E. Normally, the concentration of IDL in the plasma is low. IDL are thought to bind to heparan sulfate proteoglycans at which point their triglyceride and phospholipid are lipolysed by hepatic lipase (HL). This, in turn, results either in their uptake by hepatic receptors or

conversion to LDL. As with VLDL, IDL can be divided into subfractions.

1.5 Low Density Lipoprotein

LDL are the major cholesterol-carrying particles in the blood and function to deliver cholesterol throughout the body as well as to the liver for its metabolism, excretion or resecretion on VLDL. LDL has apo B-100 as its major protein component and, although it is derived from IDL, has a relatively low apo E concentration. Removal of LDL from the plasma occurs via an LDL receptor-mediated process, which is regulated, or via a scavenger receptors found on macrophages and fibroblasts. It is thought that when excessive amounts of LDL are cleared via this latter pathway, macrophages become cholesterol-laden foam cells which accumulate in the vascular endothelium, initiating the development of atherosclerosis.

LDL comprises several subpopulations of heterogeneous particles (the exact number depending on the method of separation used). In the early 1980s, Krauss and Burke separated LDL into four major subclasses using density gradient ultracentrifugation (DGUC) and gradient gel electrophoresis. The DGUC was later refined by Griffin et al (1990) to separate LDL into three subfractions - LDL-I (d=1.025-1.034), LDL-II (d=1.034-1.044), and LDL-III (d=1.044-1.060) and permit quantitation of these species. LDL-I is relatively large and lipid-rich whereas LDL-III is small, dense and rich in protein and phospholipid. These subfractions are believed to have differing atherogenic potential. Sufferers of CHD often have a predominance of LDL-III which is particularly atherogenic. The potential mechanisms explaining the atherogenicity of small, dense LDL-III are detailed in section 4.3.

1.6 High Density Lipoproteins

HDL are the most abundant lipoproteins in plasma but also have a significant extravascular presence. They are synthesised by both liver and small intestine, as discoid apo A containing particles and are present in the plasma in two major forms, HDL_2 and HDL_3. These can be further subclassified for example by immunoaffinity methods depending on their apo A content. Some HDL particles contain both apo AI and apo AII and thought to be of hepatic origin. Other particles contain only apo AI and appear to originate in the intestine.

HDL function as mediators of reverse cholesterol transport, the process responsible for the return of cholesterol from peripheral tissues to the liver. Free cholesterol from cells and the surface layer of triglyceride-rich lipoproteins transfer to the nascent HDL. This is esterified by lecithin:cholesterol acyl transferase (LCAT) and these cholesteryl esters move from the surface to the core of the HDL particle, which, as a result, transforms from a flattened disc into a more spherical particle. In so doing, a gradient is established allowing more free cholesterol to transfer to HDL. This cholesteryl ester is then transferred by CETP to triglyceride-rich lipoproteins, preferentially $VLDL_1$, in

exchange for triglyceride. The apo B-100 containing lipoproteins are cleared by the liver, thus completing reverse cholesterol transport. The triglyceride enriched HDL are then hydrolysed by HL forming smaller HDL_3 which are than available for free cholesterol acquisition.

An additional role for HDL is that of a repository for the small, exchangeable apolipoproteins (apoC and apo E) donating them to chylomicrons and VLDL prior to lipolysis. These apoproteins are later shed back into the HDL density range. HDL catabolism is performed by the liver via apo E-facilitated receptor uptake.

1.7 Lipoprotein (a)

Lipoprotein (a) is a more recently discovered lipoprotein whose physiological function is as yet unclear. It is positively associated with CHD and is postulated to be both thrombogenic and atherogenic. Plasma levels vary from nil to 200 mg/dl and have strong heritability. It can simplistically be described as LDL with an additional protein, apo (a), attached by a disulphide bond to the apoB. Apo(a) is synthesised by the liver and has a high degree of sequence homology with plasminogen. It is a highly glyosylated, hydrophilic protein with low affinity for lipid. Despite the similarity with LDL, Lp(a) binds less avidly to the LDL receptor.

2. THE APOLIPOPROTEINS

Apolipoproteins are responsible for the regulation of lipoprotein inter-relationships and the maintenance of lipoprotein homeostasis. They act as ligands for receptor binding and as cofactors for lipolytic enzymes.

2.1 Apolipoprotein A

The A apoproteins consist of three main apoA's - apo AI, apo AII and apo AIV. Apo AI is the main protein component of HDL accounting for 70% of its protein. It is also present to lesser extent on chylomicrons but is transferred to HDL on hydrolysis of the former. It is synthesised by both the liver and small intestine and has been shown to have several isoforms. Its main role is as the structural apoprotein for HDL and as a cofactor for LCAT. Apo AII, synthesised by the liver, is the second most abundant protein in HDL. It activates LCAT and appears to enhance the lipid binding properties of apoAI. Apo AIV is present on newly secreted chylomicrons but it is readily displaced and the majority of it is found free in the plasma. Its role is as yet uncertain but it possibly acts as an LCAT activator.

2.2 Apolipoprotein B

Apoprotein B in the delipidated state is insoluble in aqueous solutions and must therefore be denatured with, for example, sodium dodecyl sulphate or urea in order to be characterised. Two types of apo B are found in plasma - apoB-100 and apoB-48.

ApoB-100 is the main protein moiety of VLDL, IDL and LDL. It is synthesised primarily by the liver and is an obligatory component of these lipoproteins, being present as one mole per particle. It does not exchange between lipoproteins but remains with the particle from secretion to catabolism. It acts as a ligand for receptor recognition and uptake of the particle. However, conformational changes of the apoB during lipolysis of VLDL regulate the lipoprotein uptake so that it is only IDL and LDL that are cleared by this mechanism. VLDL appear to be cleared by an apoE-mediated mechanism.

ApoB-48 is as described above the major protein in chylomicrons. It is formed following editing of the apoB message so that a stop translation codon (UAA) is found at position 2153 rather than the codon (CAA) that codes for glutamine in apoB-100.

2.3 Apolipoprotein C

There are three apoC proteins - apoCI, apoCII and apoCIII. They are present on HDL, chylomicrons and VLDL, the first supplying the latter two with this protein on their entry into the circulation. ApoCI is the smallest of the three apoproteins and acts as an activator of LCAT. ApoCII is an essential cofactor of lipoprotein lipase. ApoCIII appears to have an inhibitory effect on the lipolysis and clearance of chylomicrons and VLDL. It exists in three forms depending on sialylation - $CIII_0$, $CIII_1$ and $CIII_2$, the subscript reflecting the number of sialic acid residues present.

2.4 Apolipoprotein E

ApoE, present on chylomicron remnants, VLDL and large HDL particles, is synthesised by the liver and functions as a ligand for the hepatic uptake of lipoproteins. Isoelectric focusing reveals three common apoE isoforms - E3, E2 and E4. Apo E2 and E4 arise from point mutations of the wild type, E3. The differing isoforms do have physiological effects, in particular on the metabolism of LDL precursors.

3. THE ENZYMES OF LIPOPROTEIN METABOLISM

3.1 Lipoprotein lipase (LPL)

LPL hydrolyses triglyceride in chylomicrons and VLDL and requires apoCII as a cofactor. It is found mainly in adipose tissue and skeletal muscle where it is bound to the capillary endothelium. In women LPL activity is greater in the gluteal adipose tissue than in the abdominal tissue, whereas in men the converse is true. Overall, the amounts of LPL in adipose tissue are higher in women than in men. Regular alcohol intake increases the amount of LPL in the adipose tissue, whereas exercise increases that in skeletal muscle.

3.2 Hepatic lipase (HL)

HL hydrolyses triglyceride in HDL_2 and by so doing mediates the conversion of HDL_2 to HDL_3. Additionally, it delipidates IDL to form LDL and is thought to be responsible for the interconversion between LDL subclasses. It is found on hepatic endothelial cells and concentrations in women are lower than those in men.

The triglyceride lipases are structurally related to pancreatic lipase. In addition to their role as enzymes, both have been shown to act as ligands for the binding of triglyceride-rich lipoproteins to cell membranes. Although each lipase is capable of hydrolysing each lipoprotein in vitro, in vivo they are selective - LPL preferentially acts on triglyceride-rich lipoproteins, and HL on HDL and LDL particles.

3.3 Cholesteryl ester transfer protein (CETP) and Lecithin cholesterol acyl transferase (LCAT)

LCAT and CETP are involved in reverse cholesterol transport. LCAT esterifies cholesterol with fatty acids, preferably linoleic acid, taken from lecithin. CETP catalyses the neutral lipid exchange of cholesterol esters from HDL and LDL to the triglyceride-rich lipoproteins in exchange for triglyceride.

4. HYPERTRIGLYCERIDAEMIA, LIPOPROTEIN SUBFRACTIONS AND CORONARY HEART DISEASE

4.1 Background

With respect to lipid metabolism, normal human pregnancy is characterised by an increase predominately in plasma triglyceride levels. Therefore, it is relevant to describe the effect of an increase in triglyceride concentrations on lipoprotein subfraction concentrations in non-pregnant individuals. Furthermore, it is important to recognise that documentation and understanding of such changes has assumed considerable clinical importance as there is an emerging consensus that a moderate elevation in plasma triglyceride may be an independent risk factor for coronary heart disease.

4.2 Triglycerides and Cardiovascular disease

Plasma low density lipoprotein-cholesterol (LDL-cholesterol) has long been regarded as the key lipid fraction in the pathogenesis of atherosclerosis. This view has recently been reinforced by the results of studies showing that lowering plasma LDL cholesterol concentrations using statins is an effective primary and secondary prevention measure for coronary artery disease (CAD) (Shepherd et al, 1995, Scandinavian Simvastatin Survival Study Group, 1994). However, there is accumulating evidence that elevated plasma triglycerides and related abnormalities constitute an independent cardiovascular risk factor.

Data from the PROCAM study, an eight year observational follow-up of 4559 middle-aged men, has revealed a significant and independent association between serum triglycerides and the incidence of major coronary events. In fact, the highest cardiovascular risk was seen in patients with an LDL/HDL ratio of greater than five in combination with a serum triglyceride concentration of greater than 2.3 mmol/l (Assmann et al, 1996). In a carefully-performed prospective case-control study based on a cohort from the Physicians' Health Study, serum triglyceride concentrations were a strong and independent predictor of outcome over seven years of follow-up, independent of HDL-cholesterol (Stampfer et al, 1996). Further evidence comes from a meta-analysis incorporating data from eight population-based prospective studies in more than 28,000 patients (~80% male), and controlling for HDL-cholesterol. This analysis demonstrated that for every 1 mmol increase in serum triglyceride the relative risk of coronary heart disease increased by 14% in men and 37% in women (Hokanson & Austin, 1996).

Mechanisms by which elevations in triglyceride concentrations contribute to atherogenesis include not only effects of LDL and HDL subfractions (succeeding section), but also by impacting upon thrombogenic parameters and endothelial function. A discussion of these latter two areas are detailed in sections 5.1 and 5.2, respectively. Furthermore, since normal pregnancy is associated also with alterations in thrombogenic factors and pre-eclampsia is characterised by endothelial dysfunction, the role of lipids in such changes is investigated in this thesis (Chapters III and VI).

4.3 The effect of raised triglyceride on VLDL and LDL subfraction concentrations in non-pregnant individuals

Part of the cardiovascular risk associated with elevated triglyceride levels is mediated through its influence of LDL and HDL subfractions. In order to understand these effects, it is necessary first to introduce the concept of metabolic channelling. This refers to the conversion of VLDL to LDL not via a single delipidation pathway but rather by parallel processing pathways. For example, large VLDL ($VLDL_1$) when delipidated gives rise to remnants in the smaller $VLDL_2$ and IDL density intervals and these are inefficiently converted to a class of LDL (smaller, denser) which is cleared slowly from the plasma. Newly synthesised small VLDL ($VLDL_2$), on the other hand, is rapidly and almost quantitatively delipidated to large buoyant LDL which is catabolised relatively rapidly.

Studies of normolipaemic subjects from our laboratory have demonstrated that as plasma triglyceride increases across the normal range, the hepatic synthesis and elaboration of the large $VLDL_1$ subfraction is predominately favoured such that the association of plasma triglycerides with $VLDL_1$ shows a significantly steeper slope

than that of $VLDL_2$ (Tan et al, 1996). Increased $VLDL_1$ levels, in turn, favour synthesis of small dense LDL-III by the following mechanistic scheme: an increase in the concentration of $VLDL_1$ expands the plasma triglyceride pool, and this via the action of CETP (which facilitates neutral lipid exchange between lipoprotein particles) promotes the net transfer of triglyceride into LDL-II, the major LDL species. HL then lipolyses triglyceride enriched LDL-II to generate small, dense LDL-III. It is currently postulated that an elevated HL activity (in the male range; males have twice the HL activity of females) is necessary to generate LDL-III in high amounts (Figure 1).

Small dense LDL-III particles are more atherogenic than lighter fractions. They are poorly recognised by the LDL-receptor mediated clearance mechanism and therefore stay in the plasma compartment for longer. They have a longer half life than any of the other fractions and penetrate the arterial intima more readily. They are also more readily oxidised and may contain less antioxidant, and they are more easily taken up by macrophages to create foam cells (Tribble, 1995). The overall effect is highly atherogenic. Indeed, in a recent study from our laboratory, the risk of coronary artery disease or myocardial infarction was observed to be considerably greater in those individuals with higher concentrations of LDL-III (>100mg/dl) (Griffin et al, 1994). The relative risk for coronary artery disease associated with LDL-III>100 mg/dl was 4.5 ($p<0.01$), and that for myocardial infarction 6.9 ($p<0.001$).

4.4 'Threshold' relationship between triglyceride and LDL-III

With respect to the generation of LDL-III in hypertriglyceridaemia, there is an additional complexity. Recent cross-sectional studies have prompted the suggestion that as plasma triglyceride increases, generation of LDL-III occurs only when a threshold concentration of serum triglyceride concentrations is exceeded (Griffin et al 1994, Tan et al 1996). Thus, at low-normal plasma triglyceride concentrations, there is a positive association between LDL-II concentration and plasma triglyceride. Above a certain 'threshold' plasma triglyceride value, however, (reportedly about 1.3 - 1.5 mmol/L in men), LDL-II concentration correlates negatively with plasma triglyceride, and LDL-III concentration, which had been relatively constant below this triglyceride concentration, correlates positively with plasma triglyceride. However, to the best of our knowledge no longitudinal studies have been designed to examine this phenomenon in individual subjects. The physiological changes in plasma triglyceride which accompany pregnancy provides such an opportunity.

4.5 Triglycerides and HDL concentrations

Increasing triglyceride levels also have a deleterious effect on HDL concentrations. In fact HDL-cholesterol concentrations are strongly inversely correlated with plasma triglyceride. In the presence of large triglyceride-rich $VLDL_1$ particles, increased CETP mediated exchange will generate HDL that is triglyceride enriched and these are

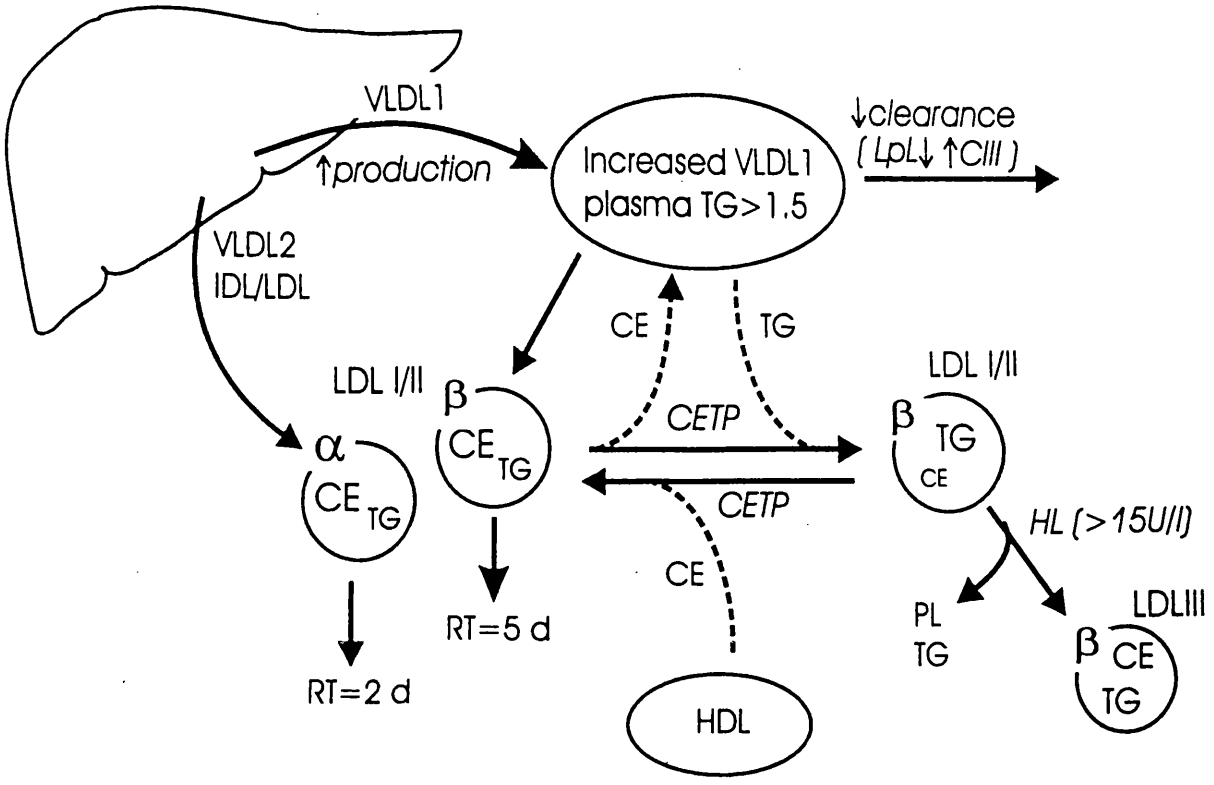


Figure 1. This hypothetical scheme links a number of observations and suggests mechanisms whereby moderate elevations of plasma triglyceride levels can adversely affect the structure and metabolism of low density lipoprotein and therefore its atherogenic potential. Two processes are thought to favour the formation of small, dense, slowly metabolised LDL. First, when the liver has an abundant supply of triglyceride it releases very low density lipoprotein particles of increased size, i.e. large $VLDL_1$. These, after delipidation, form slowly catabolised LDL. Second, a raised level of triglyceride-rich VLDL arising in part from low lipoprotein lipase activity leads to increased neutral-lipid exchange and the generation of triglyceride-rich LDL. The action of hepatic lipase removes this lipid from the core of LDL and shrinks the particle. Recent evidence has shown that smaller LDL species are more readily oxidised than larger ones and are thus more likely to penetrate the artery wall and be digested by resident macrophages.

favourable substrates for HL. This enzyme acts to remove lipid from the core of HDL, reducing its size and results in the conversion of HDL_2 to HDL_3. HDL_2 is thought to have a greater cardioprotective effect compared to HDL_3.

4.6 Hypertriglyceridaemia and the Atherogenic Lipoprotein Profile

As noted above, high plasma triglyceride levels are associated with high concentrations of small, dense LDL (LDL-III) and reduced HDL-cholesterol concentrations. This triad of raised triglycerides, small dense LDL and reduced HDL-cholesterol comprises the 'atherogenic lipoprotein phenotype' (ALP), and is the commonest lipoprotein pattern in patients with myocardial infarction (Superko 1996). The ALP is intimately linked with insulin resistance. It is often not expressed in young males and premenopausal women and hence hormonal factors have been suggested to affect the full penetrance of the phenotypic picture. Furthermore, environmental factors such as diet, exercise and the use of lipid altering medications may also affect the expression of the trait. Interest in ALP is not simply because of clustering of coronary risk factors in families but there is evidence that predominance of small, dense LDL particles appears to be common in the general population, with a prevalence of at least 30%. Thus a combination of both genetic and environmental factors identifies the individuals susceptible to premature coronary artery disease.

4.7 Insulin resistance and the Atherogenic Lipoprotein Profile

What then is the link between insulin resistance and lipid metabolism? Available evidence suggest that both insulin resistance and ALP are associated with increased risk of coronary artery disease. Insulin resistance is associated with a number of metabolic disturbances and so is the ALP. Insulin resistant individuals have higher plasma triglyceride, lower HDL cholesterol and small, dense LDL particles, almost identical to the ALP. The only difference is the disturbance in glucose metabolism in insulin resistant individuals. One might ask whether they are just part of the same spectrum of metabolic abnormality or whether one is subset of the other? Furthermore the prevalence of small, dense LDL estimated at approximately 30% in the general population is similar to the prevalence of 25% estimated for insulin resistance syndrome. Insulin suppresses the release of free fatty acids (FFAs) from adipose tissue and in insulin resistant states, the suppressive effect of insulin on fatty acid release from adipose tissue after a meal is impaired. Insulin resistance is also associated with a decreased responsiveness of LPL to the action of insulin. This is particularly important for postprandial activation of LPL with resultant elevation of plasma triglyceride. Prolonged residence of triglyceride-rich particles in the circulation leads to increased exchange of their triglyceride with cholesteryl ester in HDL through the action of CETP. The failure of suppression of FFA during the postprandial period in insulin resistant individuals aggravates the increase in VLDL-triglyceride already present. The elevation in plasma triglyceride concentrations reflects an increased number of the larger triglyceride-rich $VLDL_1$ particles both in normals and in insulin

resistant individuals. The insulin resistant individuals with higher plasma triglyceride levels would thus have a predominance of large triglyceride-rich $VLDL_1$ particles and it is these particles that determine the rate of triglyceride transfer into LDL and HDL since triglyceride-rich VLDL has been shown to be the preferred substrate for CETP action. The triglyceride in such triglyceride-enriched LDL particles may then be removed by HL, leading to small, dense LDL particles. Thus we can see that the link between insulin resistance, hypertriglyceridaemia and decreased HDL cholesterol concentrations could be mediated through disruption of the normal multifactorial role of insulin in co-ordinating postprandial lipid metabolism.

5. HYPERLIPIDAEMIA, THROMBOGENESIS AND ENDOTHELIAL DYSFUNCTION

5.1 Hyperlipidaemia and thrombogenesis

The old view of atherogenesis and thrombogenesis as two separate entities has been replaced by a 'unified field theory' that links them closer together. There is now a great deal of evidence that plasminogen activator inhibitor-1 (PAI-1) activity is enhanced (and fibrinolysis impaired) in patients with hypertriglyceridaemia. In addition, triglyceride-rich lipoproteins have been shown to promote PAI-1 release from cultured human umbilical vein endothelial cells (Stiko-Rahm et al 1990), and are the major independent predictors of PAI-1 levels in NIDDM patients (Panahloo et al 1995). Elevated PAI-1 levels may be a marker of endothelial dysfunction, and cause a profound depression of fibrinolytic activity, contributing to the pathogenesis of vascular disease (Hamsten 1995). Lipolysed remnants of triglyceride-rich lipoproteins also promote an enhanced thrombogenic tendency by increasing circulating Factor VII levels (Mitropoulos et al 1993). In epidemiological studies plasma triglyceride levels have, in addition, been demonstrated to have significant associations with coagulation factors IX and fibrinogen (Woodward et al 1997). Thus there is plentiful evidence to suggest strong links between hypertriglyceridaemia and impaired fibrinolysis.

5.2. Hyperlipidaemia and endothelial dysfunction

A considerable body of evidence relates impaired endothelial vasomotor function to CAD. Several studies implicate coronary vessel endothelial vasomotor dysfunction in the pathogenesis of myocardial ischaemia in patients with stable CAD, and particularly in the pathogenesis of unstable angina, in which platelet aggregation and thrombin formation have a more prominent role (Zeiher 1996, Levine et al 1995). Endothelial dysfunction results in abnormal vascular reactivity which in turn increases the haemodynamic stress on plaques (Vita et al 1989) and thus increases the likelihood of plaque rupture (Loree et al 1992) and thrombosis (Levine et al 1995). In patients with early coronary atherosclerosis, progressive impairment in endothelial vasomotor function is observed (Zeiher et al 1993). Endothelial dysfunction is not limited to the coronary circulation, and can also be detected in peripheral arteries (Anderson et al

1995). Furthermore, the assessment of endothelial vasomotion in these peripheral arteries has been shown to correlate with endothelial dysfunction in the coronary arteries (Anderson et al 1995) and to relate directly to coronary dysfunction (Zeiher 1996).

Endothelial vasomotor dysfunction is also evident in patients with risk factors for CAD long before structural vascular changes or clinical symptoms occur: Abnormal endothelial function has been described in hypertension (Panza et al 1993), NIDDM (McVeigh et al 1992), and obesity/insulin resistance (Steinberg et al 1996). With respect to lipids, hypercholesterolaemia has repeatedly been shown to be associated with endothelial dysfunction, whereas cholesterol-lowering therapy has been demonstrated to result in improved endothelial-dependent vasomotion (Anderson et al 1995, Treasure et al 1995).

However, there are several possible mechanisms (Sattar et al, In press) by which elevations in triglyceride-rich lipoproteins (and related metabolic perturbances) may mediate endothelial dysfunction. There is evidence to suggest that triglyceride-rich particles may be directly damaging to the endothelium; this may be principally via oxidative mechanisms. Triglyceride-rich particles can cross the endothelial barrier and enter the arterial wall, thus placing them in a position to promote *direct* endothelial damage. These particles stimulate endothelial expression of adhesion molecules and the prothrombotic factor PAI-1. By reducing LDL size and HDL cholesterol concentrations, triglyceride-rich particles may *indirectly* promote endothelial dysfunction. Small, dense LDL as mentioned above are more susceptible to oxidative damage whereas HDL particles protect the endothelium via its antioxidant properties as HDL has been found to protect against LDL oxidation (Hessler et al 1979). HDL may also play a role in reverse transport of potentially reactive hydroperoxide species for hepatic detoxification . In addition, free fatty acids, which are the major substrates for endogenous synthesis of triglyceride-rich particles, are also potentially damaging to the endothelium. This occurs via oxidative stress, by facilitating transfer of LDL across the endothelium, and by enhancing toxicity of triglyceride-rich particles. Finally, there is recent strong evidence to suggest that increased postprandial circulating concentrations of triglyceride-rich particles and remnant particles may be deleterious to the endothelium (Vogel et al 1997, Plotnick et al 1997).

6. LIPOPROTEIN METABOLISM IN PREGNANCY

6.1 Background

As described above, a complex system to transport lipids via blood has evolved in all higher species. Its function is to aid the absorption of dietary lipids, to distribute them to peripheral tissues for the synthesis of cell membranes, to store the excess in adipose tissues for later use, and to release them at a controlled rate when required to meet

energy demands. A physiological hyperlipidaemia of pregnancy has co-evolved in mammals to accelerate these processes at a time of rapid development of fetal and placental tissues and to ensure an adequate accretion of body fat to support lactation (Dugdale 1986). This condition is characterised by a marked elevation in plasma lipids, a progressive decline in serum glucose and amino acids, and a shift in maternal energy metabolism from glucose to more dependence on fatty acids and ketone bodies. This phenomenon has been recognised for nearly 150 years, although its precise metabolic foundations remain elusive (Virchow 1847).

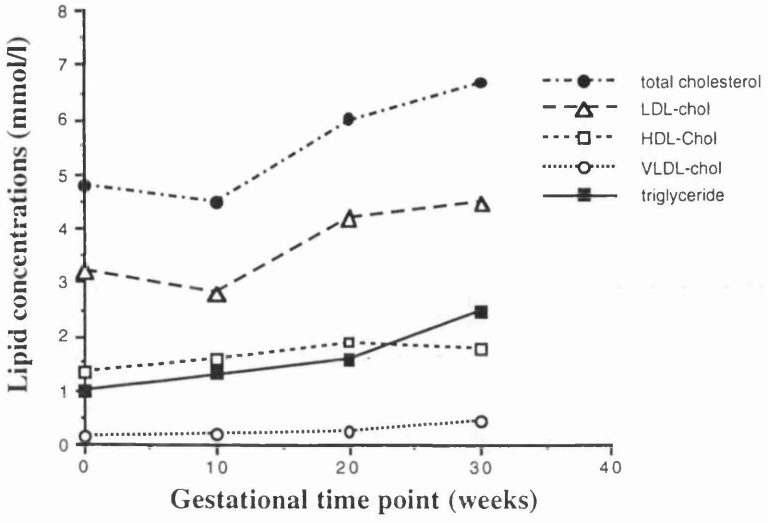
6.2 Very Low Density Lipoprotein

VLDL level rises throughout pregnancy, particularly markedly in the third trimester, and is responsible for the rise in triglyceride levels (Figure 2), (Warth et al 1975, Fahraeus et al 1985, Montelongo et al 1992). Triglyceride concentrations increase anywhere between 200-400% so that by the end of pregnancy a proportion of women will be frankly hypertriglyceridaemic (trig>2.3 mmol/l). Current evidence favours enhanced synthesis of apoB-containing triglyceride-rich VLDL, rather than reduction in catabolism, although it is likely that both mechanisms contribute. Kinetic studies are required to confirm the dominant mechanism responsible for elevated VLDL levels.

6.3 Low Density Lipoprotein

LDL cholesterol concentrations fall significantly in the first trimester probably as a result of enhanced catabolism secondary to an oestrogen mediated upregulation of hepatic LDL receptors. After eight weeks, however, LDL cholesterol levels begin to rise and do so steeply. This rise is probably due to enhanced conversion of VLDL to LDL. By term LDL cholesterol concentrations have increased by around 70% (Warth et al 1975, Fahraeus et al 1985, Montelongo et al 1992) (Figure 2). The rise in LDL triglyceride levels is more marked than LDL cholesterol and this believed to be the result of a combination of two effects. Firstly, the reduction in hepatic lipase activity, as a consequence of pregnancy hyperoestrogenaemia, could mediate LDL triglyceride enrichment. A second potential mechanism, driven by high VLDL concentrations, is an increased flux of VLDL-triglyceride to LDL in exchange for cholesteryl ester (as in Figure 1) (Montelongo et al 1992).

FIGURE 2 Typical lipid changes in normal pregnancy



6.4 High Density Lipoproteins

The hypertriglyceridaemia of pregnancy is distinguished from other hypertriglyceridaemias by an increase of HDL cholesterol rather than a fall. HDL cholesterol rises to maximum at around 25-30 weeks, the HDL_2 subfraction being mainly responsible (Fahraeus et al 1985) (Figure 2). The mechanisms likely responsible for this are complex but probably involve enhanced synthesis of apo A-I, the main protein constituent of HDL, and reduced catabolism of HDL_2 secondary to a fall in hepatic lipase activity. After 25-30 weeks HDL cholesterol and HDL_2 levels begin to fall despite little change in hepatic lipase activity and a continual rise in plasma apo A-I. Thus, other factors are involved and probably push the balance towards enhanced catabolism (Alvarez et al 1996). The higher triglyceride levels at this stage are likely to be involved by facilitating increased neutral lipid exchange between HDL and VLDL particles.

6.5 Potential Mechanisms for Pregnancy Hyperlipidaemia

The mechanism(s) for the generation of the hypertriglyceridaemia of pregnancy

appears to differ from the mechanism in the non-pregnant population. Although, pregnancy is an insulin resistant state, the rise in oestrogens is likely to contribute to the pattern of change in the lipoproteins. At the end of the first trimester, the lipoprotein pattern is similar to that observed in women on oral oestrogen containing preparations: elevated plasma triglyceride concentration with a parallel increment in HDL cholesterol, whereas LDL cholesterol are lowered (Walsh et al 1991). Increased HDL-cholesterol concentrations are likely mediated via oestrogen induced increased hepatic apo A-I synthesis and reduced hepatic lipase activity. LDL cholesterol concentrations are also likely oestrogen induced, mediated via hepatic LDL receptor up-regulation.

In the late second trimester of human pregnancy there is an increased flux of free fatty acids (FFAs) promoted by a combination of stimulation of hormone sensitive lipase (HSL) (Martin-Hidalgo et al 1994) by human placental lactogen (HPL), and relative resistance to the effects of insulin (which normally acts to suppress FFA release from adipose tissue). Normally, FFAs released from adipocytes by the action of HSL are taken up by liver and subject to two alternative routes of metabolism; one is their reassimilation into triglyceride molecules, while the other is oxidation by mitochondrial ß-oxidative pathways which results in the generation of energy and the synthesis of ketones. The delivery of substrate for triglyceride synthesis in the form of free fatty acids is known from cell culture work to be the major determinant of VLDL secretion (Dixon & Ginsberg 1993). Studies in the mouse suggest that late pregnancy is also associated with a mild impairment in β-oxidative function (Grimbert et al 1993). However, as total hepatic lipids remained unchanged, it appears that in normal murine pregnancy this decreased oxidation of fat in the liver is compensated for by the increased secretion of VLDL, in agreement with studies in other animal models (Weinstein et al 1979, Wasfi et al 1980). Furthermore, in a recent single human case study oestrogen has been demonstrated to inhibit hepatic lipid oxidation with resultant increase in serum triglyceride concentration (O'Sullivan et al, 1995). Thus the rise in plasma triglyceride-rich lipoproteins, which is maximal in the third trimester, is likely to be due to a combination of increased FFA flux and oestrogen mediated reduced hepatic β-oxidation. Overall, the perturbations in lipoprotein concentrations during pregnancy are in keeping with the concept of fatty acid supply to the placenta for trans-placental transport.

Beyond simple gross changes in the concentrations of lipoproteins, there appear to be qualitative changes in lipoprotein composition also. Studies in animal models have demonstrated that there is a specific increase in phosphatidylcholine (PC) 16:0 / 22:6 n-3 (DHA) content of maternal liver and plasma phospholipids during late pregnancy (Burdge et al, 1994). This has been shown to be due to modifications to the rate of PC synthesis and to the composition of the pools used in PC and phosphatidylethanolamine synthesis. These changes have also been observed in

human pregnancy. The simplest explanation for increased synthesis of liver PC in pregnancy is to support increased hepatic lipoprotein synthesis and secretion for the supply of lipid nutrients to the fetus to support neurological development, and adipose and mammary storage sites. This work also suggests that the supply of PUFA to the developing fetus is the result of specific adaptations to maternal hepatic phospholipid biosynthesis rather than passive transfer from the maternal diet.

7.0 PLACENTAL LIPID UPTAKE MECHANISMS

Recent research has demonstrated that the placenta has several potential lipid uptake mechanisms (Figure 3).

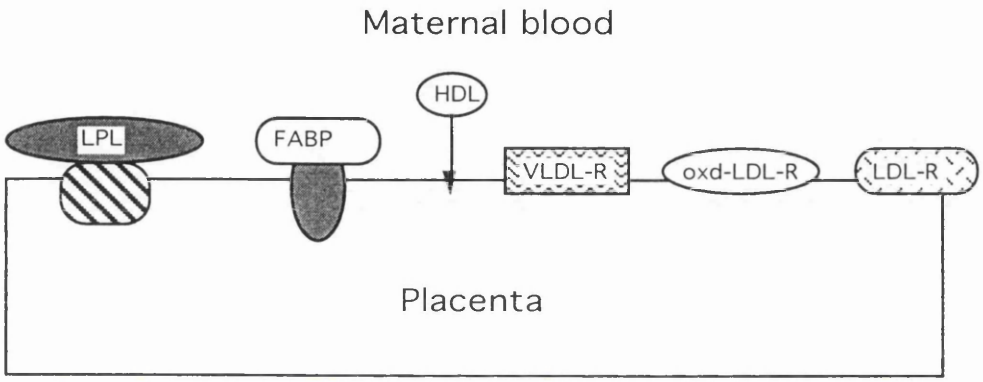


Figure 3. Placental lipid uptake mechanisms. LPL; Lipoprotein lipase;
FABP: Fatty Acid Binding Protein; VLDL-R: VLDL receptor; Oxd LDL-R: oxidised LDL receptor; LDL-R: LDL receptor

7.1 Lipoprotein Lipase

Placental lipoprotein lipase activity has been isolated from animal and human placentas (Bonet et al, 1992) Furthermore, it has been previously suggested that placental LPL hydrolysis triglycerides from maternal VLDL and not the triglycerides from chylomicrons (Dutta-Roy, 1995). This would seem to add support to the concepts of maternal remodelling of lipoproteins before delivery and may serve as protective mechanism to the fetus from the immediate impact of an unusual fatty acid composition in the maternal meal. It is noteworthy, however, that the evidence for a specific hydrolyses effect on VLDL of placental LPL is based entirely on animal studies. Furthermore, a specificity of placental LPL for VLDL would imply that it differs from LPL isolated in every other human tissue. Clearly, this requires further investigation.

Nevertheless, changes in placental LPL activity as pregnancy proceeds are consistent with a concept of placental fatty acid transport. In the guinea pig, LPL activity increases approximately 11-fold during the latter gestation. Furthermore, as pregnancy proceeds, LPL activity declines in adipose and skeletal tissue and increases in placental and mammary tissues (Martin-Hidalgo et al 1994). These changes in LPL activities in various tissues would appear to facilitate targeting of lipoproteins to placental and mammary tissues.

The majority, if not all, of the LPL activity appears to be in placental macrophages and it has been demonstrated that VLDL-triglyceride fatty acids (and possibly also chylomicrons) predominate as a source of FA uptake for placental cells, while FFA predominate for the hepatocyte. If macrophages are the main source for the placenta, it is noteworthy that they are not in direct contact with the maternal blood. However, there is the possibility of transfer of LPL from macrophages to trophoblasts, cells that are in direct contact with the blood. Accordingly, placental macrophages may play a role in the uptake of triglyceride-rich lipoprotein FA for placental storage and transfer to the fetus.

7.2 Fatty Acid Binding Protein (FABP).

Upon release by LPL hydrolyses, how are fatty acids taken up the placenta? Recent work suggests that a specific Fatty Acid Binding Protein (FABP) may be involved (Dutta-Roy et al 1995) (Figure 3). This FABP, which is ~40 kDa in size, behaves like other physiological receptors, and significantly appears to have a strong preference for long chain polyunsaturated fatty acids. As such, this placental protein may be vital to the development and growth of the human fetus.

7.3 VLDL receptors

Until recently, very little was known about VLDL-receptor localisation and regulation. VLDL-receptor is expressed in the human placenta predominantly in trophoblast cells, and in a pattern consistent with a role in placental lipid transport (Wittmack et al, 1995). VLDL-receptor expression is high at term relative to that in the first trimester, and thus is likely to be involved in transfer of lipid to the placenta for the growing fetus. Interestingly, the trophoblast VLDL-receptor is subject to up-regulation by insulin (and fasting insulin levels more than double in pregnancy) and fibrate class hypolipidemic drugs.

7.4 LDL receptors

LDL receptor activity is present in human placenta cells and the cholesterol delivered to these cells can be used for progesterone synthesis (Winkel et al 1980). Recently, scavenger receptor activity, greatly exceeding that of the LDL receptor, has been identified in human placental trophoblasts and macrophages (Bonet et al 1995). However, cholesterol assimilated via the scavenger receptor activity appears to be

disconnected from endocrine steroid genesis in trophoblasts and may function to degrade modified lipoproteins and prevent toxic effects on placental cellular function and fetal growth and development. Indeed, given the changes in plasma triglyceride concentration during pregnancy, it is likely that the LDL subfraction profile tends towards smaller, denser species (see Chapter IV). These particles are more susceptible to oxidative damage and oxidative stress is increased in pregnancy, in particular in pre-eclampsia (Uotila et al, 1993). As such an increase in the levels of modified lipoproteins during pregnancy may be a direct consequence of the increase in plasma triglyceride levels that facilitate placental lipid uptake. Accordingly scavenger receptors may function as an essential buffer against the inevitable rise in modified lipids.

7.5 High Density Lipoprotein cholesterol uptake

Uptake of free and esterified cholesterol from HDL by the placental trophoblast cells takes place through a receptor independent mechanism (Lascuncion et al, 1991). The HDL_2 subfraction is primarily involved. As such, HDL may provide a reserve or alternative supply to LDL of cholesterol for progesterone synthesis and for transport to the fetus to support growth and development.

8. PRE-ECLAMPSIA

8.1 Clinical Signs and pathogenesis

Pre-eclampsia and eclampsia are the most important causes of maternal death in the USA, Scandinavia, Iceland, Finland and the United Kingdom (Roberts & Redman 1993). They occupy the same prime position as they did nearly 40 years ago. Despite intensive research the pathogenesis remains unclear and there is no specific diagnostic test; the disorder is recognised by the concurrence of pregnancy-induced changes that regress after delivery, of which hypertension and proteinuria are the easiest to recognise and the signs by which the maternal syndrome is defined.

From recent reviews the emerging consensus is that poor placental perfusion which is the result of immunologically mediated abnormal implantation, microvascular disease, or excessive placental size, is the unique feature of pregnancies predisposed to pre-eclampsia. But how can abnormal placental perfusion lead to multiorgan systemic disease? It is proposed that the poorly perfused placenta is the origin of blood-borne materials that affect maternal systemic function by activating vascular endothelial cells (Rodgers et al 1988).

8.2 Endothelial dysfunction in pre-eclampsia

There is increasing evidence that endothelial cell injury and altered endothelial cell function play an important role in pathogenesis of pre-eclampsia (Roberts & Redman 1993). Increased levels of Factor VIII-related antigen, increased levels of fibronectin

(cFN) of fibronectin ED1+, a disturbance of plasminogen-activator inhibitor, and crucially a disturbance of the Prostacyclin (PGI_2)/ Thromboxane A_2 (TXA_2) balance, all support the hypothesis that endothelial damage is intimately involved in the pathogenesis of pre-eclampsia. Recent studies have shown that cFN serves as an early indicator of pre-eclamptic endothelial damage. Serum from pre-eclamptic women injures endothelial cells *in vitro*. Abnormalities of the haemostatic system in pre-eclampsia are compatible with a local low-grade or compensated intravascular coagulation, secondary to platelet adherence at sites of vascular endothelial damage. The critical question therefore is what is the nature of the blood-borne materials causing altered endothelial function.

8.3 Lipid changes in Pre-eclampsia: possible role in pathogenesis

In the course of normal pregnancy women have altered lipoprotein metabolism as reflected in a hyperlipidaemia that grows more marked as pregnancy proceeds. Among those in whom pre-eclampsia develops, changes in lipid metabolism have been shown to be more pronounced in that the plasma triglyceride levels are further increased (by 50% on average) compared with those women with normal pregnancies (Potter & Nestel 1979). The rise in plasma triglyceride also precedes clinical signs of pre-eclampsia (Lorentzen et al 1995). From the area of cardiovascular research there is increasing evidence that hyperlipidaemia can cause endothelial dysfunction (see section 5.2) The question arises, therefore, whether endothelial dysfunction of pre-eclampsia is causatively related to alterations in metabolism of lipids and lipoproteins

Evidence from recent studies suggests several potential mechanisms of interaction between altered lipoprotein metabolism and endothelial dysfunction in women with pre-eclampsia. Firstly, significant elevations of lipid peroxide levels occur in pre-eclampsia as compared with normal pregnancy (Maseki et al 1981, Uotila et al 1993). The source of lipid peroxide production is postulated to be placental (Wang et al 1992). The finding of elevated lipid peroxides is interesting because lipid peroxides interfere with endothelial physiology: lipid peroxides impair endothelial prostacyclin synthetase (Higgs & Vane 1983), inactive endothelium-derived relaxing factor (EDRF) (Gryglewski et al 1986), and stimulate endothelin production and release (Dekker et al 1991). Therefore, in the absence of adequate production of prostacyclin and probably the antiaggregatory auticoid EDRF, surface-mediated platelet activation may be expected to occur. Direct activation of platelets by lipid peroxides is also likely. Thus, lipid peroxides could be the blood-borne materials responsible for endothelial dysfunction in pre-eclampsia.

Secondly, a number of recent studies have demonstrated that hypertriglyceridaemic serum and triglyceride-rich lipoproteins are cytotoxic to cultured human endothelial cells (Spiedel et al 1990, Gianturco et al 1980). Therefore, the exaggerated

hypertriglyceridaemia might itself be contributory to endothelial dysfunction. In addition, if this 'excess' triglyceride is largely in the form of $VLDL_1$, the large, buoyant VLDL, then this will favour increased production of LDL-III, the small, dense, atherogenic LDL subfraction. This LDL, as described previously, is more susceptible to oxidation and therefore raised LDL-III, secondary to raised triglyceride, might also contribute to the lipid peroxide load by providing a more abundant and better substrate for peroxidation as lipoproteins circulate through the placenta. With this in mind, it is interesting to note that one of the histopathological changes seen in the placenta of pre-eclamptic women is the occlusion of vessels by fibrinoid material with adjacent foam cell invasion (Roberts & Redman 1993). These features are suggestive of the presence of oxidised LDL.

Thirdly, pre-eclamptic women have altered fatty acid metabolism, as demonstrated by recent elegant work by Henriksen's group. They have shown that sera from women with pre-eclampsia induce accumulation of triglyceride in cultured endothelial cells with resultant reduced prostacyclin synthesis (Lorentzen et al 1991). This enhanced intracellular triglyceride accumulation appears largely to be the result of raised serum free fatty acids. The authors suggest that lipolytic activity of serum is stimulated and this is the major cause for the increase in free fatty acid/albumin ratio (Endresen et al 1993).

Finally, raised triglyceride-rich lipoproteins drive enhanced neutral lipid exchange thereby explaining the lower HDL-cholesterol concentrations seen in pre-eclampsia (Potter & Nestel 1979). ApoA-I levels also are lower. There is evidence that HDL_2 stimulates PGI_2 secretion from endothelial surfaces (Kaaja et al 1995). HDL particles also have antioxidative properties and, as a result, lower HDL concentrations favour an increased lipid peroxide load and endothelial dysfunction. Interestingly, in non-pregnant individuals plasma HDL-cholesterol concentrations relate positively and significantly to endothelium-dependent relaxation of coronary vessels in patients with coronary heart disease (Kuhn et al 1991, Chowienczyk et al 1994). In addition, in a study in patients with type 2 diabetes mellitus, decreased HDL-cholesterol was the best predictor of impaired vasodilatation to acetylcholine even after adjustment for all lipid and lipoprotein concentrations (O'Brien et al 1997).

9. INTRAUTERINE GROWTH RESTRICTION

9.1 Background

Normal fetal growth occurs by a sequential process of cellular hyperplasia, hyperplasia plus hypertrophy and, lastly, by hypertrophy alone. Growth deficiency is the end result of numerous pathologies which reduce fetal cell size and, when early and severe enough, cell number. The loss of cell number is not generally corrected

after delivery and these children remain small. Broadly categorised, causes of growth deficiency include fetal, maternal, uterine and placental disorders. Risk factors for growth deficiency common to any obstetric practice include smoking and maternal vascular disease.

9.2 Lipids and Birthweight

The effect on fetal growth of hyperlipidaemia in pregnancy is not well understood at present. From available evidence, it appears that plasma triglyceride demonstrates a curvilinear relationship to birth weight in normal pregnancies. Further, apo A-I (the apoprotein principally associated with the HDL_2 subfraction) has been shown to correlate positively and apo A-II (principally associated with the smaller HDL_3 subfraction) correlate negatively with birth weight (Knopp et al, 1985).

9.3 IUGR and Lipids

Given the importance of maternal hyperlipidaemia in supplying both cholesterol and triglyceride to the rapidly developing fetus, it is conceivable that pregnancies complicated by IUGR exhibit abnormal lipoprotein metabolism. However, it would appear from the literature that such studies are sparse.

9.4 Fetal origins of coronary heart disease

The fetal origins hypothesis states that fetal under-nutrition in middle to late gestation, which leads to disproportionate fetal growth, programmes later coronary heart disease. Animal studies have shown that undernutrition before birth programmes persisting changes in a range of metabolic, physiological and structural parameters. Studies in humans have shown that men and women whose birth weights were at the lower end of the normal range, who were thin at birth, or who were small in relation to placental size have increased rates of coronary heart disease. The mechanisms underlying these associations remain unclear. The programming of blood pressure, insulin resistance, lipoprotein metabolism, blood coagulation, and hormonal settings are all active areas of research (Barker 1995).

9.5 Maternal lipoprotein metabolism and fetal birthweight

It is possible that maternal lipoprotein metabolism during pregnancy (or governing factors, such as degree of insulin resistance) may also influence fetal programming/birth weight and predict future risk of CHD. Previous literature as detailed above suggest that maternal apo AI concentrations near term correlate directly with birthweight, whereas apo AII concentrations correlate negatively with birth weight and length (Knopp et al, 1985). Total apo A or HDL-cholesterol concentrations have also been shown to be lower in mothers with pregnancies complicated by IUGR or pre-eclampsia (Potter & Nestel 1979). In addition, although babies born to mothers with gestational diabetes tend to be macrosomic, they also have an increased future risk of CHD, and at term their mothers also exhibit significantly

lower HDL-cholesterol concentrations (Montelongo et al 1992). Interestingly, HDL_2 subfractions stimulate placental progesterone synthesis and might affect fetal growth by controlling the provision of cholesterol via the placenta (Lascuncion et al 1991). Alternatively, the magnitude of insulin resistance may be a unifying hypothesis as it not only relates inversely to HDL (and thus Apo AI) metabolism, but also is greater in women with gestational diabetes and pre-eclampsia. Further support for this possibility comes from a study which demonstrated that in mothers with BMI less than 25 kg.m-2, plasma insulin and insulin response to an oral glucose tolerance test were inversely related to birth weight ($p<0.02$), after simultaneously adjusting for physiological factors and glucose concentrations (Breschi et al 1993).

Thus, it is tempting to speculate that the mother's metabolic characteristics, particularly insulin resistance (and thus CHD risk), is transmitted to the offspring through direct effects on fetal growth rather than by genetic factors. Accordingly, maternal metabolic factors may also aid in predicting future CHD risk of their children. These findings clearly deserve more detailed investigation.

10. AIMS

10.1 Lipoprotein subfraction changes in normal pregnancy

As described above, one of the mechanisms by which triglyceride influences coronary risk is through its effect on LDL subfractions, with higher triglyceride concentrations promoting increased synthesis of small, dense LDL-III particles. However, this relationship is not linear, rather recent cross-sectional studies imply a threshold effect: synthesis of LDL-III proceeding only once triglyceride concentrations go beyond a 'threshold' value. To the best of our knowledge no longitudinal studies have been designed to examine this phenomenon in individual subjects. The physiological changes in plasma triglyceride which accompany pregnancy provide such an opportunity. Thus, we have measured LDL subclasses throughout gestation using density gradient ultracentrifugation. This technique, allowed us to examine quantitatively the relationship between plasma triglyceride and perturbations in the LDL subfractions during pregnancy. This study is detailed in Chapter III

10.2 A longitudinal study of the relationships between haemostatic, lipid, and hormonal changes during normal human pregnancy.

Increased activation of both blood coagulation and fibrinolysis occurs during normal pregnancy. The responsible mechanisms are unclear, but from studies in non-pregnant individuals, may include increases in blood lipids. The associations between fasting plasma cholesterol and triglyceride, serum oestradiol, and Factor VII activity, PAI activity, t-PA antigen, fibrin D-dimer, and vWF antigen during normal pregnancy were studied and the results of this study are detailed in Chapter IV.

10.3 Lipoprotein subfraction concentrations in pre-eclampsia

In women with pre-eclampsia, plasma triglyceride concentrations climb substantially above those observed in normal pregnancy and do so well in advance of the appearance of clinical manifestations of the disorder. As described above, however, lipoprotein classes are not homogeneous entities but include subclasses of differing function and metabolic potential. Information in the literature of the concentrations of very low density (VLDL) and LDL subfractions in pre-eclampsia was lacking. This was particularly important to investigate as small, dense LDL are more atherogenic than larger LDL species. With this background we explored the hypothesis that in this disorder large triglyceride-rich lipoproteins ($VLDL_1$), and small, dense LDL (LDL-III), were significantly increased over and above the concentrations seen in normal pregnancy. The results of this investigation are detailed in chapter V.

10.4 A relationship between myometrial resistance artery behaviour and circulating lipid composition in pre-eclampsia

Substantial evidence suggests that alterations in endothelial behaviour underlie the maternal pathophysiology of pre-eclampsia, a disease with a high maternal and fetal morbidity and mortality. Researchers from Nottingham have demonstrated an alteration in endothelium dependent myometrial resistance artery function in women with this syndrome, and have induced a similar functional change in myometrial resistance vessels from normal pregnant women after incubation with plasma from women with pre-eclampsia. These findings are consistent with the hypothesis that some circulating humoral factor(s) affects maternal systemic function directly or indirectly, by activating endothelial cells with resultant vascular injury. In collaboration with this group the relationship between plasma lipid concentrations and endothelial behaviour of myometrial resistance vessels bathed in plasma from normal pregnancies and those complicated by pre-eclampsia, was examined. We measured plasma cholesterol, triglyceride, Apo AI and Apo B concentrations from normal pregnancy samples and samples from women with pre-eclampsia and related these data to observed changes in myometrial resistance artery behaviour. The results are presented in chapter VI.

10.5 Potential pathogenic roles of aberrant lipoprotein and fatty acid metabolism in pre-eclampsia

Based upon results of some of the above work, and allied with existing data from other studies in the literature, a mechanistic scheme for the generation of the hyperlipidaemia in pre-eclampsia was devised. In addition, the potential role of the hyperlipidaemia in the pathophysiology of the syndrome is detailed. These hypotheses are described in Chapter VII.

10.6 Lipoprotein concentrations in pregnancies complicated by Intrauterine Growth Restriction

Finally, since data on lipid changes in IUGR pregnancies are sparse, the aim of the final study in this thesis (Chapter VIII) was to compare lipid and lipoprotein concentrations in the third trimester from normal pregnancies and those complicated by IUGR.

10.7 Potential effects of progesterone on lipid and haemostatic changes in pregnancy

It should be noted that whilst we have examined a potential role for oestradiol in promoting the lipid and haemostatic changes during pregnancy, we have not excluded a potential impact of the significant rise in progesterone concentrations on such changes. Current literature would suggest, however, that natural progesterones, in contrast to androgenic progestogens, have minimal, if any, effect on lipid metabolism (Knopp et al 1994).

CHAPTER II

METHODS

1. Materials

Names and addresses of manufacturers and suppliers of reagents, hardware and software used for this thesis are given in Appendix 1.

2. Subject selection

2. 1 Pregnant subjects

All pregnant subjects including those with preeclampsia and IUGR were recruited from Glasgow Royal Maternity Hospital. For the purposes of studies carried out in Chapters III, IV and V, women who had no identifiable risk factors for the current pregnancy as determined by detailed obstetric history were recruited by N. Sattar at their booking visit. These women represented the normal pregnancy cohort. Each woman was followed up at home and fasting blood samples for lipid, lipoprotein and subfraction measurements were taken at 5 week intervals up to and including 35 weeks of gestation.

Women with preeclampsia were identified by Senior House Officers (Dr Astrid Bendormir and Dr Colin Berry) at Glasgow Royal Infirmary Hospital. The inclusion and exclusion criteria are described in detail in Chapter V and in Appendix 3. All patients were instructed to fast and blood samples were withdrawn around 7.30 am in the morning usually by N.Sattar although on occasions Dr's Bendormir and Berry helped out in this respect.

Women with IUGR were identified by Dr Alan Mathers (Consultant Obstetrician with an interest in Fetal Medicine, and Clinical Director of GRMH) and Dr Theresa Kelly (Senior Registrar). The inclusion and exclusion criteria for these patients are described in detail in Chapter VIII. All patients were instructed to fast and blood samples were withdrawn in the morning by Dr Theresa Kelly.

2. 2 Non-pregnant female controls

Non-pregnant female controls were recruited from laboratory staff, friends and colleagues. None were taking medication known to affect lipid or lipoprotein subclass concentrations. Once again, all blood samples were taken following an overnight fast.

3. Sample handling

In all cases, blood samples were placed on ice and spun within two hours of venesection at 3000rpm for 10 mins. Separated serum or plasma was then either frozen immediately at -70°C or stored temporarily at 4°C.

4. Ethical approval

All studies were approved by the Ethical Committee of Glasgow Royal Infirmary.

5. Laboratory techniques

The myography techniques used are described in detail in Chapter VI.

5. 1 β Quantification

Plasma total cholesterol, triglyceride, HDL cholesterol, VLDL cholesterol and LDL cholesterol measurements were performed by a modification of the standard Lipid Research Clinics protocol (1975). Five ml of plasma was placed in a Beckman ultra-clear tube (13 x 64 mm) and over layered with 2 ml of d 1.006 g/ml solution. Tubes were capped and centrifuged overnight at 35, 000 rpm (4°C) in a Beckman 50.4 rotor. The supernatant was collected from the top 25 mm into a 3 ml flask, this being the VLDL. The contents of the bottom fraction were transferred to a 5.0 ml volumetric flask. The tube was washed with saline and the wash added to the flask and the final volume adjusted to 5.0 ml with 0.15 M NaCl. A 1.0 ml aliquot of this bottom fraction was then placed in a Beckman centrifuge tube and 50 µl of precipitating agent (9.56 g Mn $CL_2.4H_2O$ + 1.05 g (approximately 5 x 10^5 units) heparin sodium salt in 25 ml 0.15M NaCl) were added and mixed. This mixture was kept at 4°C for 15 minutes and then centrifuged at 10, 000 rpm for 30 minutes. This would precipitate the LDL fraction leaving the supernatant as the HDL fractions. The cholesterol content of whole plasma, top fraction (VLDL), bottom fractions (LDL + HDL) and heparin/Mn2+ precipitated supernatant (HDL) were then determined by enzymatic colorimetric assays as described in the next section. The total plasma cholesterol, triglyceride, LDL cholesterol and HDL cholesterol were measured by kind courtesy of the routine staff of the Lipid section of the Department of Pathological Biochemistry, at the Glasgow Royal Infirmary.

Calculations

Bottom fraction cholesterol - HDL cholesterol = calculated LDL
Total cholesterol - Bottom fraction cholesterol = calculated VLDL
Measured VLDL should agree with calculated VLDL by ±0.35 mmol/l.

5. 2 Compositional Analyses

Total cholesterol and triglyceride were determined in whole plasma and in the lipoprotein fractions by enzymatic colorimetric assays on an Hitachi 717 auto analyser.

The principal of the cholesterol assay is shown diagrammatically below:

$$\text{Cholesterol esters} + H_2O \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{RCOOH}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \Delta 4\text{-cholestenone} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow{\text{POD}} \text{4-(p-benzoquinone-mono-imono)- phenazone} + 4H_2O$$

The principle of the triglyceride assay is as follows:

$$\text{Triglyceride} + 3H_2O \xrightarrow{\text{Lipase}} \text{glycerol} + 3\ \text{RCOOH}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{GPO}} \text{dihydroxyacetone phosphate} + H_2O_2$$

$$H_2O_2 + \text{4-aminophenazone} + \text{4-chlorophenol} \xrightarrow{\text{Peroxidase}} \text{4-(p-benzoquinone-mono-imino)-phenazone} + 2H_2O + HCl.$$

The assay kits used for the cholesterol and triglyceride were Boehringer Kit No 704121 and 704113 respectively and resultant colour changes were measured at 505 nm.

Free cholesterol and phospholipid were estimated with Boehringer Kit No 310328 and 691844 respectively using enzymatic colorimetric assays on a Centrifichem Encore centrifugal analyser (Baker Instruments).

Phospholipids were measured by an enzymatic colorimetric test kit (Boehringer Mannheim Cat. No. 691844), measured at 500nm, using a Centrifichem Encore analyser (Baker Instruments).

Quality controls Precinorm (Boehringer Mannheim Cat. No 781827)

Precipath (Boehringer Mannheim Cat. No. 1285874).

Free Cholesterol was measured using an enzymatic colorimetric test kit (Boehringer Mannheim Cat. No. 310328) by the CHOP-PAP method of a total cholesterol but omitting the enzyme cholesterol esterase. It was analysed using a Centrifichem Encore analyser (Baker Instruments) and read at wavelength 500nm.

Esterified Cholesterol was calculated from the total and free cholesterol as follows:-

1.68 x (total chol. -free chol.)

The correction factor of 1.68 is to account for the molecular weight of the different cholesterol esters.

Conversion Factors - The factors used to convert from mmol/l to mg/dl are as follows

cholesterol in mg/dl = 38.7 x value in mmol/l

triglyceride in mg/dl = 88.7 x value in mmol/l

5. 3 Modified Lowry Protein Assay

Protein measurements were performed according to a modified Lowry Protein Assay.

Reagents

1. Stock Reagents:
   Solution A - 2% Na_2CO_3 in 0.1M NaOH (w/v)
   Solution B - 2% NaK Tartrate (w/v)
   Solution C - 1% $CuSO_4$ (w/v)
   Folin Ciocalteu Reagent - BDH

2. Working Reagents:
   Biuret Reagent - 100 ml Solution A, 1 ml Solution B, 1 ml Solution C.
   If sample to be analysed is turbid, add 1 mg/ml sodium docecyl (Lauryl) sulphate .
   Dilute stock Folin Ciocalteu 1:1 with deionised water.

Standards

A stock solution of human albumin (Fraction V) Sigma A-8763, Lot 127F-9037 1

mg/ml is stored at 20°C.

Working standards in the range 0-50 µg (0, 15, 25, 50 µg) were prepared by taking appropriate volumes of stock standard 0-50 µl) and adjusting the final volume to 400 µl with deionised water.

Quality Control

Bovine serum albumin (Fraction V) Sigma A4503, Lot 17F-0150.

Two stock solutions of bovine serum albumin at 0.15 mg/ml and 0.30 mg/ml were stored at 20°C. One hundred µl will be equivalent to 15 µg and 30 µg respectively. The final volume in the assay was adjusted to 400 µl with deionised water.

Sample Preparation

Samples requiring dilution were adjusted to a final volume of 400 µl with deionised water. For $VLDL_1$ and $VLDL_2$ fractions, 100 µl samples as used whilst 50 µl sample was used for IDL fractions. In LDL fractions, 50 µl sample was diluted to 500 µl with distilled water and 100 µl of this dilutes ample was used for the assay.

Method

1. Two ml of Biuret reagent were added to 400 µl standard, control and samples.
2. These were vortexed and then allowed to stand for 10 minutes.
3. Two hundred µl (200 µl) working Folin Ciocalteu reagent was added with immediate mixing.
4. Allowed to stand for 30 minutes.
5. Read optical density at 750 nm within 2 hours.

5. 4 Measurement of Apo AI and ApoB.

These were measured using liquid-phase immunoprecipitation test kits (Orion Diagnostica Cat No. 67249 & 67265), read at 340 nm, in a Centrifichem Encore analyser (Baker Instruments).

5. 5 Method for LDL Subfraction Analysis (see Griffin et al, 1990)

Density Solutions

Density solutions d = 1.006 g/ml and d = 1.182 g/ml were prepared as described in the preceding section. The respective density solutions were then prepared as follows.

Solutions g/ml	Volume d = 1.006 g/ml		Volume d = 1.182
1.019	100 ml	+	8.5 ml
1.024	100 ml	+	13.6 ml
1/034	100 ml	+	18.6 ml
1.045	100 ml	+	27.8 ml
1.056	100 ml	+	42.9 ml
1.060	100 ml	+	49.3 ml

All densities were checked with a digital densitometer.

Methods

Three ml (3 ml) of fresh plasma was adjusted to a density of 1.09 g/ml by adding 0.25 g KBr and 0.3 ml of d = 1.182 g/ml solution. The sample and 6-step salt gradient were introduced sequentially into polyvinyl alcohol coated polyallomer SW-40 tubes by peristaltic pump. The gradient was prepared and centrifugation carried out at 23°C in a Beckman L8-60. The rotor was accelerated to 170 rpm and then centrifuged at 40, 000 rpm for 24 hours. On completion of the run the rotor was stopped with the brake off. After centrifugation, the LDL subfractions were eluted by upward displacement using dense hydrophobic material (Maxidens, 1.6 g/ml, Nyegaard Ltd) by a constant infusion pump (Sage Instruments, Orion Research Incorp, USA) at a flow rate of 0.69 ml/minute. The eluate was passed through a UV detector (MSE/Fisons, UK) and detected by continuous monitoring of absorbance at 280 nm. In most instances, it was possible to resolve three distinct subfractions, ie LDL-I, LDL-II and LDL-III. The individual subfraction areas beneath the LDL profile were quantified using Beckman 'Data Graphics' software (Beckman, UK). The detection system measured LDL concentration as absorbance at 280 nm and this was corrected to lipoprotein mass equivalence by applying previously calculated extinction coefficient. LDL-I 1 optical density (OD) = 2.63 mg lipoprotein/ml, LDL-II 1 OD = 2.94 mg lipoprotein/ml and LDL-III 1 OD = 1.92 mg lipoprotein ml.

The integrated areas were corrected for differences in extinction coefficient and expressed as percentage of total LDL concentrations in mg of lipoprotein/dl plasma. The total value for total LDL (d 1.019-1.063) lipoprotein mass (free cholesterol + cholesteryl ester + triglyceride + phospholipid + protein) was then used to generate individual subfraction concentration. Cholesteryl ester was calculated from (total cholesterol minus free cholesterol) times 1.69.

5. 6 Sequential Isolation of $VLDL_1$, $VLDL_2$, IDL and LDL

$VLDL_1$, $VLDL_2$, IDL and LDL were prepared from plasma by a modification of the

cumulative gradient centrifugation technique described by Lindgren et al (1972).

Density Solutions

The density solutions for the gradient was prepared from d = 1.006 g/ml and d = 1.182 g/ml solutions.

Density (g/ml)	Volume d = 1.006 g/ml		Volume d = 1.182 g/ml
1.0988	25 ml	+	27.89 ml
1.0860	25 ml	+	20.83 ml
1.0790	25 ml	+	17.72 ml
1.0722	25 ml	+	50.05 ml
1.0641	25 ml	+	12.31 ml
1.0588	25 ml	+	10.73 ml

Methods

Two ml of plasma was adjusted to a density of 1.118 g/ml with 0.341 g NaCl. This was mixed well and allowed to stand for a short time before sampling. 0.5 ml of d = 1.182 g/ml solution was pipetted into the bottom of the tube and then plasma and density solutions over layered in the order and volumes shown below using an AAII pump.

Density in g/ml	Volumes in ml
1.182	0.5
Plasma	2
1.0988	1
1.0860	1
1.0970	2
1.0722	2
1.0641	2
1.0588	2

When the gradient was prepared, centrifugation was carried out in a Beckman SW40 rotor at 39, 000 rmp at 23°C for 1 hour and 38 minutes. The top 1 ml ($VLDL_1$) was carefully removed using a long-form glass pipette at the end of the run. This was then replaced with 1 ml d = 1.0588 solution and centrifuged at 18, 5000 rpm for 15 hours and 41 minutes. At the end of this second run, the top 0.5 ml ($VLDL_2$) is removed with the long-form glass pipette. Further volumes are not replaced. IDL is removed in 0.5ml following centrifugation at 39, 000 for 2 hours and 35 minutes and LDL is removed in 1 ml following 30, 000 rpm for 21 hours and 10 minutes. The subfractions are stored at 4^0C until analysis.

5.7 Hepatic lipase assay

The technique used was a modification (Watson et al, 1995) of the post-heparin lipase activity measurement. Therefore, first the post-heparin lipase assay technique is described followed by modifications necessary for measuring pre-heparin lipase activities.

5.7.1 Post-heparin lipase assay

Plasma was incubated with a ^{14}C-labelled triglyceride/gum arabic emulsion; free fatty acids released by lipase activity were captured by albumin and extracted into a solvent. The ratio of radioactivity in the extracted fraction to the total present in blank incubations provided the basis of calculating the activity of the enzyme source. Selective measurement of lipoprotein lipase (LPL) is facilitated by preincubation of plasma with sodium dodecyl sulphate (SDS) to inactivate hepatic triglyceride lipase (HL) and the inclusion of serum as a source of LPL activator (apoC-II). HL activity is measured in 0.1 M sodium chloride to ensure inactivation of LPL.

Reagents

Glycerol Tri (1-^{14}C) oleate in toluene, 250 µCi	(Amersham CFA 258)
Triolene	(Sigma T-7140)
Gum Arabic	(Sigma G-9752)
Bovine Serum Albumin Fraction V	(Sigma A-4503)
Trizma Base	(Sigma T-3253)
Sodium Dodecyl Sulphate	(BDH 44244)
Potassium Sulfate	(Sigma P-4020)
Thrombin	(Sigma T-4265)

Preparation of Stock Reagents

Cold Triolein (20 mg/ml in toulene) - Dissolve 0.5g triolein in 25 ml toluene.

Radioactive Triolein

To 50 µCi (0.5 ml) glycerol tri (1-^{14}C) oleate add 24.5 ml toluene.
Divide into 7 x 3.5 ml aliquots in round bottomed glass flasks.
To each aliquot, add 3.5 ml cold triolein.
Dry under nitrogen.
Wash each flask three times with 3 ml heptane. Dry down under nitrogen between washes and keep the flask in a hot water bath.
Store the tubes dried down under nitrogen and sealed at -20°C.

5% Gum Arabic in 0.2 M Tris-HCl pH 8.4

Five g gum arabic in 100 ml volumetric flask, made up to volume with 0.2 M Tris-HCl (T-1503) pH to 8.4.
Filter through cotton gauze.
Divide into 18 x 5.5 ml aliquots and freeze at -20°C.

10% BSA in 0.2 M Tris-HCl pH 8.4

Weigh out 10 g BSA
Make up 20 ml of 1 M Tris-HCl (2.422 g of 20 ml).
To 20 ml of 1 M Tris-HCl, add approximately 40 ml of water, add 10 g BSA.
When the BSA has dissolved, adjust pH to 8.4.
Dilute to 100 ml with distilled water and divide into 18 x 5.5 ml aliquots and freeze at 20°C.

Extraction Solution

Methanol	:	1.4 parts (141 ml)
Chloroform	:	1.25 parts (125 ml)
Heptane	:	1.00 parts (100 ml)

Extraction Buffer

0.14 M potassium carbonate, 0.14 boric acid, pH 10.5
Dissolve 3.8699 g K_2CO_3 and 1.7312 g H_3BO_3 in 200 ml distilled water.
Adjust pH to 10.5 with 2 M KOH.

Serum

Collect 100 ml of fasted blood into EDTA from a number of individuals.
Add 0.1% w/v CaCl2 bovine thrombin 1 U/ml.
Incubate at 39°C for 30 minutes.
Remove the clot.
Dialyse against 0.15 M NaCl distilled water, pH 7.0, using 6,000-8,000 MWCO membrane.
Heat at 56°C for 30 minutes.
Dialyse against PBS.
Store as 1 ml aliquots at -20°C.

SDS Reagent

02. M Tris-HCl, 25 mm SDS (50 mm SDS for horses)
0.360 g SDS 1.21 g Tris-Base in 40 ml distilled water
Correct pH to 8.2, then make up volume to 50 ml in a volumetric flask.

Low Salt Buffer: 0.1M Tris, 0.216 M NaCl, pH 8.4

12/11 gTris Base 6.31 g NaCl in 500 ml distilled water. This gives a final NaCl concentration of 0.1M in the reaction solution.

High Salt Buffer: 0.2M Tris, 2.16 M NaCl, pH 8.4
12/11 g Tris Base, 63.11 g NaCl in 500 ml distilled water. This gives a final NaCl concentration of 1M in the reaction solution.

Assay Design

Low Salt Assay (four tubes)		High Salt Assay (two tubes)
Tubes 1, 2	Tubes 3, 4	Tubes 5, 6
-SDS	+SDS	
30 µl 0.15 M NaCl	20 µl 0.15 M NaCl	30 µl 0.14 M NaCl
10 µl PHP	20 µl preincubation mixture	10 µl PHP

NB: Each samples in duplicate
All tubes, samples substrate and serum kept on ice prior to incubation.
Include two tubes with 40 µl 0.15 M NaCl (no PHP) in each assay half to measure blank (B) and total counts (TC).

Assay Procedure

Preincubation with SDS reagent

Place 0.5 ml post heparin plasma in TMU tube and add 0.5 ml SDS reagent and vortex. Incubate in the water bath at 26°C for 60 minutes and remove 20 µl for the low salt assay. Twenty µl is removed for the assay.

Preparation of Substrate Mixture (30 minutes before required)

Radioactive triolein was placed in a glass flask and 5.5 ml gum arabic solution was added. This was sonicated on ice at 18 microns for four minutes so that no fat droplets are visible. (Sonicator tip 1/2 cm from bottom of flask). Add 5.5 ml of 10% BSA solution, mixed and vortex and keep on ice.

Reaction Mixtures

Tubes 1, 2, 3 4 : To each add 200 µl substrate
To each add 250 µl low salt buffer (0.2 M Tris/0.1 M NaCl)
To each add 50 µl serum

Tubes 5, 6 : To each add 200 µl substrate
To each add 250 µl high salt buffer (0.2M Tris/1.0 M NaCl)
To each add 50 µl 0.15 NaCl

Incubation

Cap all tubes and incubate in water bath at 28°C for 60 minutes and return to ice immediately. Add 3.25 ml extraction solution to each tube. Add 0.75 ml extraction buffer to each tube, mix on vortex. Then centrifuge at 4°C, 3 K for 30 minutes. Take 1 ml of upper phase for counting in scintillation vial. Add 10 ml Ultima Gold (Packard) scintillation fluid and 200 µl acetic acid. For blank, take 1 ml upper phase and for total counts, take 1 ml of lower phase.

Calculations of Results

$$\text{Lipase activity in µmol FFA released/ml/hour} = \frac{(\text{CPM samples} - \text{CPM blank}) \times 755.1}{\text{CPM total} - \text{background}}$$

5.7.2. Pre-heparin lipase assay

Modification of the lipase assay for the measurement of lipoprotein lipase (LPL) and hepatic lipase (HL) in preheparin plasma.

1. Samples were collected into lithium heparin tubes and kept on ice. Plasma should be separated at 4°C and frozen at -70°C within one hour of collection.

2. Sample are assayed in triplicate for LPL, HL and blanks.

3. For the measurement of LPL, plasma was preincubated with 35 mm SDS (instead of 25 mm) in 0.2 M tris base, pH 8.2 which was made up fresh on the day of assay. Incubation was for 60 minutes at 26°C as per the standard assay.

4. Forty µl (40 µl) of the plasma-SDS mixture (instead of 20 µl) was taken for the LPL incubation: the LPL substrates contains 0.2 M NaCl as per the standard assay.

5. The HL incubation contains 20 µl of plasma (instead of 10 µl) with substrate containing 2.0 M NaCl as per the standard assay.

6. Both the LPL and HL incubations were for 1.5 hours (instead of one hour).

7. Following solvent extraction, 2 ml of the upper layer (instead of 1 ml) was taken for liquid scintillation counting (added to 10 ml Ultima Gold, 200 µl of acetic acid). Use 1 ml of blank lower phase for total counts.

Calculations of Results

$$\text{Lipase activity} = \frac{(\text{CPM samples} - \text{CPM blank}) \times 755.1 \times 1/1.5 \times 1000 \times 0.5}{(\text{CPM total} - \text{background}) \times 2}$$

5.8 Surface Modification of Beckman Ultraclear Centrifuge Tube

This was a procedure for coating the interior surface of Beckman ultraclear centrifuge tubes (in particular those used in swing-out rotors) with polyvinyl alcohol. Once Coated, salt solutions are then able to run smoothly down the sides of the tubes.

6. HAEMOSTATIC MARKERS

6.1 Plasminogen Activator Inhibitor (PAI) Activity

Plasma plasminogen activator inhibitor activity levels were determined by a commercially available chromogenic substrate assay (Coatest PAI; Chromogenix, Epsom, U.K.). The assay involves the addition of a fixed amount of single-chain tissue plasminogen activator (tPA) in excess to undiluted plasma, where most of it rapidly forms an inactive complex with the fast inhibitor PAI-1. (Single-chain tPA is used in this assay since PAI-2, another inhibitor of tPA found in plasma, inhibits this single-chain form very poorly). The residual tPA then activates plasminogen to plasmin in the presence of a stimulator. The amount of plasmin formed is directly proportional to the PAI activity in the plasma sample (which under most circumstances is mostly PAI-1). Plasmin levels formed are determined by measuring the amidolytic activity of plasmin on a chromogenic substrate (S-2403), which releases p-nitroaniline, levels of which are determined using an automatic microplate reader (MR700 from Dynatech, West Sussex, UK), at 405nm. The absorbance of the sample is then compared with the standard curve generated for each test run, and a value for the level of PAI activity obtained. This is expressed in arbitrary units (AU), one AU being defined as the amount which inhibits one IU of tPA/ml under the test conditions (Chmeilewska et al 1986, Gram et al 1993). The inter assay CV performed on a normal pool on 10 occasions was found to be 8.1%. The intra assay CV calculated on 5 single assays of a normal pool was 7.9%.

6.2 Tissue Plasminogen Activator (tPA) Antigen

Plasma levels of tPA were measured with a commercially available enzyme linked immunosorbent assay (ELISA) from Biopool AB, Umea, Sweden (Tintelize #101120). The assay quantifies human single chain and two chain tPA antigen. No cross reaction with urokinase is observed. Maximal sensitivity of the assay is reported by the manufacturer as 1.5 ng/ml.

In this assay some interference may occur from plasma levels of other antibodies such as anti-goat antibodies and rheumatoid factor. To exclude false positives, each sample is applied to two wells, one containing normal goat IgG, and the other containing goat anti-human tPA IgG. The difference in assay response between these two wells is highly tPA specific. After initial binding to the pre-coated well, the second antibody which is conjugated to horseradish peroxidase is added to the wells. This will bind to

free antigenic determinants on the tPA molecules present. Unbound conjugate is washed away after a further incubation period, and the remaining peroxidase is then measured by the addition of its substrate orthophenylenediamine dihydrochloride. The colour development is proportional to the amount of tPA bound to the well. The absorbancies were read at 492 nm on an MR 700 plate reader (Dynatech, West Sussex,UK). Because of the blank reading for each test and standard, the curve had to be plotted manually and the results from each test read from the curve. Inter assay CV was 9.8%. Intra assay CV was 9.0%.

6.3 Von Willebrand Factor (VWF) Antigen

Plasma von Willebrand factor (VWF) antigen levels were measured using an in-house enzyme linked immunosorbent assay (ELISA), employing rabbit anti-human polyclonal antibodies obtained from DAKO plc, High Wycombe, UK (Short et al 1982). The standard curve was constructed using a normal pooled plasma from 20 volunteers, and the 5th British Standard (obtained from National Institute for Biological Standards and Control, South Mimms, UK) was used for pool calibration. This meant that the results could be reported as international units/decilitre (iu/dl), instead of as a percentage of the pool.

The capture antibody is coated onto the wells of a 96 well microtitre plate (Dynatech, West Sussex, UK). Diluted plasma samples and standards are then added and incubated for 30 minutes. After washing with buffer, the antibody, which is conjugated to horseradish peroxidase, is added. After this has incubated for a further 30 minutes, another washing procedure takes place. The peroxidase substrate, orthophenylenediamine which has been activated by addition of hydrogen peroxide is added and after a rapid colour development, the reaction is stopped by the addition of 1.5 M sulphuric acid. The absorbance in the wells is read using the microplate reader (MR700 from Dynatech, West Sussex, UK) at 492 nm and the results of the test samples are read against the standard curve plotted for the assay. Using the 5th British Standard in 10 assays, the CV for the inter-assay variability was 7.7%. The intra-assay variability was 6.0%, being the CV of 5 aliquots of the standard in a single assay.

6. 4 Fibrin D-dimer antigen

The measurement of plasma fibrin D-dimer, which is present in several cross-linked fibrin degradation products, was carried out using the `Dimertest' enzyme linked immunosorbent assay (ELISA) kit from Agen Biomedical Limited (Parsippany, New Jersey, U.S.A.). The cleavage of both fibrinogen and fibrin by the fibrinolytic enzyme plasmin yields fibrin(ogen) degradation products (FDPs). However, only those products from the degradation of cross-linked fibrin contain D-dimer. Elevated levels of cross-linked FDPs detected by this method indicate increased formation and lysis of

cross linked fibrin, and imply an increase in the turnover of cross-linked fibrin.

The principle of the test involves the use of the monoclonal antibody DD-3B6, which recognises D-dimer and FDPs containing it. The DD-3B6 is bound to microtitre plates to which either plasma or standard dilutions of D-dimer are added. After an incubation period to allow binding of the D-dimer to the capture antibody, the plate is washed with buffer and a second monoclonal antibody (DD-4D2) is added. This is conjugated to horseradish peroxidase and binds to the cross-linked FDPs that have been immobilised by the DD-3B6 antibody bound to the wells. After further washing, a substrate, 3-ethyl benzthiazoline sulphonic acid (ABTS) is added, which is hydrolysed by the horseradish peroxidase resulting in a coloured product which is proportional to the amount of D-dimer present in the well. The absorbance is then read spectrophotometrically on an MR700 plate reader (Dynatech, West Sussex, UK) at 405nm, and compared with the standard curve.

The CVs for this assay were higher than for other assays, with an inter assay variation of 15.8% calculated by measuring the D-dimer level of the pool in 10 different assays. The intra assay CV calculated from 5 aliquots of pool run on the same assay was 14.2%.

6. 5 Factor VIIc

FVIIc was determined by a one stage clotting assay in an ACL-300 Research coagulameter (IL, Warrington, UK). The principle of this assay involves adding to diluted patient plasma, plasma deficient in factor VII. Correction of the prolonged clotting time of deficient plasma is proportional to the concentration (activity %) of factor VII in the patients plasma. The exact activity is interpolated from a standard curve automatically measured on the instrument.

7. MYOMETRIAL RESISTANCE ARTERY BEHAVIOUR

The myography work was performed by Dr Richard Hayman, Clinical Research Fellow in Obstetrics and Gynaecology, School of Human Development, City Hospital Nottingham. The subject selection criteria, vessel collection methodology and myography techniques employed to examine the relationship between circulating lipid concentrations and endothelial function in normal pregnancy and pre-eclampsia are described in detail in Chapter VI.

CHAPTER III

A LONGITUDINAL STUDY OF LIPOPROTEIN SUBFRACTION CHANGES IN NORMAL PREGNANCY

1. INTRODUCTION

During the course of normal pregnancy, plasma triglyceride and cholesterol concentrations rise by 200-400 % and 25-50 %, respectively. Although the rudiments of this process have been the subject of many studies (Warth et al 1975, Knopp et al 1982, Ordovas et al 1984, Fahraeus et al 1985, Desoye et al 1987, Alvarez et al 1996), few were undertaken throughout the gestational period, and fewer still incorporated measurements of lipoprotein subclasses. This information is important because it has become apparent that lipoprotein particles are not homogeneous but contain discrete subfractions differing in structure, physico-chemical properties, kinetic behaviour and origin. Low density lipoprotein (LDL), for example, incorporates a spectrum of lipoproteins of differing atherogenic potential. Small, dense LDL (LDL-III) in distinction to the larger more buoyant LDL-I and LDL-II particles, exhibit enhanced oxidation potential and reduced receptor binding (Steinberg et al 1989). Once oxidised, these particles are believed to be highly atherogenic promoting foam cell formation and initiating endothelial dysfunction (Steinberg et al 1989).

The role of small, dense LDL as a risk factor for coronary heart disease (CHD) is becoming well established (Austin et al 1990, Coresh et al 1993, Tornvall et al 1993, Griffin et al 1994), and the above atherogenic mechanisms have been proposed. Furthermore, recently reported prospective studies have demonstrated that the presence of small, dense LDL particles precedes CHD (Krauss et al 1994, Gardner et al 1996) and predicts the development of non-insulin dependent diabetes mellitus (Austin et al 1995).

In man and non-pregnant women, plasma triglyceride is the major determinant of small dense LDL, accounting for 40-60% of the variability of this fraction in the plasma (Tornvall et al 1993, Griffin et al 1994, Tan et al 1995, Austin & Edwards 1996). In addition, recent cross-sectional studies (Griffin et al 1994, Tan et al 1995) have prompted the suggestion that within the relationship between plasma triglyceride and the LDL subfraction profile, there is a threshold effect. However, to the best of our knowledge no longitudinal studies have been designed to examine this phenomenon in individual subjects. The physiological changes in plasma triglyceride which accompany pregnancy provides such an opportunity.

In an earlier study of pregnant women, Silliman et al (1994) showed that the raised concentrations of plasma triglyceride which are prevalent one month prior to term are accompanied by a reduction in mean LDL size. Those women who experienced the greatest increase in plasma triglyceride exhibited the most significant shrinkage in their LDL particle diameter. By six weeks postpartum, reversal of this process was apparent, but the study design did not permit identification of the dynamics of the phenomenon or the timing of this particle size transition.

To address these issues, we have measured LDL subclasses throughout gestation using density gradient ultracentrifugation. This technique, allowed us to examine quantitatively the relationship between plasma triglyceride and perturbations in the LDL subfractions during pregnancy. Our primary hypothesis was that as pregnancy proceeded and plasma triglyceride increased, the LDL subfraction profile would be perturbed with the appearance of smaller, dense particles. A secondary hypothesis was there would exist a threshold above which LDL-III formation accelerated. We also wished to define whatever simultaneous changes in the spectral distribution of very low and intermediate density lipoprotein particles would accompany this process. To gain insight into potential mechanisms responsible for these changes we undertook serial measurements of serum oestradiol throughout pregnancy.

2. SUBJECTS, SAMPLES AND METHODS

Twelve pregnant women who registered for obstetric care at Glasgow Royal Maternity Hospital were recruited. Eight were multigravida with normal previous deliveries and four were primigravida. All women had no identifiable risk factors for the current pregnancy as determined by detailed obstetric history. During the course of pregnancy one woman emigrated and another woman had a positive test for Down's syndrome in the fetus and was excluded by mutual consent. The remaining ten women all had normal course and outcome of pregnancy, term delivery, ate a customary diet and did not receive any medication known to interfere with lipid metabolism or lipid determination. Additionally, none were phenotype apo E2/E2, an inherited trait which may generate disturbances in the plasma lipid profile even in normolipaemic subjects. The subject characteristics are given in Table 1.

For comparison, we also measured plasma lipid and lipoprotein subfraction concentrations in 20 age, and BMI matched, healthy non-pregnant females. None were taking medication known to affect lipid or lipoprotein subclass concentrations.

Table 1. Characteristics of study subjects

Parameters	Pregnant subjects Mean ±SD	Range	Non-pregnant Subjects Mean ±SD
Prim/parous	2/8		5/15
Age (yrs)	29.5±4.0	24-35	29.0±5.3
Height (m)	164±5	155-173	164±7
Weight (Kg)	62±6	55-74	61±6
BMI (Kg/m^2)	22.9±2.3	20-28	22.6±2.6
Blood pressure (mmHg)			
Systolic	104±6*	95-110	115±9
Diastolic	69±4*	58-70	77±9
Length of gestation (wks)	39.6±1.3	38-41	------
Birth Weight (Kg)	3.5±0.2	3.1-3.8	------

*Blood pressure at booking

The pregnant women were first seen at 10 weeks gestation (+/- 1 week) and thereafter at five week intervals until delivery. Each woman was seen six times. The gestational age was confirmed by ultrasound. All subjects were sampled after an overnight fast of 12 hours. Twenty-five mL of blood was collected by venepuncture into K_2EDTA (final concentration 1mg/mL), lithium-heparin (5 mL), and plain tubes (5 mL). Plasma and serum were harvested at 4C by low speed centrifugation (3000 rpm) and aliquots of plasma for lipid and lipoprotein measurements and assessment of circulating hepatic lipase activity, and serum for oestradiol determination were used immediately.

The methods employed are described in their entirety in Chapter II. Plasma total cholesterol, triglyceride and HDL cholesterol were determined by a modification of the standard Lipid Research Clinics Protocol (1975). $VLDL_1$ (Sf 60-400), $VLDL_2$ (Sf 20-60), IDL (Sf 12-20) and LDL (Sf 0-12) were prepared from plasma by a modification of the cumulative gradient ultracentrifugation technique described by Lindgren et al (1972). The cholesteryl-ester, triglyceride, free cholesterol, phospholipid and proteins of the lipoprotein were assayed as described (Chapter II) and concentrations calculated as the sum of the components (expressed as mg/dL plasma). Isolation of LDL subfractions from fasting plasma was achieved by density gradient ultracentrifugation using a discontinuous salt gradient (Griffin et al 1990). Circulating hepatic lipase (HL) activity was determined in fasting plasma using a method that has been developed in our laboratory to measure the low amounts of the enzyme normally present in the

circulation, ie, without heparinisation which is normally used to release all hepatic lipase bound to endothelial sites (Watson et al 1995).

Statistical Analysis

The lipid and lipoprotein data were parametrically distributed as judged by the examination of normal probability plots. Nevertheless, as numbers were small, data are presented as medians and ranges. Comparisons of lipoprotein composition and between changes in circulating hepatic lipase activity between 10 and 35 weeks were performed by Students paired t-test. The level of significance required in comparisons of lipoprotein composition analysis was subject to Bonferroni correction.

Associations between initial triglyceride concentration, triglyceride/oestrogen ratio and final percentage abundance LDL-III were tested by calculating the Pearson correlation coefficient (R) and the coefficient of determination (R^2) which was expressed as a percentage, (i.e. R^2 gives the percentage of variation in the dependent variable which is explained by variation in the independent variable). The significance of association between pairs of variables was determined by linear regression.

3. RESULTS

3.1. Lipids and circulating hepatic lipase

Gestational age	10	15	20	25	30	35	Non-pregnant subjects
Triglyceride (mmol/L)	0.78	1.20	1.40	1.53	1.73	2.55	0.80
	0.6-1.5	0.6-1.8	0.8-2.3	0.9-2.4	1.2-2.7	1.6-3.6	0.5-1.3
Cholesterol (mmol/L)	4.40	5.45	6.23	6.53	6.73	7.20	4.53
	3.2-6.8	4.0-7.5	4.7-7.6	5.0-8.5	5.4-8.7	5.6-8.4	3.0-6.2
HDL-Chol (mmol/L)	1.68	1.80	2.00	1.95	1.80	1.70	1.50
	1.3-2.2	1.3-2.1	1.4-2.6	1.2-2.4	1.2-2.2	1.1-2.4	1.0-1.8
Oestradiol (nmol/L)	8.4	19.0	38.7	46.2	58.1	73.0	<1.0
	3.6-22	8.8-39	17-60	34-76	40-73	47-105	<1.0-<1.0

Table 2. Median (range) of lipid and oestradiol concentrations at each gestational time point.

As pregnancy progressed from 10 to 35 weeks, median plasma triglyceride and cholesterol concentrations in our subjects rose from 0.78 to 2.55 mmol/L, and from 4.40 to 7.20 mmol/L, respectively (Table 2). Mean (SE) circulating hepatic lipase activity fell by 40% (P=0.0007) from 24.9 (1.9) to 15.8 (2.3) μmol FFA/mL/min, between 10 and 35 weeks gestation. HDL-cholesterol concentration increased from a median of 1.68 mmol/L at 10 weeks to a maximum at 20 weeks gestation of 2.00

mmol/L and thereafter fell to a concentration at 35 weeks of 1.70 mmol/L (Table 2).

3.2.VLDL subfractions and IDL composition and concentration during pregnancy

The rise in plasma triglyceride between 10 and 35 weeks encompassed the range of concentrations seen in a normal population of pre-menopausal women (Table 2) (Tan et al 1995). The concentration of the large triglyceride-rich VLDL ($VLDL_1$), increased from a median of 19 mg/dL to 109 mg/dL at 35 weeks (Table 3).

Gestational age (wks)		10	15	20	25	30	35	Non-pregnant subjects
$VLDL_1$	(mg/dL)	19	37	47	58	68	109	23
	range	12-55	12-81	26-110	27-122	27-139	38-170	5-85
$VLDL_2$	(mg/dL)	17	36	36	47	66	103	23
	range	7-45	15-67	20-77	20-112	38-114	46-168	13-44
IDL	(mg/dL)	26	48	58	72	91	124	35
	range	13-54	14-69	24-100	44-121	55-140	79-157	18-62
LDL	(mg/dL)	200	241	292	308	329	353	207
	range	135-323	128-388	206-410	204-390	272-453	244-534	150-363
LDL-I	(mg/dL)	33	37	49	53	62	67	50
	range	16-52	22-54	37-70	37-82	33-104	27-96	22-130
	(%)	17	19	18	18	18	20	25
	range	7-20	10-20	14-21	15-21	12-23	11-22	11-49
LDL-II	(mg/dL)	143	155	160	189	218	201	135
	range	95-231	88-268	103-287	95-285	65-263	59-316	72-258
	(%)	70	69	68	69	60	49	60
	range	62-75	37-72	33-73	34-73	24-69	24-72	42-76
LDL-III	(mg/dL)	28	28	32	44	79	123	31
	range	15-56	15-134	24-165	23-142	36-174	43-192	5-68
	(%)	14	15	14	13	21	32	15
	range	9-24	8-53	9-53	6-51	11-64	10-65	3-23

Table 3. Median (range) of lipoprotein subclass concentrations at each gestational time point.

There was a parallel increase in the smaller cholesterol-rich VLDL subfraction, $VLDL_2$ (17 to 103 mg/dL), so that the ratio of $VLDL_1$ to $VLDL_2$ remained constant.

Examination of $VLDL_1$ and $VLDL_2$ compositions (Table 4), indicated that the make-up of the particles was not significantly different between 10 and 35 weeks despite the increase in plasma triglyceride. Further the composition of these fractions was similar to that seen in a recent cross-sectional survey of healthy adults (Tan et al 1995).

The plasma concentration of IDL (26 to 124 mg/dL) increased in a similar proportion to increments in $VLDL_1$ and $VLDL_2$ (Table 3) and the particle became more triglyceride rich as pregnancy progressed (Table 4).

10 weeks

Subfraction	Protein	Free chol	Chol esters	Triglyceride	Phospholipid
$VLDL_1$	9.9 (0.8)	2.8 (0.4)	8.78 (0.9)	64.9 (1.6)	13.5 (1.0)
$VLDL_2$	15.4 (0.6)	3.7 (0.5)	20.6 (1.0)	43.7 (1.4)	16.6 (0.9)
IDL	21.5 (0.9)	5.0 (0.3)	39.7 (0.9)	15.1 (0.9)	18.7 (0.8)
LDL	23.3 (0.9)	9.5 (0.8)	43.1 (1.2)	6.0 (0.7)	18.1 (0.7)

35 weeks

Lipoproteins	Protein	Free chol	Chol esters	Triglyceride	Phospholipid
$VLDL_1$	7.9 (0.2)	2.5 (0.3)	11.6 (1.0)	63.9 (1.1)	14.2 (0.3)
$VLDL_2$	13.4 (0.3)	5.4 (0.4)	22.8 (1.0)	39.4 (1.3)	19.1 (0.3)
IDL	18.6 (0.2)	6.5 (0.5)	35.7 (1.1)	18.9 (0.8) *	20.3 (0.3)
LDL	24.2 (0.4)	10.3 (1.2)	35.7 (1.1) *	9.6 (0.8) †	20.2 (0.3)

Table 4. Chemical composition of apolipoprotein-B containing lipoproteins.

Values are mean (standard error) expressed as percent composition (g/100g) *$P<0.01$, † $P<0.002$ refer to the significance of difference between 10 and 35 weeks as determined by Student's paired t-test after correction for multiple comparisons by Bonferroni correction.

3.3 LDL subfraction mass, composition and profile

Total LDL mass increased during gestation in all subjects so that the median concentration increased by around 70% (200 to 353 mg/dL) between 10 and 35 weeks. Compositional analysis revealed that the lipoprotein became enriched in triglyceride and depleted in cholesteryl ester over the period (Table 4).

On examining LDL subfractions it was seen that in 4 of the 10 subjects (ED, LC, KA, CH), no significant change in the LDL subfraction pattern occurred throughout

gestation so that the relative proportions of LDL-I, LDL-II and LDL-III were relatively unaltered (see Figures 4, CH as a representative example, and Figures 5 and 6). In contrast, in the other six women (AM, AP, EG, IL, RM, AS), the LDL subfraction pattern was modified towards a smaller denser pattern in a manner suggestive of a threshold transition (see Figure 4, AM as a representative example, and Figures 5 and 6), with the proportion of LDL-III increasing at the expense of LDL-II. Once again, the proportion of LDL-I remained unchanged (Table 3). Overall, therefore, median percent LDL-I remained relatively unaltered at between 17-20%, whereas LDL-II declined from 69% to 49% as the relative proportion of LDL-III doubled (14 to 32%) (Table 3).

In line with the observed perturbations in LDL subclasses and total LDL mass, LDL-I mass increased around two-fold, from 33 to 67 mg/dL; LDL-II mass increased least by around 40% from a median of 143 to 201 mg/dL, reaching a maximum of 218 mg/dL at 30 weeks gestation; whereas in sharp contrast, LDL-III mass increased by greater than four-fold from 28 to 123 mg/dL (Table 3).

3.4. Relationship between serum oestradiol and lipoprotein perturbations.

From 10 weeks to 35 weeks of pregnancy mean serum oestradiol concentration increased steadily from a mean of 10 nmol/L to greater than 78 nmol/L. For each individual there was a strong relationship between the rise in oestradiol and the increment in plasma triglyceride (R^2 0.71-0.98, mean R^2 = 0.92; Figure 7) and plasma cholesterol (R^2 0.79-0.97, mean R^2= 0.87, data not shown). It was noted, however, that the slope of the association between increments in serum oestradiol and plasma triglyceride differed between subjects (see Table 5 and Figure 8). Additionally, the magnitude of rise in serum oestradiol during gestation correlated significantly (R^2=41.7%, p=0.044) with the change in HDL-cholesterol between 10 and 35 weeks (Figure 8)

3.5. Factors relating to LDL subclass modification

When the rate of change in plasma triglyceride for a given increment in oestrogen was calculated (see Table 5 and Figure 8) it appeared that the present cohort could be divided into two, those with a gradient of <2 and those with a gradient >2 (mmol/nmol *10^{-2}). Five of the six subjects who developed high LDL-III levels were all in the latter category, whereas three of the four women in whom LDL-III percentage remained relatively unchanged were in the former category. Similarly, the subjects could be divided on the basis of starting triglyceride (Table 5). In five of the six in whom the profile was altered towards a smaller denser LDL pattern, starting plasma triglyceride was 0.8 mmol/L or above, whereas it was below this level in the four subjects in whom no change in LDL subclass proportions was apparent. As a

result, both the 10 week triglyceride and the the ratio of increment in triglyceride corrected for oestrogen correlated with 35 weeks concentration of small, dense LDL-III, and together these two parameters accounted for 67% (p=0.021) of its variability.

Subject	Booking Trig mmol/L	Trig/ E2 gradient	LDL profile shift	LDL-III mass at 35 wks[mg/dL]	Plasma Trig @ LDL shift	$VLDL_1$ @10/35wks (mg/dL)	Gest age (wks)@ LDL shift	HL activity @10/35 wks [umolFA/mL/Hr]
CH	0.60	1.32	-	45	1.70	20/80	-	26/19
LC	0.60	1.39	-	43	1.65-	15/38	-	20/6
AP	0.65	4.63	+	192	1.3-1.4	12/123	25-30	25/27
KA	0.75	2.31	-	57	2.10-	17/58	-	28/2
ED	0.75	1.98	-	46	1.95-	17/81	-	20/12
AM	0.80	1.62	+	132	1.8-2.4	18/102	30-35	30/16
RM	1.00	3.15	+	114	2.3-2.5	35/170	25-30	32/21
AS	1.10	3.98	+	159	1.1-1.6	55/131	10-15	32/17
EG	1.50	3.49	+	149	1.7-2.3	55/162	15-20	19/4
IL	1.55	2.17	+	189	1.9-2.3	50/116	20-25	15/12

LDL III mass = 16.9 + 36.7 * Trig/E2 gradient, R^2= 48.6%, p=0.025;
LDL III mass = 14.0 + 105 * [booking triglyceride], R^2=36.7%, p=0.063
LDL III mass = -38.7 +77.7 [booking triglyceride] + 30.2 * Trig/E2 gradient, R^2=67.0%, p=0.021

Table 5. Patients characteristics in relation to LDL perturbation and LDL III mass at 35 weeks

In addition, it was notable that the 35 week concentration of $VLDL_1$ was greater than 100 mg/dL only in those individuals in whom alteration in the LDL profile was apparent ($\chi 2$, $p<0.001$), whereas hepatic lipase activity was not significantly different ($p>0.05$) between those group of individuals demonstrating no change in LDL subclass pattern compared to those exhibiting a significant increase in percentage LDL-III (Table 5).

3.6. Characteristics of threshold triglyceride levels

The alteration in the LDL subclass profile if it occurred was observed at differing gestational times and plasma triglyceride concentrations in each women (Table 5 and Figures 5 and 6); that is the "threshold" triglyceride level, at which LDL-III concentration began to increase substantially, exhibited wide variation. In subject AS, for example, the LDL pattern altered early sometime between 10 and 15 weeks corresponding to a plasma triglyceride of 1.1-1.6 mmol/L. In contrast, in subject RM, alteration in the profile was not evident until 30 weeks and therefore must have commenced sometime between 25 to 30 weeks, and at a plasma triglyceride concentration of between 2.3-2.5 mmol/L.

With respect to gestational age, the alteration in profile was evident earliest in those women (AS, EG, IL) with the highest 10 week plasma triglyceride levels. In contrast, alteration in the profile was evident at the lowest triglyceride concentrations in those women (AS and AP) exhibiting the highest triglyceride to oestrogen gradients. Additionally, AS had by far the lowest HDL-cholesterol concentration throughout pregnancy (1.35 to 1.15 mmol/L), whereas AP had the highest, around ten-fold increment (12-123 mg/dL) in $VLDL_1$ concentration (Table 5).

4. DISCUSSION

This study set out to define the alterations in lipoprotein concentration and lipoprotein subfraction proportions and concentrations occurring throughout gestation in a group of healthy women. Serum oestradiol was measured and related to the increases in plasma triglyceride concentration. The increase in plasma triglyceride, cholesterol and HDL-cholesterol during gestation are in agreement with reports by numerous other investigators (Warth et al 1975, Knopp et al 1982, Ordovas et al 1984, Fahraeus et al 1985, Desoye et al 1987, Alvarez et al 1996) and therefore our results may be applied more widely.

As pregnancy progressed and plasma triglyceride increased across the normal range, $VLDL_1$ and $VLDL_2$ increased in parallel. The significant rise in $VLDL_2$ concentration during gestation corroborates the results of one previous study (Silliman et al 1994). The results of that study allied to our data further highlight the uniqueness of the hyperlipidaemia of pregnancy. To elaborate, in the normal non-pregnant population, higher concentrations of plasma triglyceride are associated with a preferential higher $VLDL_1$ concentrations (Tan et al 1995). This particle is secreted by the liver to supply tissues with triglyceride fatty acids in the post-absorptive state. The concentration of $VLDL_2$, the principal precursor in the circulation to IDL and LDL, does not change as dramatically. In the normal adult population, a high concentration of $VLDL_1$ is often associated with a failure of insulin action and increased risk of coronary heart disease. In contrast, as pregnancy progressed and high triglyceride levels developed, $VLDL_1$

and $VLDL_2$ rose together so that their ratio, instead of increasing by two-fold, as would be predicted from population studies in the non-pregnant ($VLDL_1$ to $VLDL_2$ ratio at a plasma triglyceride of 0.5 mmol/L is 1.0 compared to 2.0 at plasma triglyceride of 2.5 mmol/L) (Tan et al 1995), remained constant.

In the absence of kinetic data, it remains unclear to what extent the increase in VLDL subclass concentrations represent increased synthesis or reduced catabolism. Oestrogen mediated increased synthesis is likely to be the predominant mechanism in early pregnancy. Micronised ethinyl oestradiol has been reported to increase large VLDL ($VLDL_1$) production rates of healthy post-menopausal women 1.8 fold (Walsh et al 1991), whereas more potent oestrogens in oral contraceptives have been shown to promote increased plasma levels of both large (3-fold) and small VLDL (2.2 fold) subclasses, by increasing production rates of both particles, fractional catabolic rates remaining unchanged (Walsh et al 1993). It follows, therefore, that the significant elevations in oestradiol seen in gestation may favour balanced elaboration of both VLDL subclasses. However, relative insulin resistance is a feature of late gestation (Herrera et al 1988) and may also contribute to increased $VLDL_1$ levels.

Reduced catabolism of VLDL (specifically $VLDL_1$), however, may be an additional factor for increased VLDL concentrations in late pregnancy as a significant reduction (around 50%) in post-heparin LPL activity and a significant negative correlation between LPL activity and VLDL levels during pregnancy have been reported previously (Alvarez et al 1996). From studies in rats (Herrera et al 1988), it is known that adipose tissue is the main site of the decreased LPL activity seen in late gestation and that this change is caused by insulin resistance.

The rise in IDL concentration during pregnancy has also been previously demonstrated (Silliman et al 1994, Warth et al 1975). It is notable, however, that the degree in elevation in IDL concentration was significantly greater (almost double) than previously reported (Tan et al 1995) in non-pregnant individuals for a similar increment in plasma cholesterol. This is in keeping with the markedly elevated $VLDL_2$ levels in pregnancy and subsequent delipidation to IDL in the circulation. In line with previous studies (Wart et al 1975, Montelongo et al 1992), as pregnancy progressed IDL and LDL particles became triglyceride enriched, reflecting in part oestrogen mediated inhibition of hepatic lipase activity with resultant reduced triglyceride hydrolysis of IDL and LDL particles, and second an increase in the interchange of neutral lipids between lipoproteins (Montelongo et al 1992, Iglesias et al 1994).

Recent work from our laboratory (Griffin et al 1994, Tan et al 1995) has suggested the presence of a critical triglyceride threshold at which significant changes in LDL subclasses may occur. The current longitudinal data from individual subjects, studied repeatedly during the appearance of the physiological hyperlipidaemia, provides additional strong support for this important concept. In all subjects with the exception

of AS, percent LDL-III (and LDL-III mass) changed little in early gestation despite increasing triglyceride concentrations. However, as gestation progressed and plasma triglyceride levels increased further, in six of the 10 subjects the LDL profile changed dramatically with the proportion of LDL-III (and therefore LDL-III mass) increasing simultaneous with a decrease in LDL-II. Because we sampled at four week intervals, we were able to determine only an approximate gestational age or plasma triglyceride concentration at which LDL profile change first occurred. Nevertheless, there appeared to be considerable variation between individuals in the gestational age and plasma triglyceride intervals at which change in the LDL profile first manifested. In general, the alteration in profile towards small dense LDL-III, was earliest in those individuals with the highest initial 10 weeks triglyceride concentrations. However, it may be significant that LDL profile alteration occurred at the lowest triglyceride concentrations in the two individuals with the highest triglyceride to oestrogen increments, and that one of these two individuals had the lowest HDL-cholesterol level, whereas the other had the greatest proportional increase in $VLDL_1$. Both low HDL-cholesterol and raised $VLDL_1$ concentrations are associated with insulin insensitivity, and although none of our subjects developed frank glucose intolerance, relative insulin insensitivity may be linked to earlier LDL profile shift during pregnancy.

In each of the six patients in whom LDL profile change took place, as plasma triglyceride increased further, the decrease in the proportion of LDL-II accompanying the dramatic LDL-III rise, caused LDL-II mass to plateau and then decline (data not shown). In the other four patients, LDL subclass pattern did not alter by 35 weeks of gestation. Significantly, these four subjects also had the lowest triglyceride increments in gestation and presumably did not reach their critical threshold triglyceride concentration. These observations therefore provide a basis for understanding the previous relationships between LDL subclasses and plasma triglyceride described in population studies.

The most widely accepted mechanism of LDL subclass formation and modulation is that of neutral lipid exchange (Griffin et al 1994, Tan et al 1995). This process involves the transfer of triglyceride from triglyceride-rich lipoprotein ($VLDL_1$ and chylomicron remnants) into the core of LDL in exchange for cholesteryl esters, a reaction mediated by cholesterol ester transfer protein (CETP). Although CETP activity has been shown to increase significantly by the second trimester of pregnancy before declining towards gestation (Iglesias et al 1994), in most normal situations the activity of this enzyme is not rate limiting (Bagdade et al 1991). In contrast, it is likely that $VLDL_1$ concentration determines the rate of of triglyceride transfer into LDL since large triglyceride-rich VLDL is a preferred substrate for cholesteryl ester transfer protein action (Eisenberg 1985). It follows that during pregnancy, progressively increasing circulating concentrations of $VLDL_1$ promote triglyceride enrichment of

LDL particles. The subsequent hydrolysis of this newly acquired triglyceride in LDL via the action of hepatic lipase, even when the activity of this enzyme is low as in pregnancy, results in the remodelling of LDL subclasses into smaller, denser species. Nevertheless, as we have now shown, remodelling of LDL particles towards smaller, denser particles does not proceed immediately but requires in each individual patient that a specific triglyceride threshold is attained.

Why should there be a threshold for the formation of small, dense LDL ? The recent model proposed by Tan et al (1995), is that a step-wise change in size from LDL-II to LDL-III occurs when HL acts upon a triglyceride-enriched LDL particle. It may be speculated that LDL-II has to have a certain minimum triglyceride enrichment so that in a single exposure to the lipase, sufficient surface and core lipid are removed to generate a conformational change in apolipoprotein B to a new thermodynamically stable state, characteristic of LDL-III. Indeed, there is recent evidence (McKeone et al 1993, McNamara et al 1996) to support the idea that apolipoprotein B configuration differs between small and large LDL.

Finally, it is noteworthy that triglyceride increments were minimal in those subjects who entered pregnancy with low basal triglyceride concentrations, whereas those subjects who entered pregnancy with higher basal triglycerides showed greatest increases and were frankly hyperlipidaemic by 35 weeks. This was not due to differences in the increments in serum oestradiol but may reflect different responses to oestrogen stimulated hyperlipidaemia. That is, pregnancy may reveal a latent hyperlipidaemia due possibly to an inherited lipolytic problem. We are not the first to suggest this possibility. Montes et al (1984), have described case examples of subjects who have triglyceride levels that are above the ninety-fifth percentile values at 36 weeks gestation and return to normal postpartum, but hyperlipidaemia is present among family members. Interestingly, these subjects also appeared to have lower HDL cholesterol concentrations when not pregnant. It would be of interest to confirm these findings in larger studies.

In conclusion, the present study makes several new and important observations: [1] As plasma triglyceride increases in pregnancy, there are parallel rise in median concentrations of $VLDL_1$, $VLDL_2$ and IDL, around 5-fold; [2] As a result of this progressive increase in plasma triglyceride, in particular in $VLDL_1$, the LDL profile is altered in some individuals towards smaller, dense particles; [3] In general, the higher the initial (booking) fasting plasma triglyceride concentration or the larger the rate of change in triglyceride for a given increment in oestradiol, the greater the probability of change in LDL profile towards smaller denser species; [4] Significantly, LDL subclass change towards smaller denser species occurs not in a gradual and progressive manner but exhibits "threshold" behaviour; and finally, [5] this "threshold" is achieved at differing gestational ages and triglyceride concentrations for different women.

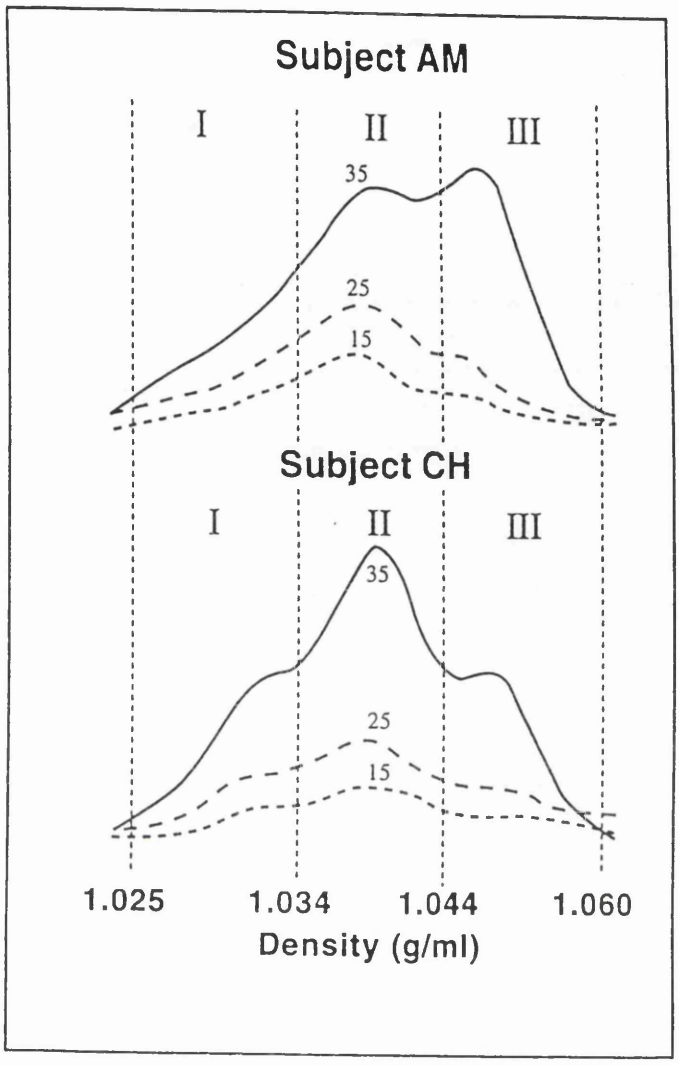


Figure 4. Representative examples of LDL subfraction density profiles as pregnancy progresses in subjects AM and CH, at 15 weeks(- - -), 25 weeks (– – –), and 35 weeks (——), of gestation. In AM, the plasma triglyceride concentration at the three gestational time points were 1.5, 1.6 and 2.4 mmol/L, respectively. The profile was perturbed towards smaller, denser pattern with the percentage abundance of LDL-III increasing at the expense of LDL-II. In CH, the triglyceride values at the three gestational time points were 0.75, 0.95 and 1.70 mmolL, respectively, and in this case, although total area under the curve increased reflecting increased LDL mass, no perturbation in the pattern was observed.

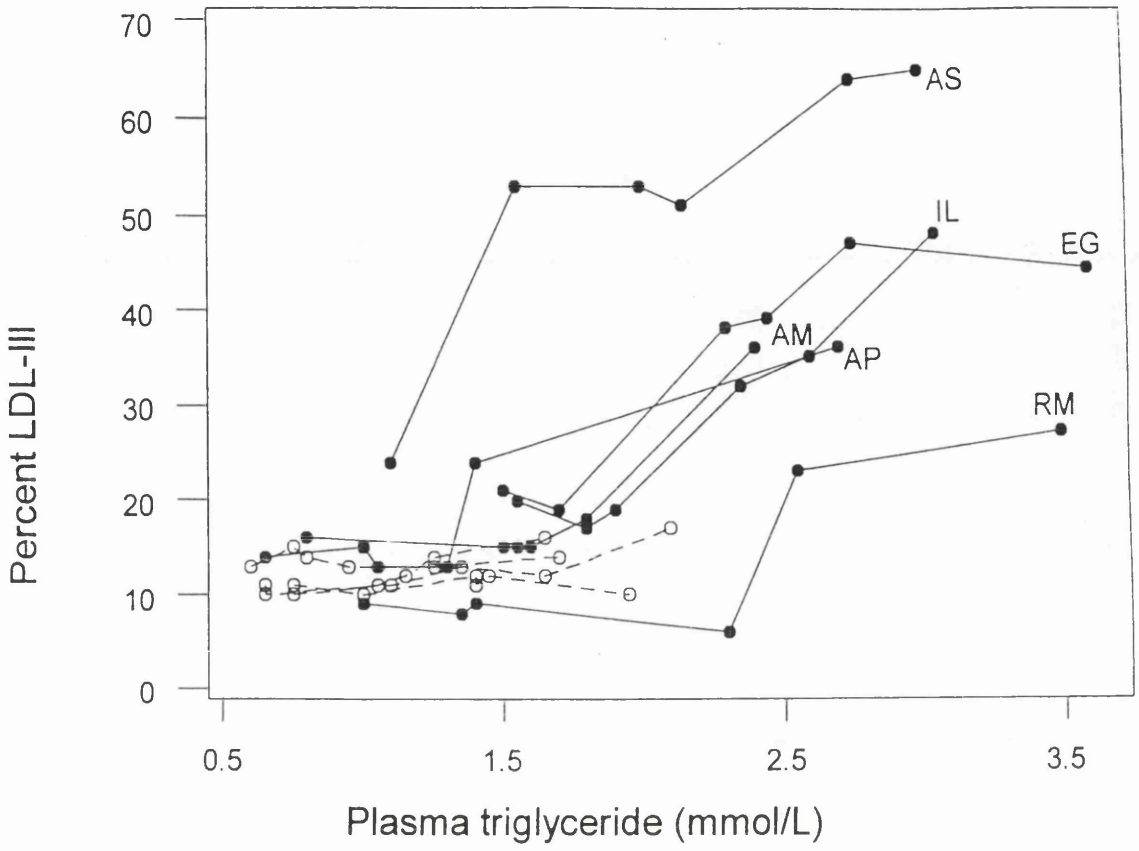


Figure 5. This graph demonstrates the sequential relationship between plasma triglyceride (mmol/L) and percent LDL-III. Lines connect the data for a given women. It was seen that percentage abundance LDL-III remained relatively constant throughout gestation in four of the 10 subjects (- - - - -). In five of the remaining six subjects (——) (IL, EG, AM, AP, RM), percent LDL-III remained relatively constant for varying lengths of time but at differing triglyceride threshold levels and gestational time points, increased in a step-wise fashion. In the final subject, AS, an immediate increment in percent LDL-III was evident. For all patients, percentage abundance of LDL-II behaved in a reciprocal manner to LDL-III and percentage abundance of LDL-I remained relatively constant across the plasma triglyceride range (see Table 3).

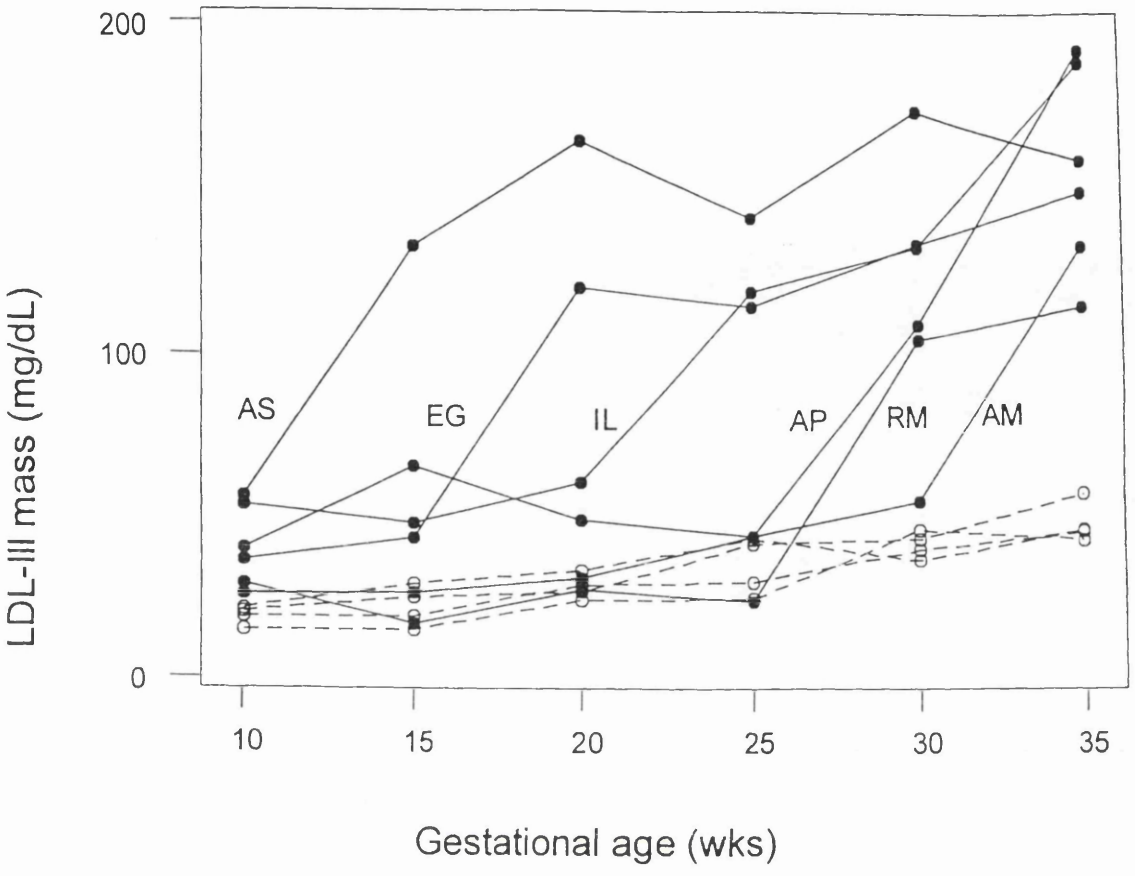


Figure 6. This graph demonstrates the sequential relationship between LDL-III mass (mg/dL) and gestational age (weeks). Lines connect the data for a given women. It was seen that LDL-III mass increased only slightly during gestation in four of the 10 subjects (- - - - -). In five of the remaining six subjects (——) (EG, IL, AP, AM, RM), LDL-III mass remained relatively unchanged for varying lengths of time but at differing gestational time points, increased dramatically. The timing of this abrupt increase in LDL-III mass corresponded to the timing of the observed perturbations in LDL profile (see Figure 5). In the final subject , AS, an immediate increment in LDL-III mass was evident. In each of the latter six patients, the decrease in the proportion of LDL-II accompanying the dramatic LDL-III rise, caused LDL-II mass to plateau and then decline as plasma triglyceride increased further (data not shown).

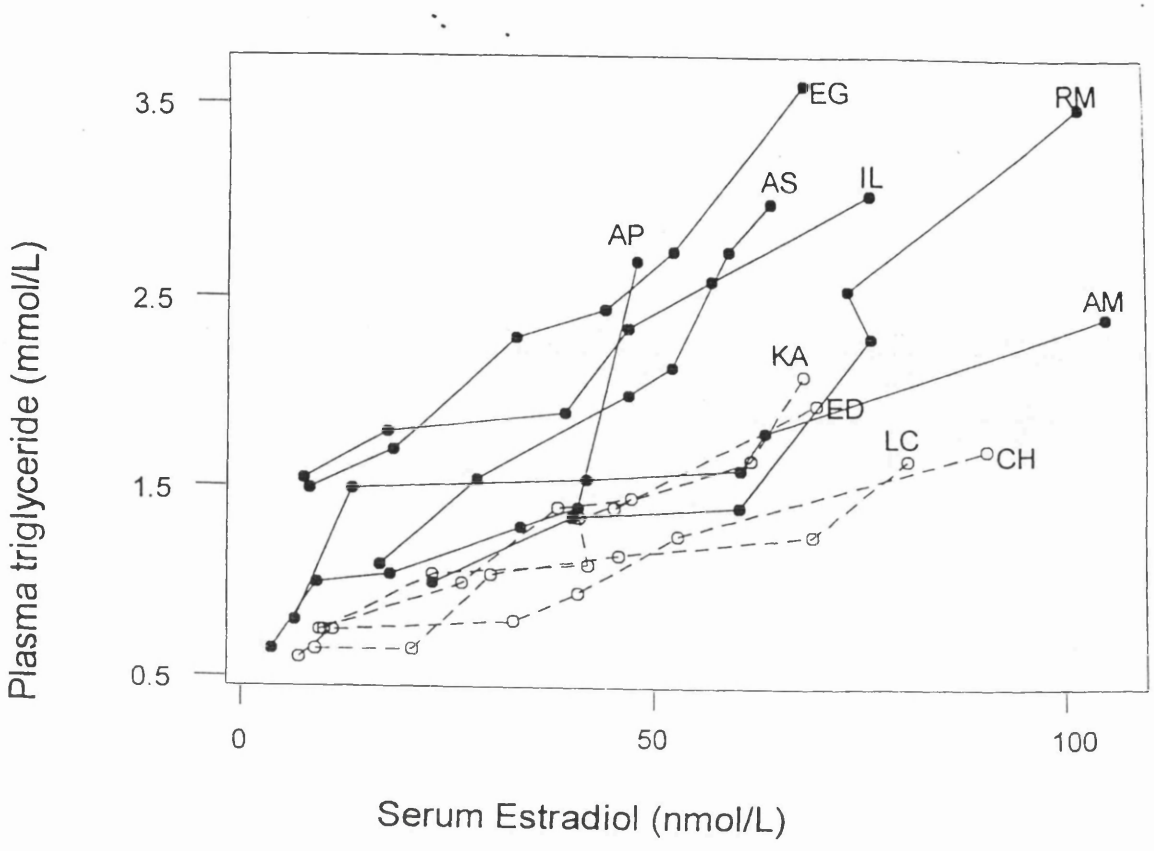


Figure 7. This graph demonstrates the relationship between plasma triglyceride (mmol/L) and serum oestradiol (nmol/L) in each of the 10 subjects as pregnancy progressed. Lines, (- - - -) if no LDL perturbation, against (——) if perturbation present, connect the data for a given women. A close correlation between increment in plasma triglyceride and serum oestradiol was observed for each individual, although the slope of the associations appeared to vary widely (see also Table 5). In general, the higher the slope of association, the greater the likelihood of LDL perturbation. It is also noteworthy that while all the subjects had similar lipid concentrations at 10 weeks gestation, they end-up at differing triglyceride concentrations. By the end of pregnancy six showed frank hyperlipidaemia (i.e. [triglyceride] >2.3 mmol/L), whereas the other four remained normolipidaemic.

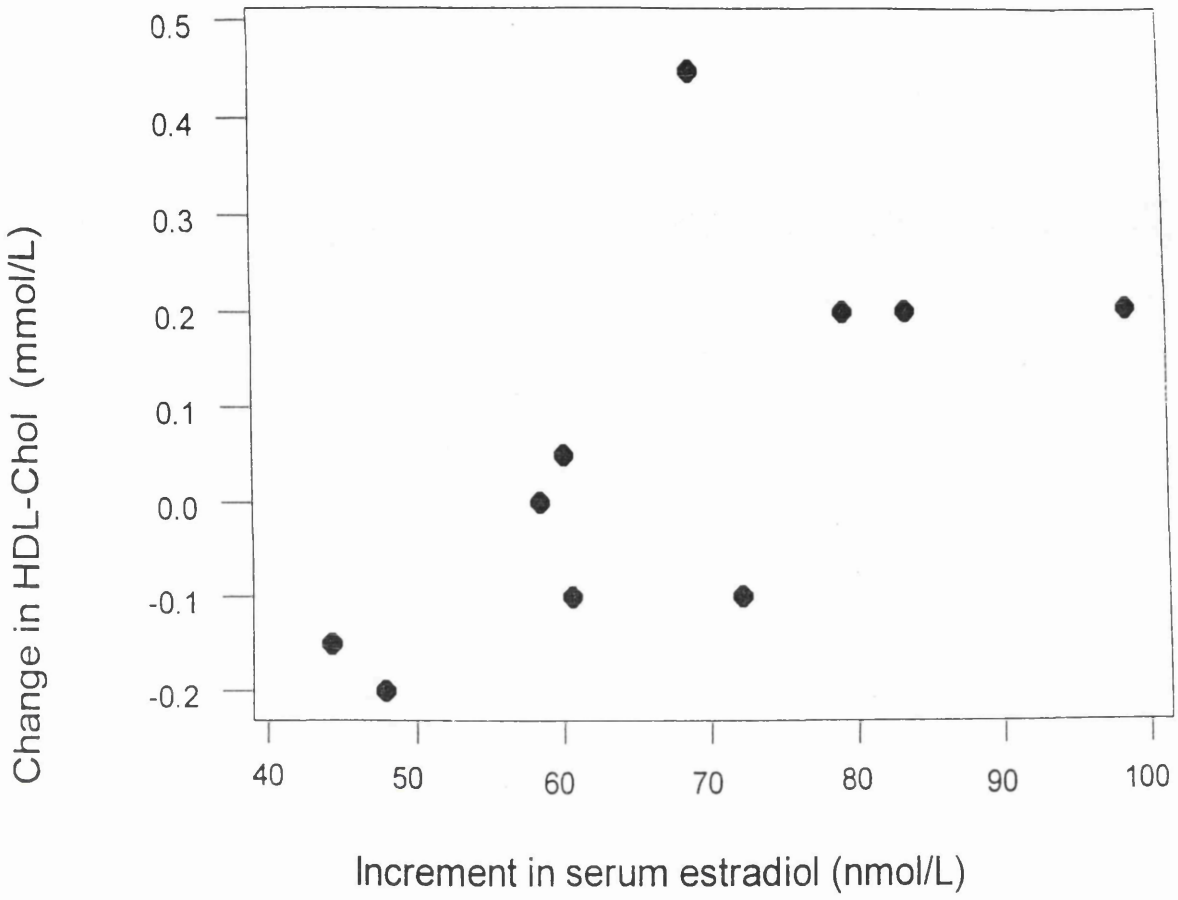


Figure 8. This figure demonstrates the significant positive (R^2=41.7%, p=0.044) relationship between the magnitude of rise in serum oestradiol during gestation with the change in HDL-cholesterol between 10 and 35 weeks.

CHAPTER IV

A LONGITUDINAL STUDY OF THE RELATIONSHIP BETWEEN HAEMOSTATIC, LIPID AND HORMONAL CHANGES DURING NORMAL PREGNANCY

1.0 INTRODUCTION

Normal human pregnancy is associated with major increases in blood coagulation factors (fibrinogen, Factors VII, X and VIIIc and von Willebrand Factor [vWF]). Fibrinolytic factors (Plasminogen activator inhibitor-1 (PAI-1), plasminogen activator inhibitor-2 (PAI-2), and tissue plasminogen activator (t-PA) antigen), and turnover of cross-linked fibrin, as measured by plasma fibrin D-dimer, are also increased (Hellgren & Blomback 1981, Beller & Ebert 1982, Stirling et al 1984, Ballageer et al 1987, Kruithof et al 1987, Greer 1994). The physiological mechanisms underlying these changes are unclear, but they may be important for the maintenance of the placento-uterine interface, or to meet the haemostatic challenge of delivery (Greer 1994). The regulation of these changes is unclear. Possible mechanisms include (a) hormonal changes in pregnancy, particularly increased oestrogen which can induce changes in coagulation and fibrinolysis when administered exogenously in oral contraceptives (Kluft & Lansink 1997) or hormone replacement therapy (HRT) (Meade 1997); and as described in Chapter I (section 5.1) (b) blood lipid changes in pregnancy, especially triglyceride which induces changes in coagulation and fibrinolysis following dietary intake (Mitropoulos 1994) (or oestradiol administration). The object of this study was to determine the associations between changes in fasting serum oestradiol, plasma triglyceride and cholesterol, and changes in Factor VII and PAI activities, and t-PA, D-dimer and vWF antigens; each of which has been related to risk of arterial and/or venous thrombosis in epidemiological studies. A detailed analysis of lipid and lipoprotein changes for the patients in this study are described in the preceding chapter (Chapter III).

2.0 SUBJECTS, SAMPLES AND METHODS

Patient recruitment, bleeding shedule, and characterisitics precisely as described in Chapter III. In addition to the serum and K_2 EDTA samples collected for oestradiol and lipid determinations as described previously, citrated plasma aliquots for the determination of haemostatic factors were collected, spun and stored immediately at -70^{o}C until analysis.

Methods are given in Chapter II. Briefly, plasma total cholesterol, triglyceride and HDL-cholesterol were determined by a modification of the standard Lipid Research Clinics Protocol. Oestradiol was determined by a quantitative immunoassay on the

Technichon Immuno-1 system ('Heterogeneous Magnetic Separation Assay', Bayer Diagnostics, UK). FVIIc was determined by a one stage clotting assay in an ACL-300 Research Coagulameter (IL, Warrington, UK). PAI activity was measured by a chromogenic assay (Chromogenix, Stockholm, Sweden), tPA (Biopool, Stockholm, Sweden), fibrin D-dimers (AGEN, Parsippany, NJ, USA), and vWF antigen (Dako, Copenhagen, Denmark) were all measured by ELISA techniques.

Statistical Analysis

The lipid, haemostatic and hormonal variables were parametrically distributed as judged by the examination of normal probability plots. Nevertheless, as numbers were small, data are presented as medians and ranges. Associations between changes in plasma lipid and haemostatic variables and oestradiol concentrations were tested by calculating the Pearson correlation coefficient (R) and the coefficient of determination (R^2) which was expressed as a percentage, (i.e. R^2 gives the percentage of variation in the dependent variable which is explained by variation in the independent variable). The significance of association between pairs of variables was determined by linear regression.

3.0 RESULTS

3.1 Lipids & Oestradiol (Table 6)

Gestational age (wks)	10	15	20	25	30	35
Triglyceride (mmol/l)	0.78	1.20	1.40	1.53	1.73	2.55
	0.6-1.5	0.6-1.8	0.8-2.3	0.9-2.4	1.2-2.7	1.5-3.6
Cholesterol (mmol/l)	4.40	5.45	6.23	6.53	6.73	7.20
	3.2-6.8	4.0-7.5	4.7-7.6	5.0-8.5	5.4-8.7	5.6-8.4
Oestradiol (nmol/l)	8.4	19.0	38.7	46.2	58.1	73.0
	3.6-22	8.8-39	17-60	33-76	40-73	47-105
FVIIc (IU/dl)	95.0	121.0	125.5	132.0	151.0	168.0
	81-138	99-137	106-149	107-179	116-179	137-193
PAI activity (% pool)	87.0	86.5	108.5	140	178.5	223.5
	33-125	72-125	59-153	79-179	129-222	181-273
tPA-antigen (ng/ml)	4.80	5.30	6.90	6.45	7.40	9.05
	2.4-6.5	3.4-7.5	4.7-7.6	5.4-8.3	5.0-10	6.8-10.8
D-dimer (ng/ml)	68.5	72.0	98.5	159.5	199.5	295.5
	29-127	30-101	47-123	66-200	127-310	196-420
vWF (IU/dl)	136.0	189.0	194.0	183.0	205.0	231.0
	93-253	78-249	81-252	134-325	89-296	109-293

Table 6. Median (range) of lipid, oestradiol and haemostatic concentrations at each gestational time point.

3.2 Haemostatic parameters

PAI activity increased by a factor of 2.56 from a median of 87 % normal pool to 226% at 35 weeks. Increments in median circulating concentrations of t-PA (4.80-9.05 ng/ml), FVIIc (95.0-168.0 IU/dl), and vWF (136.0-231.0 IU/dl) were slightly less pronounced, whereas median D-dimer concentration increased by more than 4-fold (68.5 to 295.5 ng/ml) (Table 6). The patterns of increase in plasma triglyceride, PAI activity, t-PA and D-dimer were similar, in that the maximum rise in each of these parameters occurred after 25 weeks (Table 6). In contrast, increments in FVIIc and vWF were more uniform in nature.

3.3 Relationship between serum oestradiol, plasma triglyceride and cholesterol, and Factor VII

For each individual there were strong and significant relationships (Table 7) between the rise in factor VIIc and the increment in plasma triglyceride (Figure 9), and increment in serum oestradiol (Figure 10). Furthermore, the slopes of the association between increments in factor VIIc and plasma triglyceride or serum oestradiol were similar (3-fold vs 2.35-fold). The associations between rise in factor VIIc and increment in plasma cholesterol were equally strong (Table 7).

	Triglyceride	Cholesterol	Oestradiol
FVII	0.93 (0.83-0.99)*	0.93 (0.82-0.97)*	0.93 (0.85-0.99)*
PAI-1	0.93 (0.88-0.99)*	0.82 (0.64-0.96)	0.91 (0.81-0.98)*
tPA	0.94 (0.71-0.99)†	0.79 (0.49-0.99)	0.92 (0.71-0.99)†
D-Dimer	0.92 (0.84-0.97)*	0.79 (0.71-0.96)	0.89 (0.79-0.95)†
vWF	0.63 (0.03-0.93)	0.72 (0.02-0.90)	0.68 (0.03-0.94)

Table 7: Associations in individual patients between the change in plasma concentrations of haemostatic factors and alterations in plasma triglyceride and cholesterol and serum oestradiol concentrations.

Values presented are the median (range) of the correlation coefficients (R) for the associations between the change in plasma concentrations of haemostatic factors and alterations in plasma triglyceride and cholesterol, and serum oestradiol concentrations for the 10 subjects.

*$P<0.05$ in all 10 patients; †$P<0.05$ in 8 of the 10 patients.

3.4 Relationships between serum oestradiol, plasma triglyceride and cholesterol, and PAI activity.

For each individual there were strong and significant relationships (Table 7) between the rise in PAI activity, and the increment in plasma triglyceride (Figure 11), and increment in serum oestradiol (Figure 12). The associations between changes in plasma cholesterol and PAI activity were somewhat weaker (Table 7). In contrast to the pattern seen with FVII activity, however, the range of the slopes of association between increments in PAI activity and plasma triglyceride was greater than that between changes in plasma PAI activity and serum oestradiol (4.9 fold vs 2.6 fold).

3.5 Relationship between serum oestradiol, plasma triglyceride and cholesterol, and tPA antigen.

There were strong and significant relationships (Table 7) between the rise in tPA antigen, and the increment in plasma triglyceride (Figure 13), and increment in serum oestradiol (Figure 14) in 8 of the 10 individuals. Once again, the associations between changes in plasma cholesterol and tPA antigen were somewhat weaker (Table 7). Congruous to the association between the rise in PAI activity and the increment in plasma triglyceride, the relationship between the rise in tPA antigen and the increment in plasma triglyceride also exhibited considerable variability between individuals in the slopes of association (4.7 fold difference, Figure 13).

3.6 Relationship between serum oestradiol, plasma triglyceride and cholesterol, and D-dimer

For each individual there was a strong and significant relationship (Table 7) between the rise in plasma D-dimer concentration and the increment in plasma triglyceride (Figure 15), whereas the association between alterations in plasma D-dimer and serum oestradiol concentrations were significant in 8 of the 10 subjects studied (Figure 16).

3.7 Relationship between serum oestradiol, plasma triglyceride & cholesterol and vWF

The associations between changes in vWF with increases in serum oestradiol, plasma triglyceride or cholesterol, in each individual, were considerably weaker than the associations described above (Table 7).

4. DISCUSSION

The increases in coagulation and fibrinolytic variables during normal pregnancy which were observed in the present study were similar to those described in previous studies (Hellgren & Blomback 1981, Beller & Ebert 1982, Stirling et al 1984, Ballageer et al 1987, Kruithof et al 1987, Greer 1994), as were the increases in serum oestradiol and

plasma lipids (Montelongo et al 1992, Silliman et al 1994) To our knowledge, the present study is the first to report strong and similar correlations in individual patients between the increments in Factor VII and PAI activities, t-PA and D-dimer antigens, and serum oestradiol and plasma triglyceride. These results are consistent with the hypothesis that oestradiol-induced hypertriglyceridaemia may be one mechanism through which increases in these coagulation and fibrinolytic variables occur during pregnancy.

There is much evidence that hypertriglyceridaemia in non-pregnant subjects is associated with increases in Factor VII and PAI activities: in part, this may reflect causal effects of triglyceride-rich lipoproteins on both activation of Factor VII, and synthesis and release of PAI-1 from endothelial cells (Juhan-Vague & Alesi 1993, Mitropoulos 194). Exogenous oral oestrogens increase Factor VII activity even in low doses (HRT) (Kluft & Lansink 1997, Meade 1997) and it has been suggested that this may be related to increased plasma triglyceride levels (Lowe 1996). While lower doses of exogenous oestrogens (HRT and contraceptives) decrease plasma levels of PAI-1 (Lowe 1996, Kluft & Lansink 1997, Meade 1997) pregnancy results in far higher levels of endogenous oestradiol. Thus a stimulatory effect on PAI-1 in pregnancy cannot be excluded, and further research is required to examine the effects of varying oestradiol doses on PAI-1 synthesis in vitro. It should be noted that the assay of PAI activity used in the present study is relatively specific for PAI-1, and relatively insensitive to PAI-2 which increases greatly during pregnancy (Kruithof et al 1987). It should also be noted that the relationship between PAI-1 and triglyceride may be related to insulin resistance (Juhan-Vague & Alesi 1993).

The increase in plasma t-PA antigen during pregnancy was only slightly lower than the increase in PAI activity. This may reflect a primary increase in PAI which binds t-PA, and circulates as t-PA-PA1 complexes. These complexes are detected by the t-PA assay used in the present study. Additionally and /or alternatively, it may reflect endothelial release of t-PA. While the increase in t-PA antigen during pregnancy was accompanied by a similar increase in vWF antigen, suggesting a related change in endothelial release, the correlation between the t-PA increment and the oestradiol and triglyceride increments was much stronger than the correlation between the vWF increment and the oestradiol and triglyceride increments. This finding suggests that either oestradiol-induced hypertriglyceridaemia is more important in endothelial release of t-PA than vWF in normal pregnancy, or that the increase in t-PA may be secondary to a primary increase in PAI-1 due to oestradiol-induced hypertriglyceridaemia.

The correlations between increases in plasma fibrin D-dimer, oestradiol and triglyceride during normal pregnancy suggest that the changes in Factor VII associated with oestradiol-induced hypertriglyceridaemia may result in increased fibrin turnover in vivo. There is evidence in non-pregnant subjects that increasing Factor VII activity

is associated with increased thrombin activity in vitro, as measured by plasma levels of fibrinopeptides, fragments F1+2, or thrombin-antithrombin complexes (Miller 1997, Lowe 1997). Further studies are required to examine the relationships between oestradiol, triglyceride, Factor VII and D-dimer.

These findings may be relevant not only to the possible mechanisms of change in coagulation and fibrinolysis during pregnancy, but also to the pathogenesis of atherosclerosis and thrombosis. Pregnancy is an interesting transient model of hyperlipidaemia and the development of an "atherogenic" lipoprotein profile is also related to changes in coagulation and fibrinolysis, which have been related to thrombotic risk in epidemiological studies (Lowe et al 1997). A notable finding in the present study was the inter-individual variability in increments in coagulation and fibrinolytic variables with elevations in oestradiol and triglycerides during pregnancy, suggesting the possibility of gene-environmental interactions (Green & Humphries 1994). Clearly, further studies of individual changes in hormones, lipids and haemostatic variables during pregnancy may offer insights into the regulation of coagulation and fibrinolysis, and their relationships to atherothrombosis.

In conclusion, the results of this study show that in individual women undergoing normal pregnancy, there are significant correlations between oestradiol, triglyceride, Factor VII, PAI, t-PA and fibrin D-dimers, but not vWF, suggesting that oestradiol induced triglyceride changes may be responsible for the changes in coagulation.

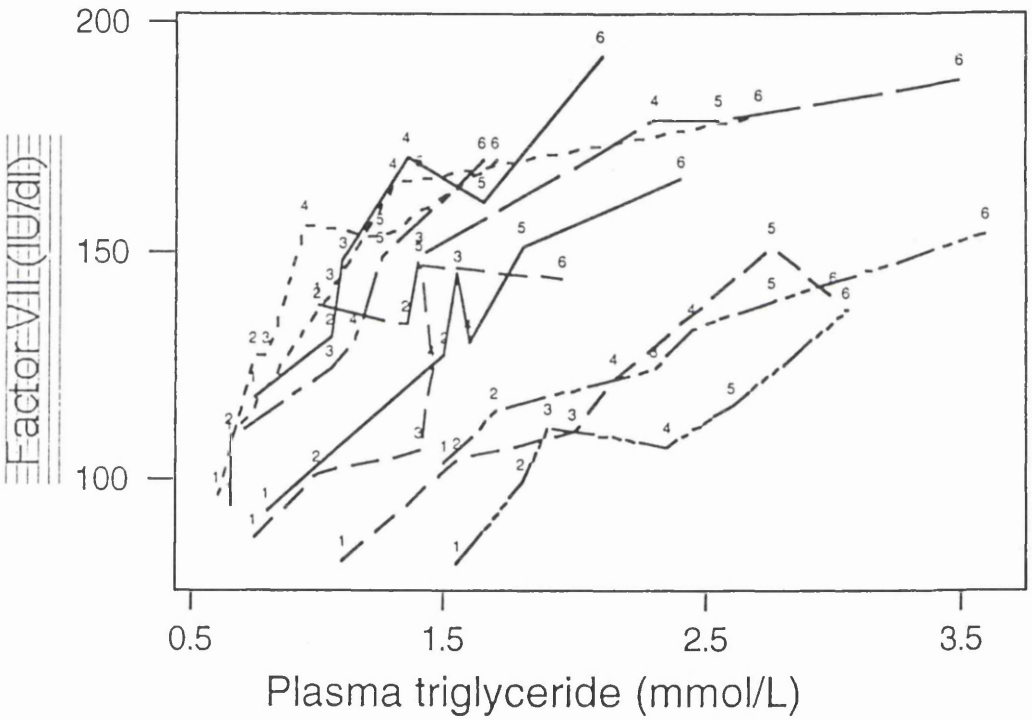


Figure 9. This graph demonstrates the sequential relationship between plasma triglyceride (mmol/l) and FVIIc in each of the 10 subjects as pregnancy progressed. Lines connect the data for a given women. A significant and strong linear correlation (Table 7) between increment in plasma triglyceride and FVIIc was observed in each of the individuals studied.

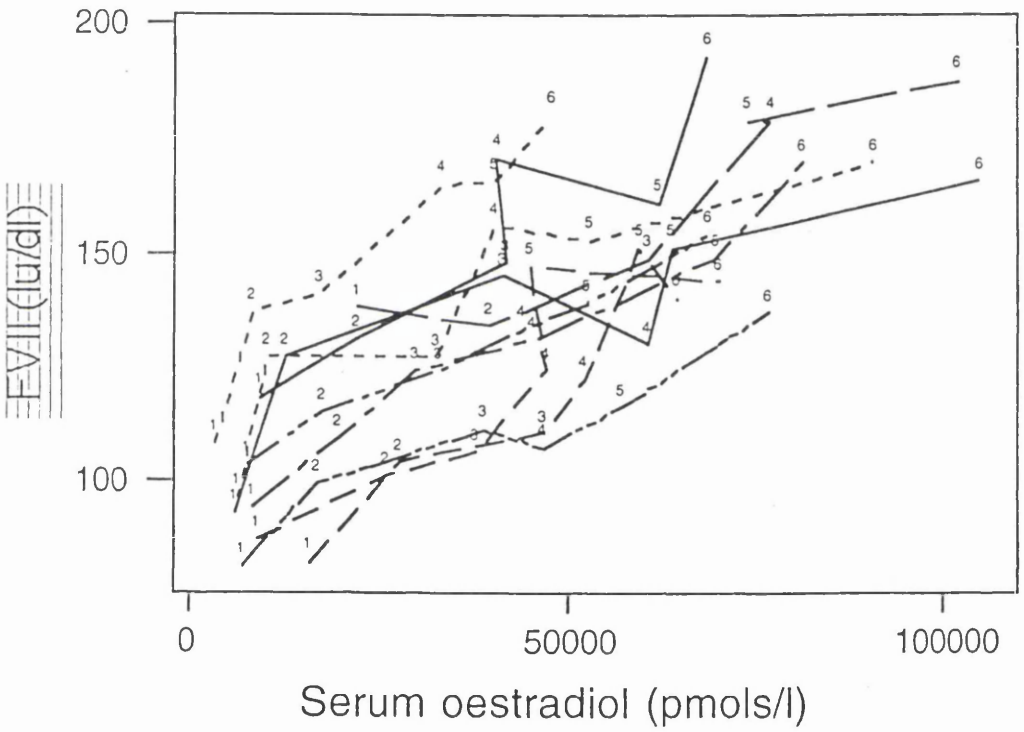


Figure 10. This graph demonstrates the sequential relationship between serum oestradiol (mmol/l) and factor VIIc in each of the 10 subjects as pregnancy progressed. Lines connect the data for a given women. A significant and strong linear correlation (Table 7) between increments in the two parameters was observed in each of the individuals studied.

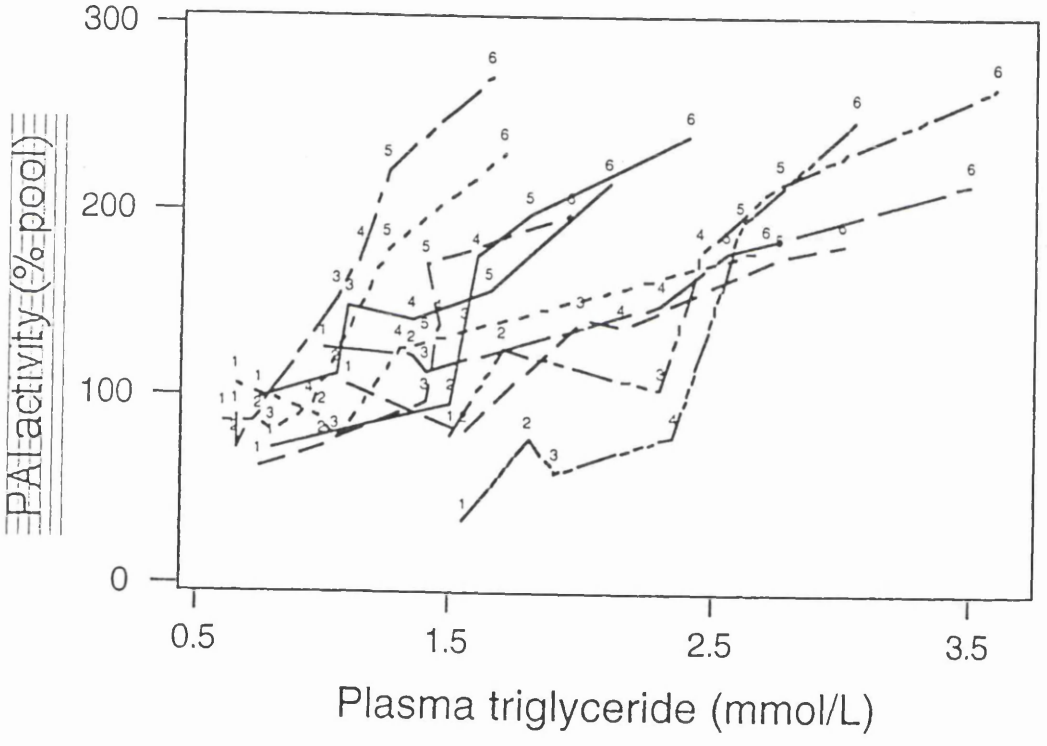


Figure 11. This graph demonstrates the sequential relationship between plasma triglyceride (mmol/l) and PAI-1 activity in each of the 10 subjects as pregnancy progressed. Lines connect the data for a given women. A significant and strong linear correlation (Table 7) between increments in the two parameters was observed in each of the individuals studied, although the slope of the associations appeared to vary markedly (4.9 fold difference).

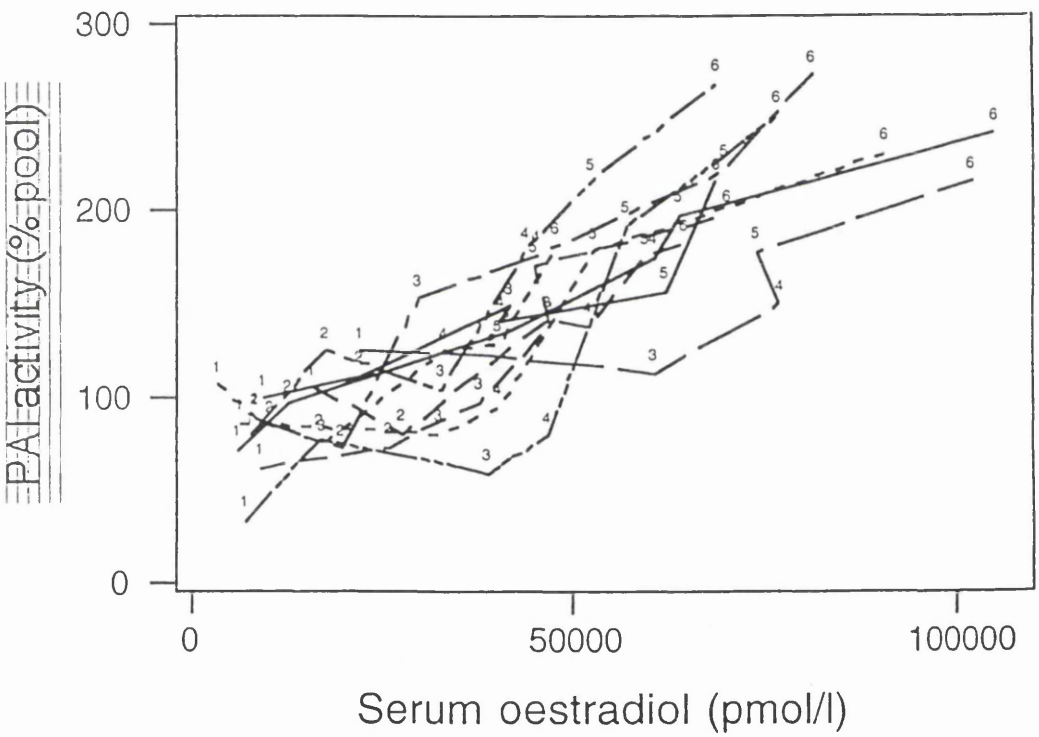


Figure 12. This graph demonstrates the sequential relationship between serum oestradiol (mmol/l) and PAI-1 activity in each of the 10 subjects as pregnancy progressed. Lines connect the data for a given women. A significant and strong linear correlation (Table 7) between increments in the two parameters was observed in each of the individuals studied.

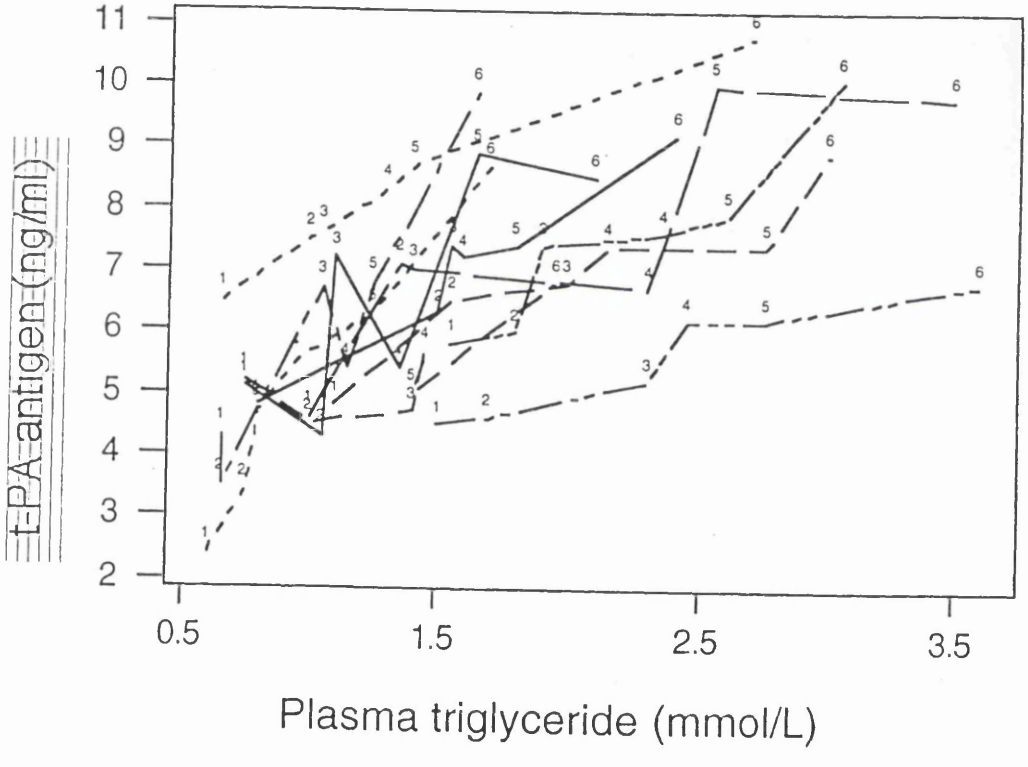


Figure 13. This graph demonstrates the relationship between plasma triglyceride and t-PA.Ag levels in each of the 10 subjects as pregnancy progressed. Lines connect the data for a given women. A significant and strong linear correlation (Table 7) between increments in the two parameters was observed in each of the individuals studied, although the slope of the associations appeared to vary widely.

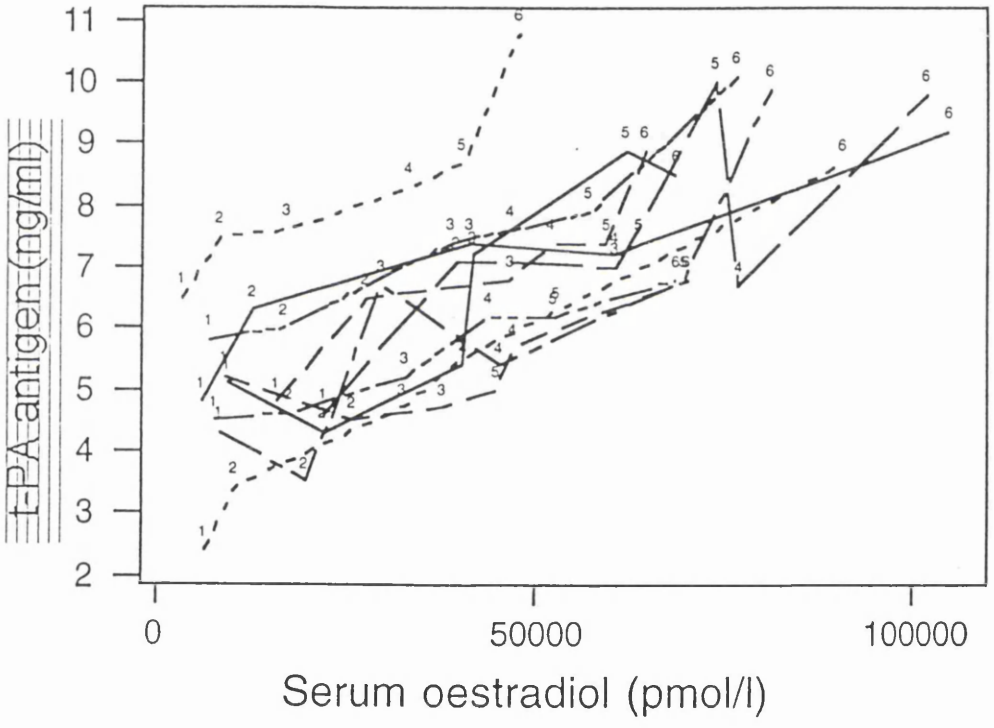


Figure 14. This graph demonstrates the relationship between serum oestradiol and t-PA.Ag levels in each of the 10 subjects as pregnancy progressed. Lines connect the data for a given women. A significant and strong linear correlation (Table 7) between increments in the two parameters was observed in each of the individuals studied.

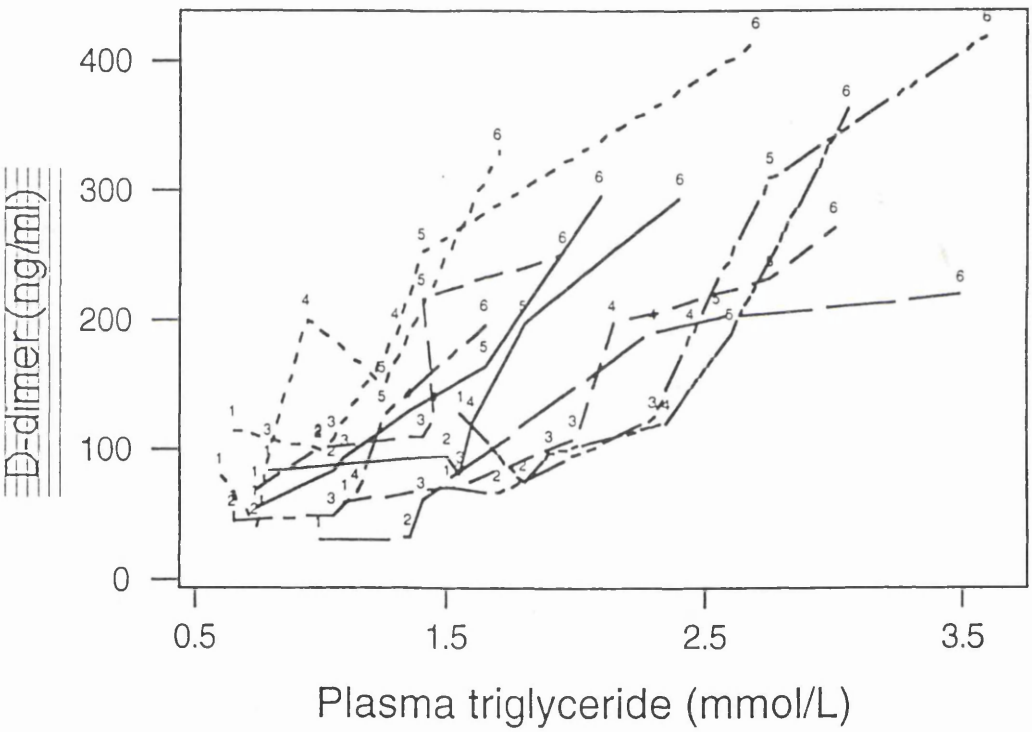


Figure 15. This graph demonstrates the sequential relationship between plasma triglyceride and D-dimer levels in each of the 10 subjects as pregnancy progressed. Lines connect data for a given women. A significant and strong linear correlation (Table 7) between increments in the two parameters was observed in each of the individuals studied.

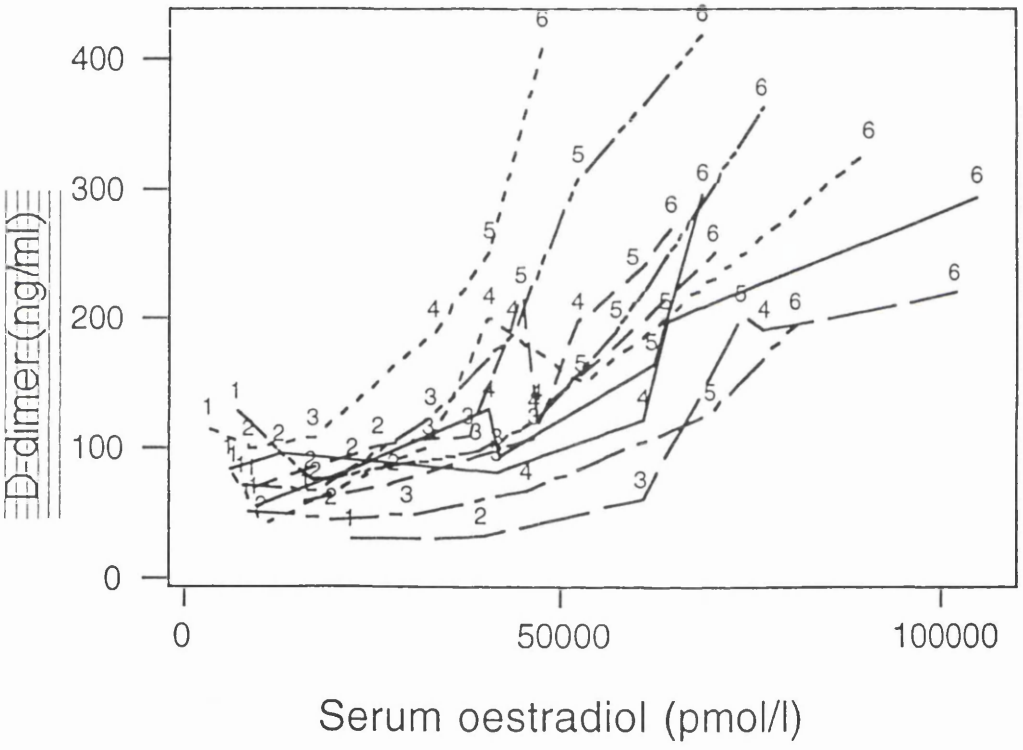


Figure 16. This graph demonstrates the sequential relationship between serum oestradiol (mmol/l) and D-dimer in each of the 10 subjects as pregnancy progressed. Lines connect the data for a given women. A significant and strong correlation (Table 7) between increments in the two parameters was observed in 8 of the 10 individuals studied.

CHAPTER V

LIPOPROTEIN SUBFRACTION CONCENTRATIONS IN PRE-ECLAMPSIA

1.0 INTRODUCTION

As discussed in detail in Chapter I, current concepts on the pathophysiology of pre-eclampsia suggest endothelial cell injury and altered endothelial cell function play a pivotal role. It is proposed that the poorly perfused placenta is the origin of a humoral factor that affects maternal systemic function, directly or indirectly, by activating endothelial cells with resultant vascular injury. The characteristic pathologic lesion seen in the utero-placental bed of patients with pre-eclampsia is a necrotising arteriopathy consisting of fibrinoid necrosis, accumulation of foam cells or lipid laden macrophages in the decidua, fibroblast proliferation, and a perivascular infiltrate. This lesion has been termed 'acute atherosis.' (Roberts & Redman 1993).

Work in the field of cardiovascular research has shown that serum lipids have a direct effect on endothelial function and that abnormal serum lipid profiles are associated with endothelial dysfunction (Chapter I, section 5.2). As such, the potential role of abnormal lipid metabolism in the genesis or expression of pre-eclampsia is the subject of increasing interest. Lipid and lipoprotein levels undergo dramatic changes in pregnancy presumably to supply lipid nutrients to the growing fetus. Plasma concentrations of triglyceride and cholesterol increase around 300% and 50%, respectively (Chapter III). In some situations the mechanisms regulating this physiologic hyperlipidaemia may malfunction. In women with pre-eclampsia, plasma free fatty acids and triglyceride concentrations climb substantially above those observed in normal pregnancy and do so well in advance of the appearance of clinical manifestations of the disorder (Lorentzen et al 1995). Branch and co-workers (1994) have shown that antibodies to an epitope of oxidized low density lipoprotein (LDL) are increased in pre-eclamptic patients, and as a result suggested that oxidized LDL may contribute to the foam cell formation in the decidua by mechanisms analogous to those involved in atherosclerosis. It should be noted, however, that other groups (Kurki et al 1996) have found that antibodies to oxidized LDL fail to predict the risk of pre-eclampsia. More recently, first trimester serum cholesterol concentrations have been shown to be significantly associated with the risk of pre-eclampsia (van den Elzen 1996).

Lipoprotein classes are not homogeneous entities but include subclasses of differing function and metabolic potential. At present there is no information on the concentrations of very low density (VLDL) and LDL subfractions in pre-eclampsia.

This is particularly important to investigate as small, dense LDL are more atherogenic than larger LDL species. Poor recognition by the LDL receptor-mediated clearance mechanisms allows them to stay in the plasma compartment for longer, thereby increasing their likelihood of penetrating the arterial intima more readily. Further, small, dense LDL are also more readily oxidized and may contain less antioxidant; interestingly, decreased levels of vitamin E, an endogenous antioxidant in LDL, have been reported in severe pre-eclampsia (Wang et al 1991). As a result, small dense LDL are more readily taken up by macrophages to create foam cells. There is also considerable evidence to suggest that oxidized small, dense LDL may be the prime candidate associated with endothelial dysfunction (Chin et al 1992).

In addition, Arbogast et al (1994) have hypothesised that VLDL toxicity may help to explain endothelial dysfunction in pre-eclampsia. Previous data (Gianturco et al 1980) suggest that large triglyceride-rich VLDL subfraction ($VLDL_1$) exhibits greater cytotoxicity than the smaller, cholesterol-rich VLDL ($VLDL_2$) fraction. With this background this study explored the hypothesis that in this disorder large triglyceride-rich lipoproteins ($VLDL_1$), and small, dense LDL (LDL-III), were significantly increased over and above the concentrations seen in normal pregnancy.

2. SUBJECTS, SAMPLES AND METHODS

Eight women with pre-eclampsia and eight women with uncomplicated pregnancies were studied. The patient details are shown in Table 8.

	Cases n=8	Controls n=8	P-Value
Age	32 (25-37)	32 (26-35)	NS
Parity (Prim/parous)	8/0	7/1	NS
Gestational age (wk)	32 (26-36)	32 (27-34)	NS
Weight (kg)	73 (57-99)	72 (63-94)	NS
SBP (mmHg)	155 (130-210)	130 (110-140)	0.01
DBP(mmHg)	105 (90-115)	75 (70-90)	0.0016
Platelets ($x10^9/l$)	238 (111-377)	-----	
Urate (umol/L)	392 (243-482)	-----	
Fetal Weight (Kg)	2.7 (1.0-3.2)	3.4 (2.7-3.9)	0.013

NS = not significant. Values are given as medians (ranges).

Table 8 Characteristics of Normotensive Pregnant Women and Pre-eclamptic Women

Cases, all primigravida, were recruited with confirmed pre-eclampsia (proteinuric pregnancy-induced hypertension) as defined by the International Society for the Study of Hypertension in Pregnancy (ie, diastolic blood pressure was greater than 110 mm Hg on one reading or exceeded 90 mm Hg on repeated readings; proteinuria was defined as 0.3 g/24 hours or greater, or 2+ or greater on dipstick testing, in the absence of infection or renal disease). Consecutive patients eligible for this study, where obtaining a fasting sample was possible, were recruited and all those approached agreed to participate. All patients were healthy before onset of pre-eclampsia and were not receiving any medication known to interfere with lipid metabolism or lipid determination.

Eight normal pregnant women matched as a group for pre-pregnancy weight, age, and gestational age served as controls. All had normal course and outcome of pregnancy, term delivery, and customary diet, and did not receive any medication known to interfere with lipid metabolism or lipid determination. Additionally, none of the patients or controls were phenotype apo E2/E2, an inherited trait known to generate disturbances in the plasma lipid profile even in normolipaemic subjects. Further, none of the patients or controls were in labour at the time of sampling.

All subjects were sampled after an overnight fast of at least 10 hours. Twenty mL of blood was collected by venepuncture into K_2EDTA (final concentration 1mg/mL) and lithium heparin tubes. Plasma was harvested at 4C by low speed centrifugation and aliquots of plasma for lipid and lipoprotein measurements were used immediately. Plasma for pre-heparin hepatic lipase was stored at -70C until analyses. The assay methods are described in their entirety in Chapter II. Briefly, plasma total cholesterol, triglyceride, and HDL-cholesterol measurements were performed by a modification of the standard Lipid Research Clinics Protocol. $VLDL_1$, $VLDL_2$, IDL, and LDL were prepared and quantified as described earlier by cumulative flotation gradient ultracentrifugation. The cholesterol, triglyceride, free cholesterol, phospholipid, and proteins of the lipoprotein fractions were assayed and concentrations calculated as the sum of the components (expressed as mg/dL plasma). Isolation of LDL subfractions from fasting plasma was achieved by density gradient ultracentrifugation using a discontinuous salt gradient. Pre-heparin hepatic lipase activity was determined in fasting plasma as described in Chapter II.

Statistics

As the principal parameters of interest, ie, $VLDL_1$ and LDL-III, exhibited a lack of normality, data are presented as median and range. Where appropriate, data were tested for statistical significance using Mann-Whitney U-test (Minitab, CA, USA). Comparisons between lipoprotein compositions were assessed by Student unpaired t-test and because of the multiple comparisons made, statistical significance was taken as the 0.01 (99 percent) level. The association between plasma concentrations of

plasma triglyceride and mass LDL-III was performed using Spearman Rank correlation.

3. RESULTS

3.1 Lipid and lipoprotein concentrations

	Cases n=8	Controls n=8	P-value
Triglyceride (mmol/l)	3.68 (2.35-5.00)	1.93 (1.25-2.90)	0.004
Cholesterol (mmol/l)	6.88 (3.95-8.15)	7.35 (5.45-8.80)	NS
HDL-Cholesterol (mmol/l)	1.43 (1.05-1.65)	1.70 (1.45-2.15)	0.021
VLDL1 (mg/dl)	184 (111-385)	68 (27-135)	0.0016
VLDL2 (mg/dl)	146 (63-240)	76 (38-93)	0.014
IDL (mg/dl)	93 (60-160)	123 (70-184)	NS
Total LDL (mg/dl)	279 (172-410)	345 (174-453)	NS
LDL-I (mg/dl)	22 (12-33)	62 (23-86)	0.004
LDL-II (mg/dl)	55 (32-190)	211 (64-276)	0.010
LDL-III (mg/dl)	170 (16-356)	55 (11-126)	0.024
Hepatic lipase activity (umol FA/ml/Hr)	28.5 (18-50)	18 (10-25)	0.041

Values are given as medians (ranges). NS = not significant

Table 9 Lipid and Lipoprotein Concentrations, and Pre-heparin Hepatic Lipase Activity of Cases and Controls

The groups were comparable with respect to age, parity (although one control was parous), weight, and gestational age (Table 8). Median plasma triglyceride in patients with pre-eclampsia was almost double that in the control group. This was reflected in an almost three-fold increase in $VLDL_1$ and a two-fold increase in $VLDL_2$, whereas total plasma cholesterol, IDL and total LDL concentrations were the same in patients and controls (Table 9). Women with pre-eclampsia demonstrated significantly lower concentrations of LDL-I and LDL-II, the larger species, and markedly elevated plasma concentrations of small, dense LDL-III. The concentration of LDL-III correlated positively with plasma triglyceride concentration ($r^2 = 0.504$, p=0.002, Figure 1). In line with the observed LDL profile, LDL particles were protein enriched (25.6 % [1.6] versus 23.3% [1.2], mean [SD] of g protein/100 g total lipoprotein mass for pre-eclamptics vs controls respectively, p=0.006, see below, Table 10). High density lipoprotein-cholesterol concentration was also statistically significant lower in the pre-eclamptic group (p= 0.021). Pre-heparin hepatic lipase activity was significantly elevated in the pre-eclamptic group (p= 0.041, Table 9)

3.2 Lipoprotein compositions

Cases

Lipoprotein	Protein	Free chol	Chol esters	Triglyceride	Phospholipid
$VLDL_1$	6.7(0.9)†	3.4(1.6)	11.0(3.5)	62.6(3.0)	16.3(1.7)
$VLDL_2$	13.4(1.3)	7.0(2.6)	17.9(2.5)†	40.9(3.5)	20.8(1.0)
IDL	19.9(1.9)	5.7(2.2)	32.3(2.5)	20.2(3.4)*	21.8(1.6)
LDL	25.6(1.6)*	6.2(1.9)	38.3(2.2)	9.4(2.6)	20.5(1.6)

Controls

Lipoprotein	Protein	Free chol	Chol esters	Triglyceride	Phospholipid
$VLDL_1$	9.2(1.3)	3.0(0.67)	10.4(2.9)	62.2(4.1)	15.2(3.0)
$VLDL_2$	15.7(2.0)	4.3(1.8)	27.7(4.7)	32.7(7.6)	19.7(1.8)
IDL	20.0(2.2)	7.7(2.2)	36.6(4.2)	14.6(2.6)	21.1(1.7)
LDL	23.3(1.2)	9.9(4.1)	37.3(1.7)	9.0(1.9)	20.5(1.2)

Data are presented as mean (standard deviation) expressed as percent composition (g/100g); Chol esters = cholesteryl esters; Free chol = free cholesterol.

*P<0.01, †P<0.001 refer to the significance of difference between cases and controls as determined by Student unpaired t-test.

Table 10. Chemical Composition of Apolipoprotein-B Containing Lipoproteins

When the composition of these particles was determined (Table 10) the following was found: the cholesteryl-ester to triglyceride ratio (the lipid composition of the hydrophobic core of the particle) was reduced in $VLDL_2$ (0.44 [0.10] versus 0.93 [0.44], mean [SD], for pre-eclamptics and controls respectively, p=0.019) and IDL (1.66 [0.37] versus 2.60 [0.66], p= 0.005) but not in $VLDL_1$ (0.17 [0.07] vs 0.18 [0.05], p =0.77) and LDL (4.36 [1.30] versus 4.30 [1.00], p=0.91). Put another way, both $VLDL_2$ and IDL were triglyceride enriched suggesting that both $VLDL_2$ and IDL were increasingly products of $VLDL_1$ catabolism in pre-eclampsia.

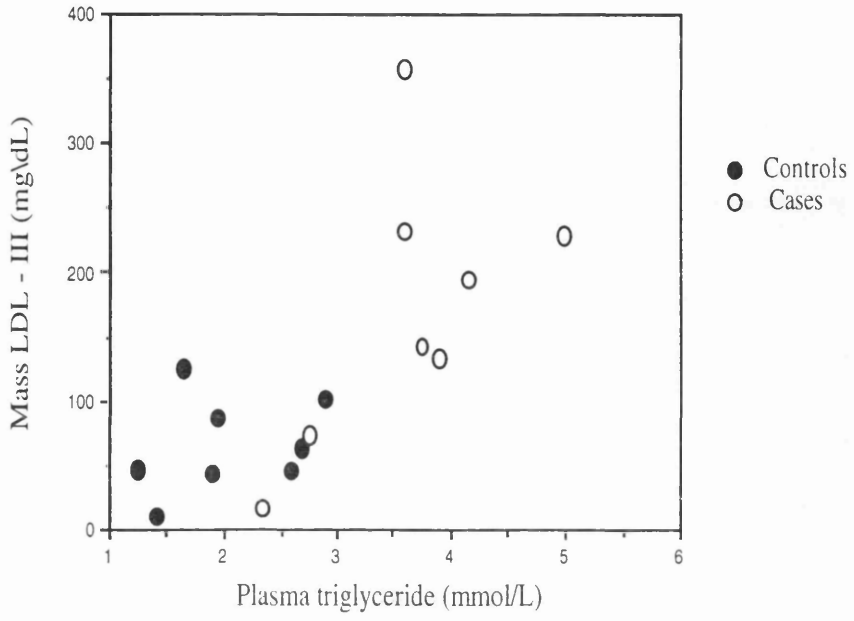


Figure 17 The relationship between plasma concentrations of small, dense LDL-III (mg/dL) and triglyceride (mmol/L), in cases (O) and controls (●), $r^2=0.504$, $p=0.002$.

4. DISCUSSION

The finding of significantly elevated plasma concentrations of triglyceride and lower HDL-cholesterol concentrations in women with pre-eclampsia is in good agreement with previous larger studies (Lorentzen et al 1995, Kaaja et al 1995). The results of this study have demonstrated, for the first time, that raised plasma triglyceride concentrations in pre-eclampsia are associated with substantially elevated plasma concentrations of $VLDL_1$ and importantly of small, dense LDL-III. These lipoprotein subfraction changes (and raised hepatic lipase activity) are in keeping with the

metabolic pattern associated with the insulin resistance syndrome of which hypertriglyceridaemia is a key component (See Chapter I, section 4.7).

The close correlation in our study between mass LDL-III and plasma triglyceride concentration is in good agreement with previous studies of non-pregnant individuals. Using multivariate analysis, we and others (Griffin et al 1994, Tan et al 1995, Austin & Edwards 1996) have shown that in the non-pregnant situation plasma triglyceride is the major determinant of the concentration of small LDL, accounting for anywhere between 40-60% of its variability. Thus, the higher plasma triglyceride concentrations seen in women with pre-eclampsia may account for the substantially increased (around three-fold) levels of small, dense LDL.

A model has been published (Tan et al 1995) in which the generation of LDL-III is linked to increased triglyceride exchange into LDL followed by hepatic lipase-induced lipolysis of the particle. In that study it was seen that in women, but not in men, hepatic lipase activity was an independent predictor of the plasma concentration of small, dense LDL. Therefore, the higher hepatic lipase activity we observed in our pre-eclamptic cases may also contribute to the smaller, denser LDL profile.

It is as yet unclear why plasma triglyceride concentrations are elevated in pre-eclampsia. It may be hypothesized that higher free fatty acid levels in conjunction with reduced hepatic β-oxidation may be involved (See Chapter VII). Equally, enhanced peripheral or hepatic insulin resistance may play a role (Kaaja et al 1995) as could reduced catabolism of triglycerides, ie, reduced lipoprotein lipase activity secondary to cytokine inhibition (See Chapter VII).

Whatever the mechanism, increased concentrations of triglyceride-rich lipoproteins in the circulation are potential contributors both directly, and through the generation of small, dense LDL, indirectly, to endothelial dysfunction and therefore the expression of pre-eclampsia.

Small, dense LDL are known from atherosclerosis studies to be more susceptible to oxidation than their larger counterparts and there is ample evidence of oxidative stress in pre-eclampsia (Wang et al 1991). Consistent with this finding, antibodies to an epitope of oxidized LDL have been shown to be significantly elevated in pre-eclamptic patients (Branch et al 1994). There is also evidence of enhanced type II phospholipase A_2 (PLA_2) concentrations in the plasma of women with severe pre-eclampsia (Lim et al 1995). This may be associated with an increased intrinsic LDL-PLA_2 activity, which is necessary for oxidation to occur (Witztum 1993). Therefore, the observation that small dense LDL is markedly elevated in pre-eclampsia may be the key link in explaining the recently reported relationship (Hubel et al 1996) between serum triglyceride levels and oxidation products in pre-eclamptic patients, and as such may play an important role in the genesis of endothelial dysfunction.

Moreover, oxidized LDL inhibits endothelial prostacyclin synthesis, inactivates (and reduces synthesis and release of) endothelial derived relaxing factor (Chin et al 1992), and simultaneously increases endothelin production and release. These changes promote platelet activation with resultant enhanced thromboxane release. Lysophosphatidylcholine in oxidized LDL also stimulates expression of vascular endothelial-cell adhesion molecule-1 (Kume et al 1992) a monocyte adhesion molecule expressed by activated endothelial cells and previously demonstrated to be elevated in pre-eclampsia (Lyall et al 1994) and promotes rapid adhesion of neutrophils to endothelium by up-regulation of CD11b/CD18 adhesion receptors on neutrophils (Lehr et al 1995). Large triglyceride-rich VLDL are also known to be cytotoxic and to promote synthesis and release of plasminogen activator inhibitor-1 (Hamsten & Eriksson 1994).

As many of these changes have also been documented and implicated in the pathogenesis of pre-eclampsia, the question arises whether endothelial dysfunction of pre-eclampsia is causally related to alterations in metabolism of fatty acids and triglyceride.

CHAPTER VI

A RELATIONSHIP BETWEEN MYOMETRIAL RESISTANCE ARTERY BEHAVIOUR AND CIRCULATING LIPID COMPOSITION

1.0 INTRODUCTION

In the previous Chapter (V) it was shown that in pre-eclampsia, plasma triglyceride and small, dense LDL concentrations are substantially higher and HDL-cholesterol concentrations substantially lower than concentrations observed in normal pregnancy. In addition, Lorentzen et al (1995) have reported that FFA and triglyceride concentrations climb higher in pre-eclampsia well in advance of clinical manifestations of the disease.

In turn, Chapter I section 5.2 cites plentiful evidence from studies in non-pregnant individuals revealing the capacity of plasma lipids to promote endothelial changes. Briefly, hypercholesterolaemia has repeatedly been shown to be associated with endothelial dysfunction, whereas cholesterol-lowering therapy has been demonstrated to result in improved endothelial-dependent vasomotion (Anderson et al 1995, Treasure et al 1995). In contrast, plasma high density lipoprotein-cholesterol (HDL-cholesterol) concentrations are positively related to endothelium-dependent relaxation (Zeiher et al 1994, Kuhn et al 1991). Subsequently, it has been proposed that the higher HDL-cholesterol concentrations observed in pre-menopausal women may be responsible for their observed protection against the adverse effects of hypercholesterolaemia on endothelium-dependent vasodilatation (Chowienczyk et al 1994). There is also plentiful evidence to suggest that triglyceride-rich particles, partly through oxidative pathways, and particularly in the post prandial state, may be damaging to the endothelium (Sattar et al, In press).

By collaborating with colleagues in Nottingham, an opportunity to examine the relationship between lipids and endothelial function in normal pregnancy and pre-eclampsia, presented. The Nottingham group have demonstrated an alteration in endothelium dependent myometrial resistance artery function in women with pre-eclampsia (Ashworth et al 1997a), and have induced a similar functional change in myometrial resistance vessels from normal pregnant women after incubation with plasma from women with pre-eclampsia (Ashworth et al 1997b). Their findings are consistent with the hypothesis that some circulating humoral factor(s) affects maternal systemic function directly or indirectly, by activating endothelial cells with resultant vascular injury. Furthermore, there is evidence from other in vitro work that a circulating factor in women with pre-eclampsia is capable of causing endothelial

dysfunction (Roberts & Redman 1993, Baker et al 1995).

In this study, we examined the relationship between plasma cholesterol, triglyceride Apo AI (the apoprotein associated with HDL particles) and Apo B (the apoprotein associated with triglyceride-rich lipoproteins) concentrations and endothelial behaviour of myometrial resistance vessels bathed in plasma from normal pregnancies and those complicated by pre-eclampsia.

Our hypothesis was that, in line with the situation in non-pregnant individuals, plasma cholesterol and/or apo AI (HDL-cholesterol) concentrations would associate strongly with endothelial function in normal pregnancy and pre-eclampsia. For completeness, we also measured apo B and non-fasting triglyceride concentrations

2. SUBJECTS, SAMPLES, METHODS

Ethical approval was obtained from Nottingham City Hospital and University Hospital Nottingham Ethics Committees prior to commencement of the study. Written informed consent was obtained from all patients before enrolment into the trial. This applied to both those donating plasma and those donating vessels at the time of Caesarean section.

Study Group

The normal pregnant women from whom all the vessels were obtained (n= 20) had singleton pregnancies and were undergoing elective lower segment Caesarean section at term. Indications for operative delivery were malpresentation, previous Caesarean section and/or maternal request. Neither these pregnancies, nor any proceeding pregnancy were complicated by pre-eclampsia, intrauterine growth restriction or any other medical complications (e.g. diabetes, epilepsy etc.).

Plasma was collected from 10 nulliparous patients with pre-eclampsia and 10 gestational-age matched nulliparous normal pregnant women. Pre-eclampsia was defined as a clinical syndrome developing in a previously normotensive woman after 20 completed weeks gestation (with no pre-existing renal disease), characterised by the presence of significant proteinuria (greater than 300mg/litre in a 24 hour urine collection and in the absence of a urinary tract infection) in the presence of hypertension (a blood pressure of greater than 140/90 mm Hg on two separate occasions at least 4 hours apart), both of which resolve by the 6th post partum week (Davey & MacGillivary 1988). Blood was obtained from these patients at 8 am, into EDTA tubes onto ice, and was usually, but not invariably, collected from fasting subjects. These were centrifuged at 1500 x g at 4^{o}C for 15 minutes to precipitate the cellular components of the blood. Plasma was then aliquoted into sterile eppendorf tubes, labelled and stored at - 80 ^{o}C until required.

Vessel collection

At Caesarean section, following delivery of the baby and placenta, a full thickness biopsy of myometrium was taken from the upper margin of the lower segment uterine incision. Uterine biopsies were placed into physiological salt solution (PSS) at 4^{o}C, through which a mixture of 95% oxygen and 5% carbon dioxide had been bubbled prior to sample collection. Microscopic dissection was performed in 4^{o}C PSS within 2 hours of the Caesarean section, resistance arteries of 200 - 550 mm diameter, corresponding to uterine radial arteries in the myometrium, being removed. The dissected vessels were then separated into two groups and incubated with 2% plasma (in PSS with heparin (1 IU/ml)) from either the pre-eclampsia or normal pregnant groups. Once carbonated in 5% CO_2 / 95% O_2 these vessels were then stored for up to 20 hours at 0-4 ^{o}C prior to experimentation. It has been previously shown that storage of the vessels for up to 48 hours had no measurable deleterious effect on vessel response to a variety of vasoconstrictors and vasodilators in the experimental protocol used, provided that dissection had occurred within 2 hours of the biopsy specimens being taken (Ashworth et al 1996).

Myography

Following incubation, the paired vessels were mounted on two parallel 50 mm wires on a Mulvany wire myograph, the experiments being performed in PSS, with the operator blind as to which sample the vessels had been incubated in.

After an hour's equilibration to 37^{o}C, the vessels were normalised to allow calculation of that diameter at which the transmural pressure was equal to 100 mm Hg (L_{100}), and set at a diameter of 0.9 L_{100}. This was an identical protocol to other studies performed previously at our unit. Vessels were contracted with vasopressin to a concentration of 10^{-8}M at which point a steady state contraction was achieved. Those vessels exhibiting significant vasomotion (>20% of the contraction) or those failing to achieve a contraction of greater than 50 mm Hg were excluded from the study. In accordance with other studies, work performed in the Nottingham unit (Ashworth et al 1996) has demonstrated that other vasoconstrictors (such as norepinephrine, prostaglandin F2α, angiotensin II, or 5-hydoxytryptamine, which are found in vivo in the vascular beds under investigation), were not capable in vitro, of providing a consistent, repeatable, sustained contraction in human myometrial resistance vessels. These contracted vessels were then exposed to incremental doses of the endothelium dependent agent bradykinin from $1x10^{-10}$ M to $3.3x10^{-6}$ M, relaxation being expressed as a percentage of the size of the original contraction.

Solutions and chemicals

The physiological salt solution is a modified Krebs solution, its constituents being

(mmol/L): NaCl (119), KCl (5.9), $CaCl_2$ (2.5), $MgSO_4$ (1.17), $NaHCO_3$ (25), KH_2PO_4 (0.2), and EDTA (0.026). The glucose concentration was 5 mmol/L and the pH of the solution buffered to 7.4. All chemicals used were Analar reagents supplied by Merck Ltd, Poole UK, except calcium chloride and EDTA which were obtained from Sigma Chemicals, St Louis, USA. Sigma Chemicals also supplied vasopressin, bradykinin, and norepinephrine.

Lipid analysis

The plasma samples were assayed for cholesterol, triglyceride, Apo A1 and Apo B as described in detail in Chapter II. It is important to note that although the non-fasting nature on some of the samples causes variability in triglyceride concentrations, cholesterol, apo AI and apo B concentrations are negligibly affected and thus their non-fasting measurements provide an accurate reflection of fasting concentrations.

Statistical analysis

Values are given as mean with standard error (SE), or as median (range) for non-parametrically distributed data. In all cases two arteries from each patient were studied, and mean data used for analysis. Maternal age, birth weight and individualised birth ratio (birth weight corrected for gestational age, parity, maternal body mass index, and maternal ethnic origin, Sanderson et al 1994), blood pressure at booking and delivery, and gestational age at delivery were analysed by the unpaired students t-test, or the Mann-Whitney unpaired two-tailed U test where appropriate.

Concentration response curves were analysed by comparison of the percentage relaxation for each woman at each dose of bradykinin, using repeated measures analysis of variance to compare the results obtained from different groups.

Statistical significance was accepted when $p<0.05$.

3. RESULTS

3.1 Demographic details

	Normal pregnant median (range)	Pre-eclampsia median (range)	P-Value Mann-Whitney U
Age	23 (22-26)	28 (25-30)	0.23
BMI	22 (21-25)	24 (22-25)	0.78
MAP (booking) mm Hg	88 (84-90)	84 (76-92)	0.73
MAP (delivery) mm Hg	92 (84-94)	112 (76-92)	0.0001
Proteinuria g/dl	0 (0-0)	1.2 (0.64-1.54)	0.0001
IBR	46 (27-82)	11 (2-36)	0.03
	mean (standard dev)	mean (standard dev)	Students t test
Gestation at venepuncture (days)	261 (20)	243 (25)	0.09
Gestation at delivery (days)	274 (13)	243 (27)	0.03

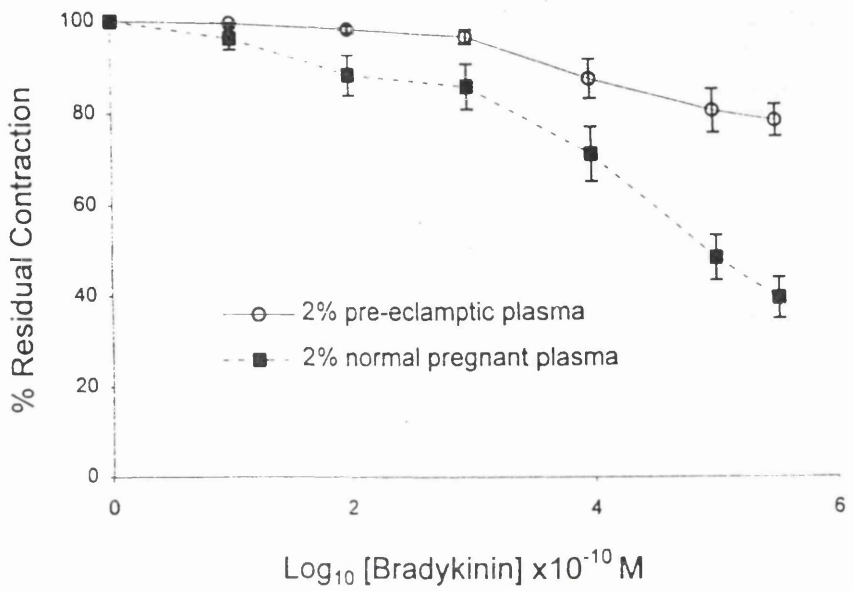
Table 11 Demographic details of the patients from whom the plasma was obtained. BMI : body mass index; MAP: mean arterial pressure; IBR: individualised birth weight ratio.

There was a statistically significant difference between the normal pregnant plasma and pre-eclamptic plasma groups in the mean arterial pressure at delivery (p= 0.0001 Mann Whitney U); proteinuria (p= 0.0001 Mann Whitney U); and individualised birth weight ratio (p=0.03).

3.2 Percent residual contraction of myometrial vessels with plasma from normal and pre-eclamptic pregnancies.

Vessels from each patient (n=20) were divided into two groups prior to incubation with 2% plasma from patients with pre-eclampsia or 2% plasma from normal pregnant women. Incubation of vessels with 2% plasma from women with pre-eclampsia resulted in a significant loss of endothelium dependent relaxation when compared with vessels incubated in 2% plasma from normal pregnant women ($p<0.0001$ repeated measures ANOVA, Figure 18).

Figure 18.



X axis: Log 10 [Bradykinin] x 10 -10 M
Y axis: % residual contraction of vessels (reciprocal of % relaxation)

All vessels were obtained from normal pregnant women undergoing elective lower segment Caesarean section at term (n=20). Vessels from the same patient were divided into two groups prior to incubation with 2% plasma from patients with pre-eclampsia or 2% plasma from normal pregnant women. Incubation of vessels with 2% plasma from women with pre-eclampsia resulted in a significant loss of endothelium dependent relaxation when compared with vessels incubated in 2% plasma from normal pregnant women or without plasma ($p<0.0001$ repeated measures ANOVA).

3.3 Lipid concentrations of normal pregnant plasma and plasma from women with pre-eclampsia.

There was no significant difference in the lipid profiles measure between the normal pregnant women and the women with pre-eclampsia ($p>0.15$ in all parameters measured, Mann Whitney U).

	Normal pregnant median (range)	Pre-eclampsia median (range)	P-Value Mann-Whitney U
Cholesterol (mmol/l)	6.4 (5.6-6.9)	6.7 (5.7-7.7))	>0.5
Triglyceride (mmol/l)	2.25 (1.71-3.55)	3.13 (2.33-4.43)	0.24
Apo AI (g/l)	1.9 (1.6-1.9)	1.6 (1.4-1.9)	>0.5
Apo B (g/l)	1.1 (0.9-1.3)	1.2 (1.0-1.4)	>0.5
Apo AI/B	1.7 (1.3-2.1)	1.3 (1.2-1.7)	>0.5

Table 12 Lipid concentrations of normal pregnant plasma and plasma from women with pre-eclampsia. Apo AI: apolipoprotein AI; Apo B: apolipoprotein B; Apo AI/B: ratio between apo AI/B.

3.4 Relationship of percent residual contraction of vessels with plasma apo AI. (Figures 19 and 20).

We found a significant association between the concentration of circulating Apo AI (indicative of HDL cholesterol) and the endothelium-dependent behaviour of the vessels (Spearman rank correlation. $p=0.025$). On subgroup analysis of those vessels incubated with plasma from women with pre-eclampsia this correlation persisted (Spearman rank correlation. $p=0.036$). This was not the case in vessels incubated in plasma from normal pregnant women (Spearman rank correlation $p=0.25$). No other significant associations between lipid parameters and endothelium dependent vessel behaviour were found.

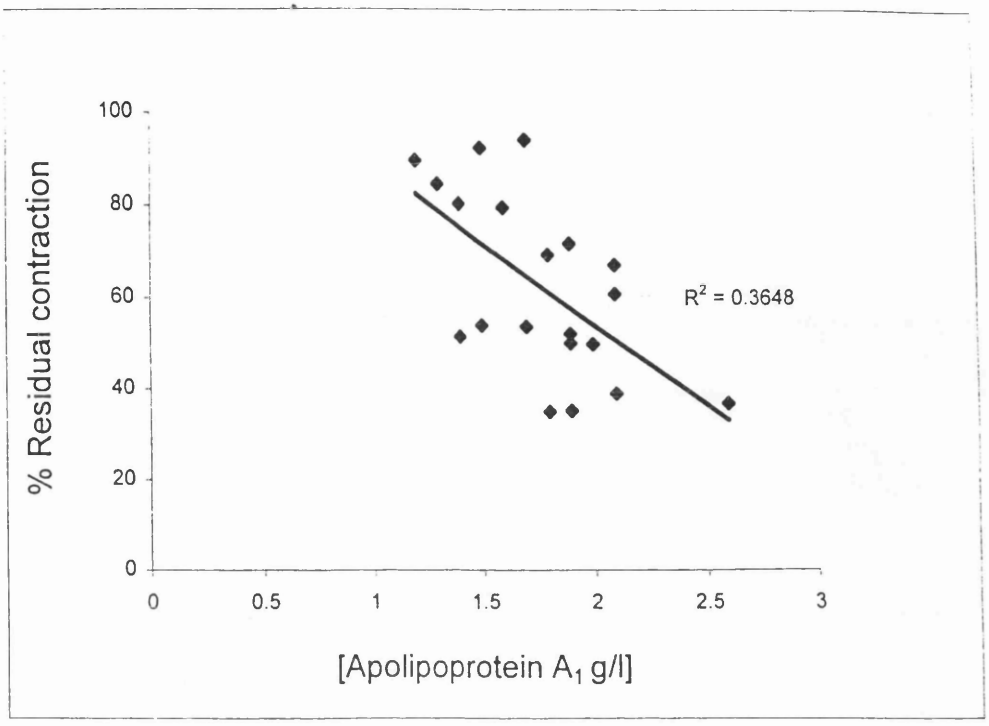


Fig. 19 % residual contraction of vessels at 3.3 x 10-6 M Bradykinin plotted against [apolipoprotein A1] for all vessels (those incubated with plasma from normal pregnant women and plasma from women with pre-eclampsia).

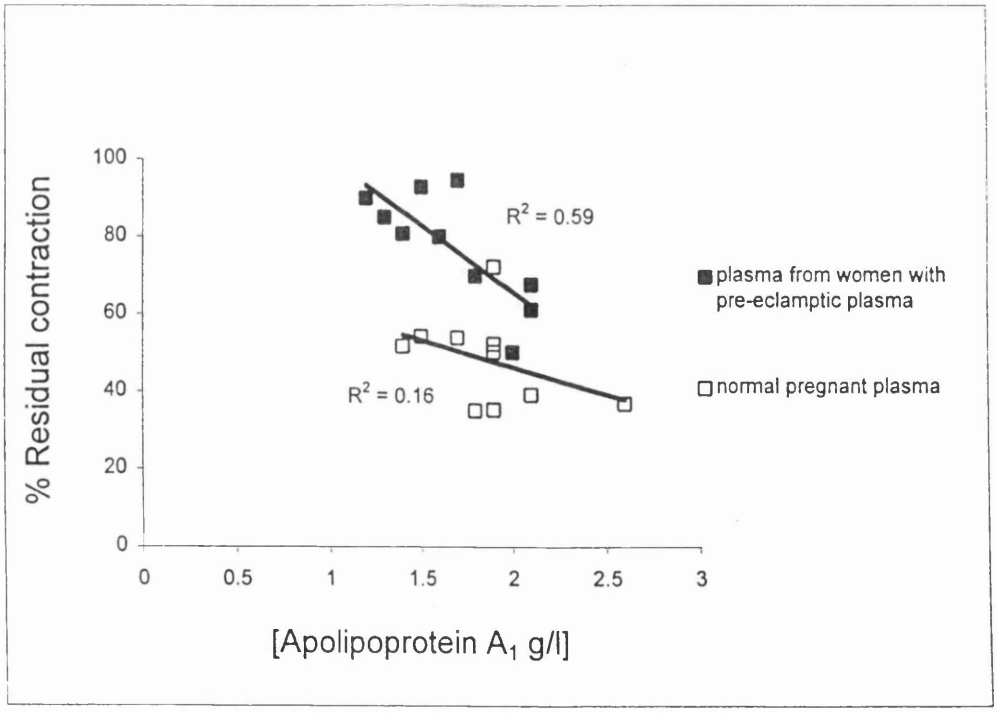


Fig. 20 % residual contraction of vessels at 3.3 x 10-6 M Bradykinin plotted against [apolipoprotein A1] for vessels:
a) incubated with plasma from women with pre-eclampsia (closed squares)
b) incubated with plasma from normal pregnant women (open squares)

4. DISCUSSION

It has been demonstrated, in an in vitro setting, that the plasma from women with pre-eclampsia is capable of inducing a functional change in the behaviour of myometrial resistance arteries of normal pregnant women, such that these vessels mimic the behaviour of similar sized resistance arteries from women with pre-eclampsia.

In this study, we examined whether plasma lipid (triglyceride, cholesterol, Apo AI or Apo B) concentrations in normal pregnancy and pre-eclampsia contributed to the observed endothelial behaviour. In contrast with data from several previous studies (Potter & Nestel 1979, Lorentzen et al 1995, Kaaja et al 1995), and the results presented in Chapter V, we did not observe a significant difference between the plasma triglyceride, Apo AI and Apo B concentrations between the pre-eclamptic and normal pregnant groups. A notable difference in this study, and one that may help to explain this lack of statistical difference in triglyceride concentrations is that not all the samples were collected in the fasting state. As a result, variability in triglyceride measurements was greater. Furthermore, although we had attempted to match the groups for gestational age at sampling, there was a tendency for samples to be collected from the pre-eclamptic group at an earlier gestation (261 vs, 243 days $0.1< p>0.05$). This may have been relevant since triglyceride levels increase most dramatically in late pregnancy, and also at this stage of pregnancy HDL-cholesterol (and Apo AI) concentrations, which reach a maximum at around 25-30 weeks, are declining towards first trimester levels (See Chapter III).

Despite the lack of a statistical difference in the lipid concentration between the groups, a significant correlation between endothelium dependent relaxation and the plasma concentration of Apo AI ($p=0.025$), was observed. Furthermore, and perhaps more importantly, a significant correlation ($R^2=0.59$, $p=0.036$) between the endothelium dependent relaxation in vessels incubated with plasma from women with pre-eclampsia and the plasma content Apo AI was seen (Figures 19 and 20). In contrast, plasma triglyceride, cholesterol, and Apo B concentrations did not relate significantly to endothelial behaviour.

Interestingly, these results in pregnant patients are broadly consistent with data from non-pregnant individuals. Plasma HDL-cholesterol concentrations have been demonstrated to relate positively and significantly to endothelium-dependent relaxation of coronary vessels in patients with coronary heart disease (Zeiher et al 1994, Kuhn et al 1991). In addition, in a study in patients with type 2 diabetes mellitus, decreased HDL cholesterol was the best predictor of impaired vasodilatation to acetylcholine even after adjustment for all lipid and lipoprotein concentrations (O'Brien et al 1997). Thus, in non-pregnant individuals there is good evidence for a role of HDL in favourably modifying endothelial function.

Endothelial protection by HDL may relate to its antioxidant properties as HDL has been found to protect against LDL oxidation (Hessler et al 1992). HDL antioxidant actions may be related to its protein components (which bind transition metals) (Kunitake et al 1992), and to two intrinsic anti-oxidative enzyme systems: platelet activating factor acetylhydrolase (Stafforini et al 1993) and paraoxonase (Mackness et al 1991). HDL may also play a role in reverse transport of potentially reactive hydroperoxide species for hepatic detoxification as described by Tribble (1995). Impaired removal of these hydroperoxides (when HDL-cholesterol concentrations are low) could be particularly deleterious under conditions favouring a prolonged residence time and accumulation of susceptible sub-populations of particles, for example, small dense LDL (Tribble 1995). Indeed, both oxidative stress (as shown by increased lipid peroxide concentrations) (Uotila et al 1993), and high concentrations of small, dense LDL (Chapter V) are characteristics of pre-eclampsia. There evidence also that HDL particles stimulate prostacyclin secretion from endothelial surfaces (Kaaja et al 1995). Thus, there are also abundant possible mechanisms by which HDL particles may favour endothelial dilatation.

An alternative possibility for the findings in this study is that rather than a direct influence of Apo AI on endothelial behaviour, some other factor(s) dictating Apo AI metabolism and thus Apo AI concentrations may relate to endothelial behaviour. In the non-pregnant situation, Apo AI and HDL-cholesterol concentrations are largely governed by the efficiency of triglyceride catabolism. Interestingly, in non-pregnant subjects it has recently been demonstrated that post-prandial hypertriglyceridaemia (a test of the efficiency of triglyceride catabolism) transiently impairs endothelium dependent flow-mediated vasodilatation (Vogel et al 1997). Furthermore, in this study mean change in post-prandial flow-mediated vasoreactivity correlated with change in 2-hour serum triglyceride concentration. Therefore, it would be interesting to examine the relationship in future studies between triglyceride metabolism and endothelial behaviour in women with pre-eclampsia.

Whatever the mechanism our data demonstrate that plasma Apo AI (and therefore HDL) concentrations are associated with altered endothelial behaviour of the endothelium in pre-eclampsia. Clearly, future studies are needed to examine in greater detail the relationship between endothelial behaviour and lipid concentrations in pre-eclampsia.

CHAPTER VII

HYPOTHESIS: MECHANISM FOR GENERATION OF HYPERLIPIDAEMIA IN PRE-ECLAMPSIA, AND A DISCUSSION OF ITS POTENTIAL PATHOGENIC ROLE.

1. INTRODUCTION

In the preceding 2 chapters (V and VI) lipoprotein subfraction concentrations were examined in pre-eclampsia, and lipid concentrations, specifically apo AI concentrations, were related to endothelial function in pre-eclampsia as measured in myometrial resistance vessels. In this current chapter, a hypothesis which brings together indirect evidence for the participation of cytokines and lipid peroxides in the development of the exaggerated hyperlipidaemia of pre-eclampsia is presented. This hypothesis incorporates a growing body of evidence based on clinical studies on pre-eclampsia, but also on possible animal models, that focus attention on the liver and its handling of fatty acids as a key link in the pathogenic chain.

2. Liver dysfunction in pre-eclampsia

Liver dysfunction occurs frequently in pre-eclampsia. The severity of hepatic disturbance is variable ranging from mild elevations in the plasma concentrations of liver enzymes to more serious forms associated with clinical jaundice and gross hepatic impairment such as HELLP syndrome (haemolysis, elevated liver enzymes and low platelet count). Similarities with acute fatty liver of pregnancy (AFLP) suggest that these disorders form a spectrum of disease with common clinical and laboratory abnormalities (Minakami et al 1988).

3. Equine hyperlipaemia and Reyes syndrome

In a series of studies in our laboratory we sought to explain the pathogenesis of a condition common in certain breeds of horses, e.g. ponies, known as equine hyperlipaemia (Watson et al 1992). Normally, free fatty acids released from adipocytes by the action of hormone sensitive lipase (HSL) are taken up by liver and subject to two alternative routes of metabolism; one is their reassimilation into triglyceride molecules, while the other is oxidation by mitochondrial β-oxidative pathways which results in the generation of energy and the synthesis of ketones (Figure 21). Horses as a species have a relative inability to undertake β-oxidation and so the liver if presented with fatty acids generates triglyceride for storage and for secretion as very low density lipoprotein (VLDL). The delivery of substrate for triglyceride synthesis in the form of free fatty acids is known from cell culture work to be the major determinant of VLDL secretion (Dixon et al 1993).

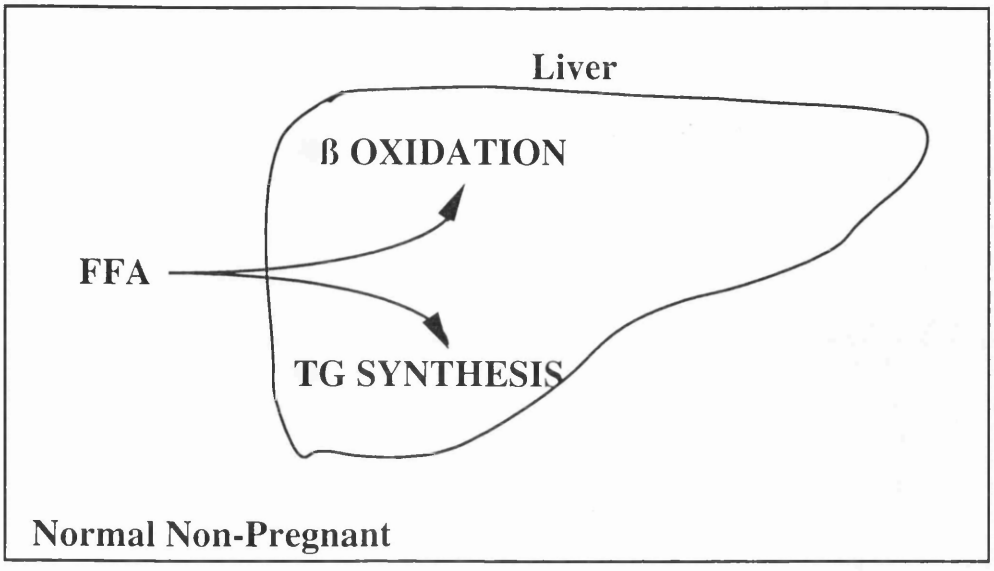


Figure 21. Normally FFAs entering the liver have two routes of metabolism; they are either subjected to β-oxidation resulting in CO_2 (and a small amount of ketones) or they are esterified and secreted as triglyceride in VLDL.

In conditions of stress, e.g when horses are transported over long distances, there is a massive release of fatty acids from adipocytes and when these reach the liver they stimulate triglyceride synthesis with consequent hypersecretion of VLDL and a marked hypertriglyceridaemia ensues. However, this pathway of triglyceride elimination from the liver eventually becomes saturated and as a result triglyceride accumulates in the hepatocytes giving the characteristic histological picture of hepatic steatosis. Particularly pertinent to the present discussion is the observation that equine hyperlipaemia is more prevalent in pregnancy, particularly in animals with abdominal obesity (Watson et al 1992).

Similarly, the hepatic steatosis observed in Reye's syndrome is thought to be the result of a combination of increased flux of FFAs (Chaves-Carbello et al 1979) and impaired hepatocyte β-oxidation of fatty acids due to post-infectious (Heubi et al 1987), and in some cases salicylic acid mediated, mitochondrial dysfunction (Deschamps et al 1991). Indeed, impaired mitochondrial oxidation is implicated in many other examples of microvesicular steatosis (Grimbert et al 1993).

4. 1 FFA flux and β-oxidation in normal pregnancy

In the late second trimester of human pregnancy there is an increased flux of free fatty acids promoted by a combination of stimulation of HSL by human placental lactogen (HPL) (Martin-Hidalgo et al 1994), and relative resistance to the effects of insulin (Silliman et al 1994) (which normally acts to suppress FFA release from adipose tissue). Studies in the mouse suggest that late pregnancy is also associated with a mild impairment in β-oxidative function (Grimert et al 1993). However, as total hepatic lipids remained unchanged, it appears that in normal murine pregnancy this decreased oxidation of fat in the liver is compensated for by the increased secretion of VLDL, in agreement with studies in other animal models (Weinstein et al 1979, Wasfi et al 1980) (Figure 22). Recently, in a single human case study oestrogen has been demonstrated to inhibit hepatic lipid oxidation with resultant increase in serum triglyceride concentration (O'Sullivan et al 1995).

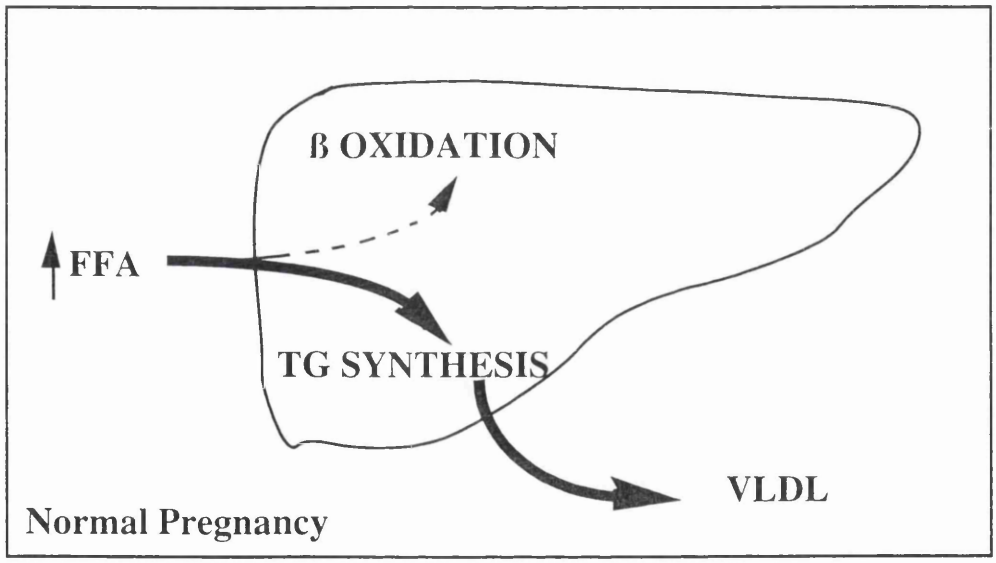


Figure 22. Late pregnancy is associated with an increased flux of FFAs and impaired fatty acid oxidation. The net effect is increased delivery of FFAs into triglyceride synthesis. However, in normal pregnancy this increased triglyceride synthesis is compensated for by increased secretion of VLDL. Consequently, hepatic triglyceride content remains unchanged.

4.2 FFA flux and β-oxidation in pre-eclampsia

In women with pre-eclampsia there is evidence to suggest that an increased flux of FFAs occurs over and above that seen in normal pregnancy and, significantly, this phenomenon is observed long before the onset of clinical disease (Lorentzen et al 1994). It has also been shown that the FFA/albumin ratio in women with pre-eclampsia is twice that observed in normal pregnancy (Endersen et al 1992) and serum from affected women causes triglyceride accumulation in cultured endothelial cells with resultant impaired release of prostacyclin (Lorentzen et al 1991). Although the higher FFA/albumin ratio in pre-eclampsia may be partly explained by lower plasma albumin concentrations, enhanced adipocyte lipolysis with resultant higher circulating concentrations of FFAs is likely to be the major cause, as intravascular lipolytic activity did not account for the changes observed in plasma FFA concentrations pre-eclamptic women prior to clinical manifestations of their disease (Lorentzen et al 1994).

In pre-eclampsia, it is tempting to postulate that, as in the equine model, increased FFA flux overlaid on the impaired β-oxidation in late pregnancy promotes the excessive synthesis of hepatic triglyceride and thus VLDL hypersecretion. Plasma triglyceride concentrations are significantly raised in women with pre-eclampsia compared to unaffected controls matched for gestational age (Potter & Nestel 1979), and it has been further demonstrated that raised plasma triglyceride concentrations preceded the onset of clinical disease (Lorentzen et al 1994). This chain of events fits with earlier work and with findings from our laboratory of a specific accumulation of large, triglyceride-rich VLDL in the circulation of pre-eclamptic women (Chapter V). There is also the possibility that β-oxidation is further impaired in subjects with pre-eclampsia since ketonaemia is not a feature despite fatty acid levels being increased. Also in AFLP there is more direct evidence for inhibition of fatty acid oxidation (Treem et al 1994, Wilcken et al 1993).

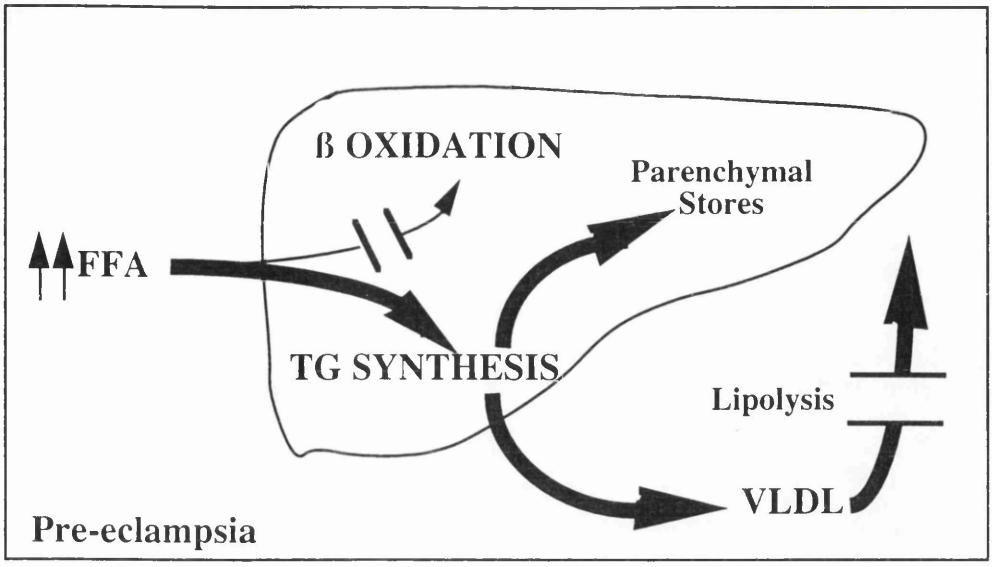


Figure 23. In pre-eclampsia, FFA flux is further enhanced and β-oxidation may be further impaired. The liver responds by further increasing secretion of VLDL, but eventually this mechanism becomes saturated resulting in the deposition of triglyceride within the parenchyma.

When the liver's ability to produce VLDL is saturated it is likely that this is followed by accumulation of triglyceride in hepatocyte stores. Significant amounts of microvesicular fat droplets have been demonstrated in cases of pre-eclampsia, with and without liver dysfunction, increasing in density in HELLP syndrome and maximal in cases of AFLP (Minakami et al 1988) (Figure 23). Furthermore, in the last study, the density of hepatocellular fat correlated positively with plasma urate concentration and negatively with the platelet count, established markers of severity and progression of pre-eclampsia.

Data on plasma triglyceride and high density lipoprotein (HDL)-cholesterol concentrations in HELLP syndrome and AFLP are currently lacking. However, in a study of plasma lipoprotein concentrations in Reye's syndrome (Chaves-Carbello et al 1979), increased plasma triglyceride concentrations and reduced HDL-cholesterol concentrations were observed to be most marked in fatal cases. Thus, plasma lipoprotein concentrations may be useful indices of severity in Reye's syndrome and by extrapolation may be worth investigating as prognostic indicators for pre-

eclampsia.

Although we have emphasised the synthetic aspects of the control of plasma VLDL concentrations drawing on animal work and limited clinical data, it is possible that impaired adipose tissue lipoprotein lipase activity also contributes to the exaggerated hypertriglyceridaemia observed in pre-eclampsia.

5. Cytokines and lipid peroxides

Increased plasma cytokine concentrations, particularly TNF-α (Visser et al 1994, Vince et al 1995), IL-1 (Greer et al 1995) and IL-6 (Greer et al 1995), have been implicated in the pathophysiology of pre-eclampsia. It is of interest that these same cytokines can induce adipocyte lipolysis (TNF-α, IL-1) (Chajek-Schaul et al 1989, Feingold et al 1991), promote *de novo* hepatic fatty acid synthesis (TNF-α, IL-1, IL-6) (Chajek-Schaul et al 1989, Feingold et al 1991, Greenberg et al 1988), and impair hepatic fatty acid oxidation and ketogenesis (IL-1, TNF-α) (Memon et al 1992). The result is increased hepatic triglyceride synthesis (Figure 24). Furthermore, the significantly higher TNF-α (Visser et al 1994) and TNF-R levels (Vince et al 1995) reported in patients with the HELLP syndrome, support a role for TNF-α in liver dysfunction. TNF-α and IL-6 also reduce lipoprotein lipase activity (Greenberg et al 1988, Saxena et al 1990), thereby impairing removal of triglyceride-rich lipoproteins from the circulation.

Lipid peroxides have been shown to be increased (and antioxidant defences depleted) in pre-eclampsia (Maseki et al 1981, Uotila et al 1993) and these could induce hepatic dysfunction. It has been demonstrated that free radicals, at biologically attainable levels, induce hepatic mitochondrial dysfunction promoting microvesicular fatty damage (Saibara et al 1994). Further evidence to support a role for lipid peroxide mediated hepatocyte fat accumulation and dysfunction comes from the common finding of burr cells (as produced *in vitro* under peroxidising conditions) in pre-eclamptic women with liver damage (Stark et al 1993).

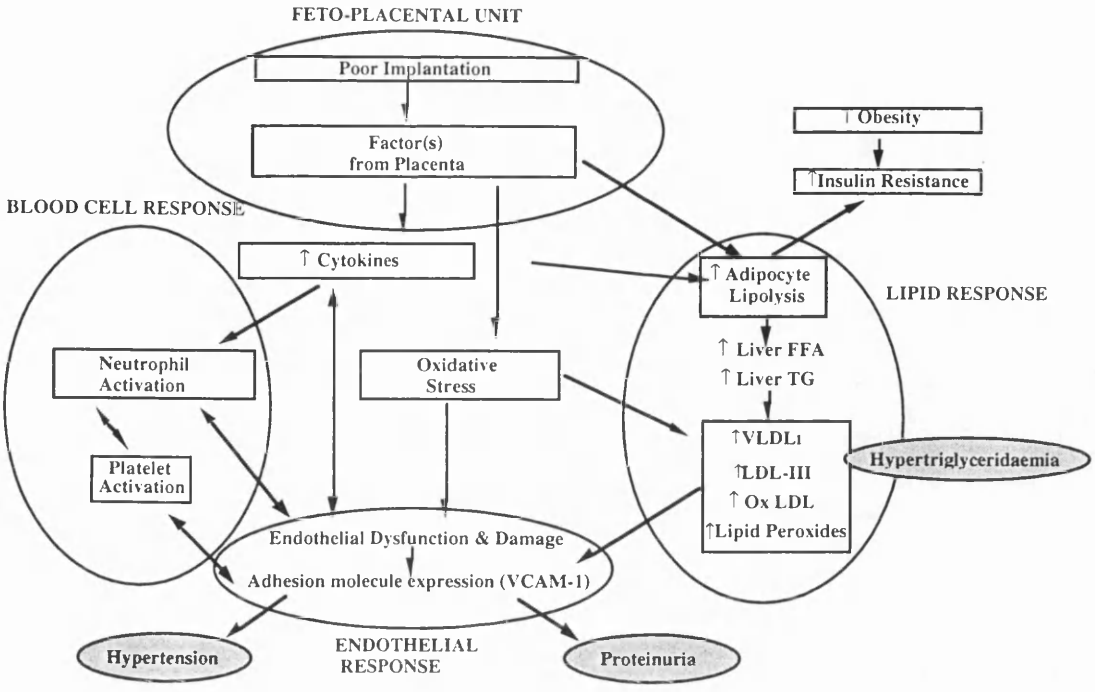


Figure 24. This diagram summarises the potential role of a disturbance in fatty acid and lipoprotein metabolism in the pathogenesis of pre-eclampsia. Increased plasma cytokine (IL-1, TNF-α) concentrations resulting from activation of macrophages / neutrophils either directly or indirectly through endothelial activation, or by the placenta itself, may enhance peripheral lipolysis which is already stimulated in normal pregnancy by HPL. This results in an increased flux of free fatty acids to the liver. As shown in Figure 23 these are channelled predominantly into hepatic triglyceride synthesis and so there is increased secretion (over and above that of normal pregnancy) of large, triglyceride-rich VLDL particles, which are also removed less efficiently than normal. Accumulation of triglyceride occurs in the hepatocyte when this pathway is saturated, and this response is a possible explanation of the fatty changes in liver seen in pre-eclampsia and AFLP. Increased concentrations of triglyceride-rich lipoproteins in the circulation may contribute both directly and, through the generation of small, dense LDL, indirectly to endothelial dysfunction and therefore expression of pre-eclampsia in the mother. It is important to note that multiple inter-relationships exist between these systems, e.g. hyperlipidaemia may result in platelet activation and oxidised LDL may induce IL-1ß release, but these have

not been shown for clarity.

6. Obesity/ Insulin resistance

It has been observed that there is a higher risk of pre-eclampsia with increasing obesity (Eskenazi et al 1991, Sibai et al 1995) and that insulin levels are higher in hypertensive pregnancies (Kaaja et al 1995). These findings are understandable if abnormalities in fatty acid metabolism are important in these disorders. Obesity, in particular abdominal, rather than gluteal-femoral, is associated with insulin resistance and hypertriglyceridaemia (Kissebah et al 1989). Insulin resistance in adipocytes causes lipolysis to proceed in a relatively unregulated fashion so that the flux of FFA to the liver is enhanced. Also, the accumulation of fat in the abdomen is associated with hypertrophy of the adipocytes, which renders them more responsive to lipolytic stimuli (Mauriege et al 1991). The release of fatty acids from omental fat cells directly into the portal circulation exposes the liver to higher FFA concentrations than would be the case if the same amount of fat were distributed at peripheral sites and this has consequences for triglyceride synthesis and VLDL secretion (Despres 1991). Furthermore, the increased hepatic uptake of FFA may itself reduce portal insulin extraction by the liver resulting in higher peripheral insulin levels (Bjorntorp 1994, Jensen et al 1989).

7. Latent genetic defect in β-oxidation

A latent genetic defect in fatty acid oxidation in AFLP (and perhaps also the HELLP syndrome) unmasked by the increased flux of FFAs in pregnancy has been proposed (Treem et al 1994, Wilcken et al 1993). This is based upon several cases of AFLP reported in women heterozygous for a partial defect in hepatic fatty acid oxidation due to long-chain 3-hydroxyacyl-coenzyme A dehydrogenase deficiency, and the similarities of the clinical and pathological pictures of patients with AFLP to those with inherited disorders of fatty acid oxidation.

8. Consequences of hypertriglyceridaemia in pre-eclampsia

There is increasing evidence in cardiovascular research that hyperlipidaemia, can cause endothelial dysfunction (See Chapter I section 5.2). Recent studies have demonstrated that hypertriglyceridaemic serum and triglyceride-rich lipoproteins are cytotoxic to cultured human endothelial cells (Spiedel et al 1990). Along with the raised plasma triglyceride concentration due to the increased secretion of large triglyceride-rich VLDL evident in women with pre-eclampsia as explained above, there is an associated increase in the plasma concentration of small, dense LDL. This is a predictable consequence of the elevation in plasma triglyceride (McNamara et al 1992). Data from our laboratory (Chapter V) support the finding of raised plasma concentrations of small, dense LDL in pre-eclampsia. Such LDL are known to be more susceptible to oxidation than their larger counterparts (deGraaf et al 1991) and both lipoprotein species i.e. large VLDL and small, dense LDL (particularly when oxidised), have the

capacity to cause endothelial dysfunction, (Figure 24); for example, oxidised LDL and VLDL, stimulate expression of VECAM-1 (Kume et al 1992), a monocyte adhesion molecule expressed by activated endothelial cells and found to be increased in pre-eclampsia (Lyall et al 1994). Oxidised LDL has also been shown to promote rapid adhesion of neutrophils to endothelium by upregulation of neutrophil adhesion receptors (CD11b/CD18) (Lehr et al 1995), while multiple oxidation products in LDL induce IL-1β release from human blood mononuclear cells (Thomas et al 1994). Large triglyceride rich VLDL is known to promote synthesis and release of PAI-1, while oxidised LDL inhibits endothelial prostacyclin synthesis. Additionally, oxidised LDL simultaneously increases endothelin production and release, and inactivates EDRF (Stewart & Monge 1993). As a result of these changes, oxidised LDL and large VLDL promote activation of platelets with resultant enhanced thromboxane release (Stewart & Monge 1993), (Figure 24).

The observed lower plasma HDL-cholesterol levels in pre-eclampsia may also contribute to the reduced prostacyclin levels seen in this condition, as there is evidence that HDL particles stimulate PGI_2 secretion from endothelial surfaces (Kaaja et al 1995). In addition, in the same study it was demonstrated that in pre-eclampsia, serum HDL (especially the larger HDL_2 subfraction) cholesterol concentrations correlated directly with 2,3-dinor-6-keto $PGF_{1\alpha}$ excretion, the urinary metabolite of PGI_2, while serum triglyceride concentrations correlated positively with 2,3-dinor-thromboxane B_2 excretion (Kaaja et al 1995). Lower plasma HDL concentrations may reduce antioxidative protection for other lipoproteins (Mackness & Durrington 1995).

As many of these changes in endothelial cell and blood mononuclear cell function and prostaglandin synthesis have been documented and implicated in the pathogenesis of pre-eclampsia (Greer 1994), the question arises as to whether endothelial dysfunction of pre-eclampsia is causally related to, and exacerbated by, alterations in the metabolism of fatty acids and triglyceride (Figure 24). The findings in the preceding chapter (VI) of a significant relationship between plasma apo AI concentrations and endothelial behaviour in pre-eclampsia support this concept.

9. Lipids, 'acute atherosis' and 'glomerular capillary endotheliosis'.

It is noteworthy that the characteristic pathological lesion seen in the utero-placental bed of patients with pre-eclampsia is a necrotising arteriopathy consisting of fibrinoid necrosis, accumulation of foam cells or lipid laden macrophages, fibroblast proliferation and a perivascular infiltrate. This lesion has been termed 'acute atherosis' (Roberts & Redman 1993) and shares many features with coronary atherosclerosis. It is also significant that lipoproteins have been implicated in the generation of renal disease (Walli et al 1993). Observations in experimental animals and in patients with genetically determined and acquired hyperlipidaemias suggest that lipids can damage

the kidney and lead to glomerulosclerosis. *In vitro* studies of human glomerular cells have been useful in providing information on lipid-induced glomerular damage. Thus, there are strong indications that lipoproteins may play a role in the development of mesangial cell damage and progressive renal disease (Walli et al 1993). Indeed in pre-eclampsia, renal morphology reveals hypertrophy of the cytoplasmic organelles in endothelial and occasionally mesangial cells, particularly the lysosomes, which undergo marked enlargement and vacuolisation (due to accumulation of free neutral lipids). These reactive changes have been termed 'glomerular capillary endotheliosis' (Garber et al 1994), and these histopathological changes are clearly in keeping with a disorder of lipoprotein metabolism.

10 . Hypertriglyceridaemia and fetal nutrition?

The exaggerated hypertriglyceridaemia in pre-eclampsia may benefit fetal nutrition since it is increasingly recognised that triacylglycerol fatty acids, and not albumin bound FFAs, are the predominant source of fatty acids for the placenta where they are released for trans-placental transport by lipoprotein-lipase mediated hydrolysis (Herrera et al 1988, Bonet et al 1992). Direct receptor mechanisms such as the newly described VLDL receptor may also play a role (Gafvels et al 1994). This permits fatty acids to be targeted more efficiently towards the placenta. In women with pre-eclampsia and mothers of intra-uterine growth retarded fetuses, it has been shown that placental lipoprotein lipase activity is significantly greater than in normal pregnancy (Biale 1985). Conversely, peripheral adipose tissue lipoprotein lipase activity may be impaired further in pre-eclampsia due to the actions of the cytokines (Greenberg et al 1988, Saxena et al 1990). Thus, the feto-placental unit may initiate chemical signals promoting the generation of an exaggerated hypertriglyceridaemia for the reward of receiving an increased supply of fatty acids in the form of triglyceride-rich lipoproteins. This suggestion, however, must remain tentative as we await further definitive data.

11. Conclusion

This review brings together indirect evidence for the participation of cytokines and lipid peroxides in the development of the hypertriglyceridaemia in, and the hepatic manifestations of pre-eclampsia (see Figure 24). Increased plasma cytokine (IL-1, TNF-α) concentrations resulting from activation of macrophages/neutrophils either directly or indirectly through endothelial activation, or the placenta itself, may enhance the peripheral lipolysis already activated in normal pregnancy by HPL. This, in association with cytokine mediated *de novo* hepatic fatty acid synthesis, results in an increased flux of free fatty acids to the liver. These are channelled predominantly into hepatic triglyceride synthesis and leads to an increased secretion (over and above that of normal pregnancy) of large, triglyceride-rich VLDL particles, which are also removed less efficiently than normal. Accumulation of free triglyceride occurs in the hepatocyte when there is saturation of this pathway. This triglyceride accumulation is

sufficient to explain the fatty changes in the liver seen in pre-eclampsia and AFLP. Futhermore, we have demonstrated that increased concentrations of triglyceride-rich lipoproteins in the circulation may contribute both directly and, through the generation of small, dense LDL, indirectly to endothelial dysfunction and therefore expression of pre-eclampsia in the mother, despite potentially benefiting fetal nutrition.

Several aspects of this proposed scheme for the pathogenesis of pre-eclampsia are readily testable and experiments are currently underway in both our laboratory and others. Furthermore, if confirmed, this mechanism immediately suggests novel therapeutic strategies for the management of this life-threatening condition. Manipulation of fatty acid metabolism perhaps through the administration of drugs that inhibit free fatty acid fluxes may have potential clinical use.

CHAPTER VIII

LIPID AND LIPOPROTEIN SUBFRACTION CONCENTRATIONS IN PREGNANCIES COMPLICATED BY IUGR

1. INTRODUCTION

Previous studies have shown that in pre-eclampsia, plasma lipids climb substantially above levels seen in normal pregnancies (Chapter V). It has been proposed that such lipid changes may play a role in the endothelial damage characteristic of pre-eclampsia (Chapters VI and VII). Pregnancies complicated by intrauterine growth restriction (IUGR) without pre-eclampsia have similar placental pathology to pre-eclampsia despite the absence of the maternal systemic manifestations of hypertension and proteinuria. In both conditions there is failure of trophoblast invasion of the maternal spiral arteries, vascular damage and placental infarction.

Given the physiological role of gestational hyperlipidaemia in supplying both cholesterol and triglyceride to the rapidly developing fetus, it is conceivable that pregnancies complicated by IUGR exhibit abnormal lipoprotein metabolism in an attempt to compensate for the placental insufficiency. Alternatively, abnormal lipoprotein metabolism may be a factor underlying poor fetal growth. However, it would appear from the literature that such studies are sparse.

The aim of the final study in this thesis, therefore, was to perform a cross-sectional study of lipid and lipoprotein concentrations in the third trimester from normal pregnancies and those complicated by IUGR without pre-eclampsia.

2. SUBJECTS, SAMPLES AND METHODS

Eight women with IUGR and 10 women with uncomplicated pregnancies were studied. The patient details are shown in Table 13. The study was approved by the Ethical Committee of Glasgow Royal Infirmary, and all women written gave informed consent.

	IUGR pregnancies n=8	Pregnant Controls n=10	P-Value
Age	26.3 (6.4)	29.5 (4.0)	0.46
Parity (Prim/parous)	4/4	2/8	0.16
Body Mass Index (Kg/m^2) @ booking	24.4 (5.3)	22.6 (2.6)	0.46
Gestational age (wks) @ sampling	33.4 (3.4)	34.7 (1.2)	0.33
Gestational age (wks) @ delivery	34.5 (3.2)	39.6 (1.3)	0.003
Birth weight (grams)	1765 (657)	3482 (279)	<0.001

Table 13 Characteristics of normotensive pregnant women and women with pregnancies complicated by IUGR without pre-eclampsia.
Values are given as mean (SD).

Mothers with IUGR as assessed by ultrasound and without clinical signs of pre-eclampsia were recruited (i.e. all had normal blood pressure and absence of proteinuria). IUGR was defined as estimated fetal weight <5th percentile for gestation and associated decreased liquor volume (oligohydrammnios). In addition, three of the eight patients had abnormal vascular blood flow on Doppler ultrasound. Patients with suspected genetic/anatomical anomalies likely to be contributory to reduced fetal growth were excluded. Consecutive patients eligible for this study, where obtaining a fasting blood sample was possible, were recruited and all those approached agreed to participate. All patients were healthy before pregnancy and had customary diet and were not receiving any medication known to interfere with lipid metabolism or lipid determination. Ten normal pregnant women matched as a group for pre-pregnancy weight, age, and gestational age served as controls. All had normal course and outcome of pregnancy, term delivery, and customary diet, and did not receive any medication known to interfere with lipid metabolism or lipid determination. Additionally, none of the patients or controls were phenotype apo E2/E2, an inherited trait known to generate disturbances in the plasma lipid profile even in normolipaemic subjects. Further, none of the patients or controls were in labour at the time of sampling.

All subjects were sampled after an overnight fast of at least 10 hours. Twenty mL of blood was collected by venepuncture into K_2EDTA (final concentration 1mg/mL) and lithium heparin tubes. Plasma was harvested at 4C by low speed centrifugation and

aliquots of plasma for lipid and lipoprotein measurements were used immediately. The assay methods are described in their entirety in Chapter II. Briefly, plasma total cholesterol, triglyceride, and HDL-cholesterol measurements were performed by a modification of the standard Lipid Research Clinics Protocol. $VLDL_1$, $VLDL_2$, IDL, and LDL were prepared and quantified as described earlier by cumulative flotation gradient ultracentrifugation. The cholesterol, triglyceride, free cholesterol, phospholipid, and proteins of the lipoprotein fractions were assayed and concentrations calculated as the sum of the components (expressed as mg/dL plasma).

Statistics

Data are presented as mean and standard deviation. Where appropriate differences were tested for statistical significance using Student unpaired t-test or the Chi square test (Minitab, CA, USA).

3. RESULTS

3.1 Lipid and lipoprotein concentrations

	IUGR n=8	Controls n=10	P-value
Cholesterol (mmol/l)	5.00 (1.23)	7.14 (1.01)	0.0016
Triglyceride (mmol/l)	2.19 (0.31)	2.59 (0.69)	0.13
HDL-Chol (mmol/l)	1.71 (0.40)	1.76 (0.36)	0.80
LDL-Chol (mmol/l)	2.28 (1.07)	4.20 (0.85)	0.0012
$VLDL_1$ (mg/dl)	94.9 (35.8)	105.8 (42.7)	0.57
$VLDL_2$ (mg/dl)	60.9 (18.1)	102.9 (36.2)	0.007
IDL (mg/dl)	58.8 (27.3)	116.5 (27.0)	<0.001
Total LDL (mg/dl)	183.4 (56.2)	368.1 (87.2)	<0.001

Table 14 Lipid and lipoprotein concentrations in women with IUGR and normal pregnancy controls.

Values are given as mean (SD).

Women with pregnancies complicated by IUGR had significantly lower median cholesterol, $VLDL_2$, IDL and LDL concentrations compared with control patients (Table 14 and Figure 25). There were no significant differences in concentrations of triglyceride, HDL and $VLDL_1$. The relative percent increments compared to non-pregnant values (as reported in Chapter III) in each of the parameters that exhibited differences were as follows: Cholesterol (10% vs 58%, IUGR patients vs pregnant controls, respectively, $VLDL_2$ (165% vs 347%), IDL (68% vs 252%), LDL (-11% vs 78%).

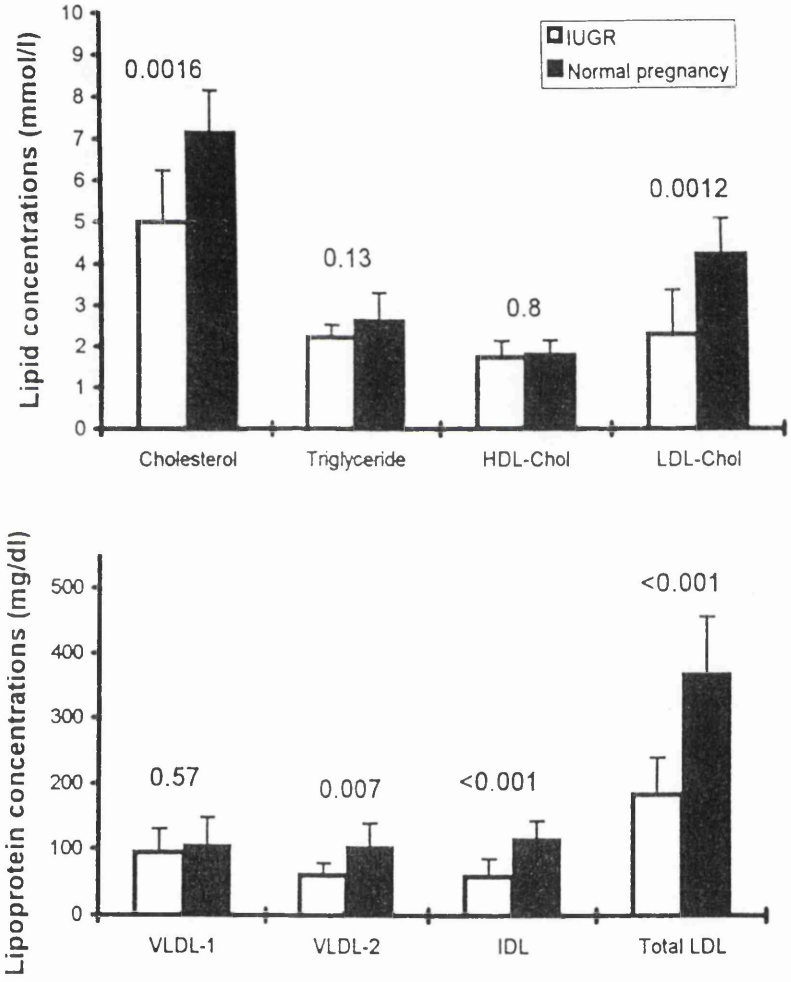


Figure 25. Lipid and lipoprotein concentrations in women with IUGR and normal pregnancy controls. Columns(bars) represent mean(SD).

3.2 Lipoprotein compositions

When the composition of these particles was determined (Table 15) the following was found: the cholesteryl-ester to triglyceride ratio (the lipid composition of the hydrophobic core of the particle) was reduced in $VLDL_2$ in IUGR. In addition, LDL particles were enriched in cholesteryl-ester and depleted in free cholesterol.

IUGR

Subfraction	Protein	Free chol	Chol esters	Triglyceride	Phospholipid
$VLDL_1$	7.2 (0.5)	3.6 (0.3)	8.64 (0.6)*	65.2 (1.0)	15.5 (0.4)
$VLDL_2$	12.4 (0.8)	4.7 (0.3)	19.1 (1.0)*	43.0 (1.2)	20.7 (0.6)
IDL	18.9 (1.1)	5.3 (0.5)	35.1 (1.4)	18.7 (1.0)	22.0 (0.7)
LDL	23.5 (0.7)	6.5 (0.7)*	40.8 (1.2)†	8.0 (0.8)	21.1 (0.6)

Controls

Lipoproteins	Protein	Free chol	Chol esters	Triglyceride	Phospholipid
$VLDL_1$	7.9 (0.2)	2.5 (0.3)	11.6 (1.0)	63.9 (1.1)	14.2 (0.3)
$VLDL_2$	13.4 (0.3)	5.4 (0.4)	22.8 (1.0)	39.4 (1.3)	19.1 (0.3)
IDL	18.6 (0.2)	6.5 (0.5)	35.7 (1.1)	18.9 (0.8)	20.3 (0.3)
LDL	24.2 (0.4)	10.3 (1.2)	35.7 (1.1)	9.6 (0.8)	20.2 (0.3)

Data are presented as mean (standard deviation) expressed as percent composition (g/100g); Chol esters = cholesteryl esters; Free chol = free cholesterol.
*$P<0.01$, †$P<0.001$ refer to the significance of difference between cases and controls as determined by Student unpaired t-test.

Table 15 Chemical Composition of Apolipoprotein-B Containing Lipoproteins

4. DISCUSSION

In this study, we set out to establish the pattern of lipid and lipoprotein concentrations in the third trimester in women with pregnancies complicated by IUGR. The most prominent finding in this study was of a significantly lower (p=0.0016) cholesterol concentrations in IUGR cases, principally the result of substantially lower (p=0.0012) LDL-cholesterol concentrations. In addition, $VLDL_2$ and IDL concentrations were also significantly lower in women with IUGR. In contrast, triglyceride, $VLDL_1$ concentrations, and HDL concentrations were similar to control population.

What are the potential mechanisms for this observation? Information on booking first term lipid levels was not available so it is possible that women destined to develop IUGR have lower starting cholesterol values. However, preliminary data from our laboratory which compared first trimester cholesterol levels in a group of women destined to have pregnancies complicated by IUGR [n=7; mean cholesterol 5.42 (1.42) mmol/l, birthweight 2232 (423) grams; mean (SD)] with those having normal pregnancies [n=794; mean cholesterol 5.5 (0.97) mmol/l; birthweight 3394 (552) grams] would suggest that this is not the case. Therefore, it would appear that in pregnancies complicated by IUGR there is a failure of an appropriate rise in LDL concentrations. This failure for LDL to rise could be the result of increased LDL catabolism and / or a reduction in synthesis. The observed significantly lower $VLDL_2$ and IDL concentrations, the synthetic precursors to LDL in the circulation, would imply a failure of appropriate LDL synthesis. We may speculate that in order to maintain energy supply (most efficiently delivered via triglycerides) to the growing fetus, triglyceride synthesis (in the form of $VLDL_1$) is maintained at the expense of cholesterol in the form of $VLDL_2$, IDL and in particular LDL. As LDL is required to supply cholesterol to the placenta for hormonal synthesis, lower LDL levels could contribute to the lower oestrogen levels seen in pregnancies complicated by IUGR.

Whatever the mechanism, the results of this study demonstrate that LDL levels which normally increase by around 70% in uncomplicated pregnancies (Chapter III), fail to rise appropriately in pregnancies complicated by IUGR, and as a result may play a role in the pathogenesis of growth retardation. Finally, if our results are confirmed in larger studies and longitudinal investigations, then serial LDL-cholesterol measurements (when LDL-cholesterol fails to rise appropriately) may be of use in identifying mothers with IUGR pregnancies.

CHAPTER IX

CONCLUSIONS

1. INTRODUCTION

At the time the thesis was initiated, it was a novel thought that unique changes in lipoprotein metabolism might be central to the pathophysiology of pre-eclampsia rather than an epiphenomonen. We drew on our knowledge of other situations, particularly the hyperlipidaemic changes that lead to accelerated atherosclerosis, to postulate that similar mechanisms may be in operation in pre-eclampsia. Therefore, the primary objectives of the thesis were:

i) to establish mechanisms underlying the physiological hyperlipidaemia of normal pregnancies and the pathological hyperlipidaemia of pre-eclampsia.

ii) to determine the consequences of the altered lipoprotein metabolism in pre-eclampsia for the promotion of the characteristic endothelial dysfunction present in this disorder.

iii) to establish the relationship between lipid changes and haemostatic factors during normal gestation.

iv) to determine lipid and lipoprotein concentrations in pregnancies complicated by IUGR, a condition in which the placental appearance is similar to that seen in pre-eclampsia but where the maternal systemic problems of hypertension and proteinuria are absent.

The first part of this project entailed a detailed longitudinal examination of plasma lipoprotein subfraction concentrations and compositions in normal pregnancy. Plasma lipids and lipoprotein subfractions were quantified in 10 normal pregnant women at 5 weekly intervals from 10 to 35 weeks of gestation, together with circulating hepatic lipase and serum oestradiol concentrations. Concentrations of $VLDL_1$, $VLDL_2$ and IDL increased in parallel as plasma triglyceride increased with advancing gestation, and LDL mass increased by 70%. Thus it appears that pregnancy promotes hypersecretion of $VLDL_1$ and $VLDL_2$ particles to provide for both maternal and feto-placental triglyceride and cholesterol needs.

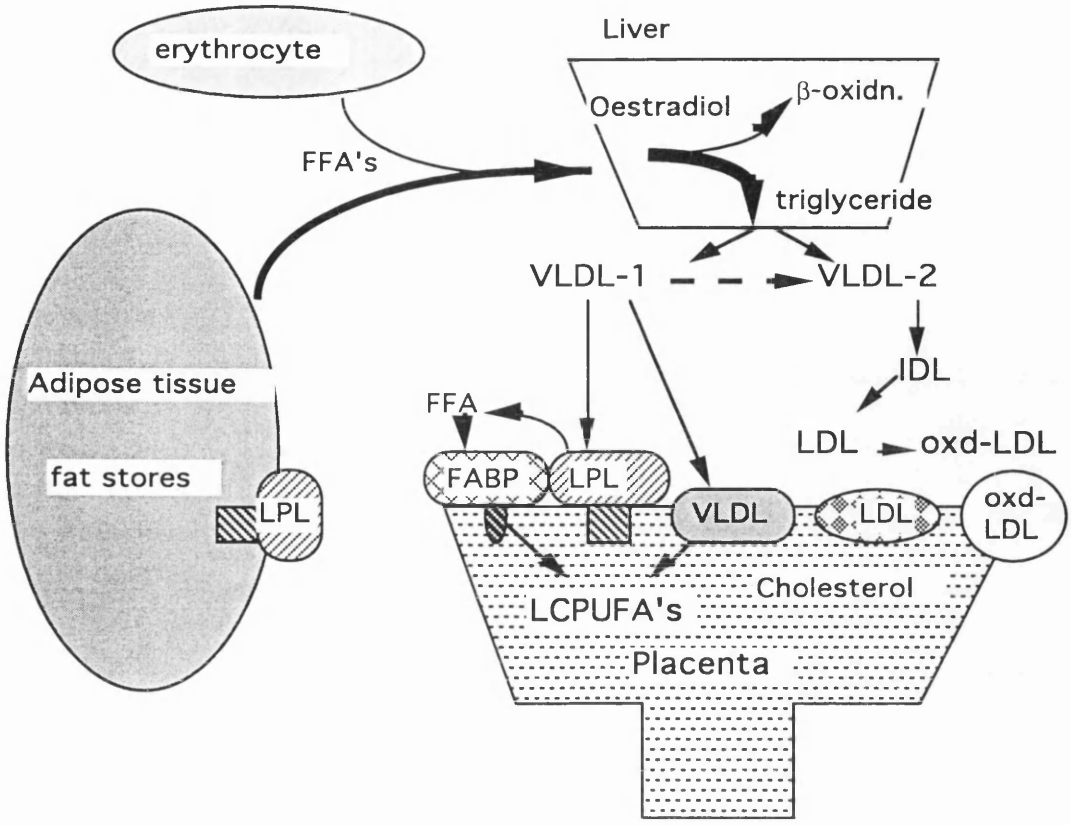


Figure 26 Pregnancy, partly via increased lipolysis and oestrogen induced changes, promotes hypersecretion of $VLDL_1$ and $VLDL_2$ particles to provide for both maternal and feto-placental triglyceride and cholesterol needs and as a result, plasma $VLDL_1$ and $VLDL_2$, IDL and LDL rise together. The placenta expresses several different receptors and enzymes which act in concert to facilitate uptake of circulating lipids.

In addition, in six of the 10 women studied, the LDL subfraction pattern was modified towards a smaller denser pattern in a manner indicative of a "threshold" transition. The concept of a threshold was of critical importance to our understanding of this important aspect of lipoprotein physiology. This "threshold" transition, if it occurred, did so at varying gestational ages and triglyceride concentrations for different women [Chapter III].

Second, as part of our longitudinal assessment of lipid changes during normal pregnancy, we took the opportunity to measure also the circulating concentrations of coagulation and fibrinolytic markers and relate these to changes in plasma lipids and oestradiol [Chapter IV]. The data suggest that gestation associated increments in PAI activity, FVIIc, t-PA antigen and D-dimers, factors associated with vascular disease in the non-pregnant, may be linked to changes in the circulating concentration of triglyceride and oestradiol. However, a notable finding in that study was the inter-individual variability in increments in coagulation and fibrinolytic variables with elevations in oestradiol and triglycerides during pregnancy, suggesting the possibility of gene-environmental interactions. Clearly, further studies of individual changes in hormones, lipids and haemostatic variables during pregnancy may offer insights into the regulation of coagulation and fibrinolysis, and their relationships to atherothrombosis.

Next, plasma concentrations of VLDL, and LDL subfractions and pre-heparin hepatic lipase activity were compared in women with pre-eclampsia and healthy, age, gestational age and weight-matched controls [Chapter V]. Women with pre-eclampsia exhibited higher median plasma triglyceride,$VLDL_1$ and $VLDL_2$ concentrations, whereas total plasma cholesterol, IDL and total LDL concentrations were the same in cases and controls. Furthermore, women with pre-eclampsia demonstrated markedly elevated median plasma concentrations of small, dense LDL, LDL-III. Consistent with studies in non-pregnant populations, the concentration of small, dense LDL correlated positively with plasma triglyceride concentration. Since, both $VLDL_1$ and small, dense LDL-III are damaging to the endothelium, we postulated that elevated concentrations of these lipoprotein species may contribute to the expression of pre-eclampsia.

Fourth, we have related lipid, specifically apoAI levels, to endothelial behaviour in myometrial vessels bathed in plasma from normal and pre-eclamptic pregnancies [Chapter VI]. These results are consistent with data from studies in non-pregnant individuals where HDL cholesterol has been positively and strongly related to endothelial function. This relationship may reflect a direct effect of HDL particles since they are antioxidative and cardioprotective. Alternatively, rather than a direct influence of Apo AI on endothelial behaviour, some other factor(s) dictating Apo AI metabolism and thus Apo AI concentrations may relate to endothelial behaviour. In particular, the efficiency of triglyceride catabolism which in the non-pregnant situation, is a major determinant of Apo AI and HDL-cholesterol concentrations, may be involved.

In Chapter VII, a mechanistic scheme for the generation of the hyperlipidaemia in pre-eclampsia was devised. It was suggested that increased plasma cytokine (IL-1, TNF-

α) concentrations resulting from activation of macrophages/neutrophils either directly or indirectly through endothelial activation, or the placenta itself, may enhance the peripheral lipolysis already activated in normal pregnancy by HPL. This, in association with cytokine mediated *de novo* hepatic fatty acid synthesis, would then result in an increased flux of free fatty acids to the liver. These are channelled predominantly into hepatic triglyceride synthesis and lead to an increased secretion (over and above that of normal pregnancy) of large, triglyceride-rich VLDL particles, which are also removed less efficiently than normal. Accumulation of free triglyceride occurs in the hepatocyte when there is saturation of this pathway. This triglyceride accumulation is sufficient to explain the fatty changes in the liver seen in pre-eclampsia and AFLP, which as previously discussed may be a variant of the pre-eclampsia process. Furthermore, it was suggested that increased concentrations of triglyceride-rich lipoproteins in the circulation may contribute both directly and, through the generation of small, dense LDL, indirectly to endothelial dysfunction and therefore expression of pre-eclampsia in the mother, despite potentially benefiting fetal nutrition.

Finally, we examined lipid changes in pregnancies complicated by IUGR [Chapter VIII]. The results of this study demonstrated that LDL levels which normally increase by around 70% in uncomplicated pregnancies (Chapter III), fail to rise appropriately in pregnancies complicated by IUGR. The mechanisms for this observation are unclear but clearly, given the importance of LDL particles in delivering cholesterol to the placenta for hormonal synthesis and for transport to the fetus, such low levels may contribute to poor fetal growth. In addition, these data suggest that LDL-cholesterol measurements may be useful in identifying mothers with IUGR pregnancies.

In conclusion, the results of our novel studies have given deeper insight into the unique features associated with the hyperlipidaemia of normal pregnancy, particularly with reference to lipoprotein subfraction concentrations. Some of this information is clearly relevant to our understanding of the factors which govern generation of small, dense LDL in the non-pregnant population, and atherosclerosis. Furthermore, data presented in this thesis suggest that in some situations (pre-eclampsia and IUGR) mechanisms regulating this physiologic hyperlipidaemia may malfunction, with potential ramifications for the clinical expression or diagnosis of these conditions.

2. FUTURE WORK

Clearly, novel data have been generated from work performed in this thesis. However, the results have also suggested many more lines of investigation. In this final section, therefore, I will detail potential future studies which would help yield deeper insights into the conditions discussed.

2.1 Kinetic studies

In order to establish more precisely whether the gestational hyperlipidaemia is due predominantly to increased synthesis of lipoproteins or their reduced catabolism, kinetic (stable isotope) studies are necessary. The detailed methodology has already been established in the laboratory. Basically, the rates of $VLDL_1$ and $VLDL_2$ synthesis and catabolism are measured by endogenous labelling of their primary protein component, apoB, with a non-radioactive amino acid tracer, trideuterated leucine (D_3-leucine). This tracer is administered through an intravenous catheter as a short constant infusion which is 'primed' by a small bolus. D_3-leucine is incorporated by the liver into apoB, which is assembled into VLDL and secreted into the circulation. Blood samples are obtained from the subject at frequently selected time points during and after the administration of the tracer. From these samples $VLDL_1$ and $VLDL_2$ is prepared and the amount of D_3-leucine incorporated determined by GC-MS. The change in isotopic-enrichment within the individual apoB-containing lipoprotein over time, provides a measure of the metabolism of that lipoprotein.

Kinetic studies could also be performed in women with pre-eclampsia and IUGR to confirm our belief that the former condition is characterised predominately by an increase in synthesis of $VLDL_1$ whereas the latter condition is characterised by a breakdown of the $VLDL_2$ to IDL to LDL synthetic chain.

2.2 Adipocyte lipolysis

From the mechanistic scheme presented in Chapter VI, it appears that enhanced adipocyte lipolysis may be pivotal in the chain of events leading to the metabolic perturbances and thus pathophysiology of pre-eclampsia. The factor(s) responsible for stimulating adipocyte lipolysis is unknown but could be of placenta origin. Using established laboratory techniques and in collaboration with Professor C.Redman's group in Oxford (who have isolated syncytiotrophoblast villous membranes in the maternal plasma of women with pre-eclampsia) it would be interesting to examine the potential of these placental factors to stimulate adipocyte lipolysis.

2.3 Lipoprotein lipase assays

Another possible mechanism for the exaggerated hyperlipidaemia of pre eclampsia is reduced VLDL catabolism secondary to reduced adipose / skeletal tissue LPL

activities. LPL activity may be reduced in these tissues to facilitate 'targeting' of lipids towards fetus. No such data in the literature are evident and assays for tissue LPL measurements are readily available.

2.4 Long term health of women with history of pregnancies complicated by pre-eclampsia / IUGR

Women who develop pre-eclampsia appear to have a predisposition towards central obesity and insulin resistance, these features being important to the development of the exaggerated hyperlipidaemia seen in that condition. As a result, such women may be at heightened risk of cardiovascular disease in later life. In contrast, women who develop IUGR tend to have low weights at pregnancy onset. Although, these women exhibit similar placental morphology to that seen in pre-eclampsia, their low weights (and by association low abdominal adipose tissue stores) may protect them from the systemic manifestations of hypertension and proteinuria. Data on the long term health of these women are sparse. Therefore, it would be interesting to characterise the metabolic profiles in groups of women with a history of pre-eclampsia or IUGR relative to those with a history of uncomplicated pregnancies.

2.5 IUGR and lipids

In Chapter VIII it was demonstrated that LDL levels which normally increase by around 70% in uncomplicated pregnancies (Chapter III), fail to rise appropriately in pregnancies complicated by IUGR, and this has potential implications for the pathogenesis of growth restriction. Larger studies are needed to confirm our preliminary observations. Furthermore, longitudinal studies would be useful.

2.6 Importance of future work

The importance of these disorders is not simply in relation to pregnancy as it is clear that disturbances in fetal growth and development have long term implications for adult health, particularly, with regard to metabolic and vascular disease. Thus, maternal programming of the fetus may occur with possible transmission of metabolic characteristics such as insulin-resistance. Furthermore there are implications for the mother, with the potential of increased metabolic conditions and vascular events in later life. Thus the purpose of the proposed programme of work is to elaborate the mechanism of increased lipids and lipoproteins in normal pregnancy and pre-eclampsia and how this influences the plasma lipoprotein concentrations in these situations and other disorders associated with metabolic dysfunction in pregnancy including gestational diabetes, insulin-dependent diabetes and IUGR. In addition, the consequences of the dyslipidaemia required to be studied to understand the relationship between these metabolic disturbances and abnormalities in endothelial function. Finally, it is important to determine the effects of the changes in lipids and lipoproteins associated with pregnancy and its complications on maternal health by way of long term follow-up studies.

GLOSSARY

AFLP	Acute fatty liver of pregnancy
ALP	Atherogenic lipoprotein profile
Apo	Apolipoprotein
BMI	Body mass index
BP	Blood pressure
CETP	Cholesteryl ester transfer protein
CHD	Coronary heart disease
EDRF	Endothelial derived relaxing factor
FABP	Fatty acid binding protein
FFAs	Free fatty acids
FVII	Factor VII
HDL	High density lipoprotein
HELLP	Haemolysis, Elevated Liver enzymes, Low Platelet count
HL	Hepatic lipase
HPL	Human placental lactogen
HSL	Hormone sensitive lipase
IBR	Individualised birth ratio
IDL	Intermediate density lipoprotein
IUGR	Intrauterine growth restriction
Lp (a)	Lipoprotein (a)
LpL	Lipoprotein lipase
LCAT	Lecithin cholesterol acyl transferase
LDL	Low density lipoprotein
MAP	Mean arterial pressure
PAI	Plasminogen activator inhibitor
TNF	Tumor necrosis factor
tPA	Tissue plasminogen activator
VLDL	Very low density lipoprotein
vWF	von Willebrand Factor

APPENDIX 1

Manufacturers and Suppliers of Reagents, Hardware and Software

Baker Instruments Ltd
Rushman Park Whitehall Lane
Egham, Surrey
TW20 9NW, UK

Beckman Instruments (UK) Ltd Analytical Sales and Service Operation
Progress Road, Sands Industrial Estate
High Wyncombe, Bucks
HP12 4JL, UK

BDH Laboratory Supplies
McQuilkin & Co
21 Polmadie Avenue, Glasgow
G5 OBB, UK

Bio-Rad Laboratories
2000 Alfred Nobel Drive
Hercules
CA 94547, USA

Boehringer Mannheim UK (Diagnostics and Biochemicals) Ltd
Bell Lane
Lewes, East Sussex
BN71LG, UK

Chromacol Ltd
3 Little Mundells, WGC
Herts,
AL7 1EW, UK

Fisons Instruments
Crewe Road
Wythemshaw, Manchester
M23 9B, UK

Genzyme Biochemicals
50 Gibson Drive, Kings Hill
West Malling, Kent
ME19 6HG, UK

VA Howe & Company Ltd
Beaumont Close
Banbury
OX16 7RG, UK

Imed
San Diego
CA 92131-1192, USA

Innogenetics NV
Canadastraat 21-Haven 1009
B-2070 Zwijndrecht
Belgium

Isotec Inc
A Matheson USA Company
3858 Benner Road, Miamisburg
OH 16801-3008, USA

Nycomed Pharma AS
Oslo, Norway

Orion Diagnostica
Espoo, Finland

Packard Instrument Company
Canberra Packard Ltd
Brook House, 14 Station Road
Pangbourne, Berks
RG8 7DT, UK

Sigma Chemical Company
Fancy Road
Poole, Dorset
BH17 7TG, UK

Appendix 2

PREGLIP STUDY

LONGITUDINAL SUBFRACTION CHANGES

RECORD FILE FOR

Patient's Name: _____

Tick when completed:

- Demographics ☐
- Consent Form ☐
- Screening Results ☐
- Inclusion Criteria ☐
- Exclusion Criteria ☐
- Sampling Protocol ☐
- Results ☐

PREGLIP STUDY : DEMOGRAPHIC DETAILS

Date of Assessment: _____

Patient's Name:

Forename: _____

Surname: _____

Date of Birth: _____

Address: _____

_____

Post Code: _____

Telephone No: _____

General Practitioner: _____

Consultant: _____

Booking Weight: _____ kg **Height (without shoes):** _____

Estimated Date of Delivery: _____ **LMP:** _____

Outcome: _____

Fetal Weight: _____

PREGLIP STUDY : CONSENT FORM

GREATER GLASGOW HEALTH BOARD

GLASGOW ROYAL INFIRMARY UNIT - RESEARCH ETHICS COMMITTEE

FORM OF CONSENT FOR PATIENTS IN CLINICAL RESEARCH PROJECT

Brief Title of Project:

Assessment of Lipoprotein Metabolism in Normal Pregnancy
(lipoprotein subfraction changes)

Patient's Summary (Purpose of study, nature of procedure, discomfort and possible risks in terms which the patient can understand):

I understand that Professors Greer and Shepherd and their colleagues are investigating in detail changes in blood fat and cholesterol levels in normal pregnancy. My participation would involve the donation of a blood sample at 8, 12, 16, 20, 24, 28, 32, 36 and possibly 40 weeks of pregnancy. Giving a blood sample can be associated with minor discomfort.

The study may be of little or no benefit to me directly but the results may be of help to others in the future.

Participants can withdraw from the project at any time and this will not affect my regular care and treatment.

CONSENT

I, (Name) _____

of (Address) _____

_____

agree to take part in the Research Project Study Programme described above.

Dr/Mr _____ **has explained to me what I have to do,**

how it might affect me and the purpose of the Research Project/Study Programme.

Signed: _____ **Date:** _____

Witness: _____ **Date:** _____

PREGLIP STUDY : SCREENING RESULTS

BP

Systolic	
Diastolic	

Urea and Electrolyte

Na^+	
K^+	
Cl^-	
HCO_3	
Urea	
Cr	

Liver Function

Bil	
ALB	
AST	
ALT	
LDH	
CPK	

Blood Glucose

GLU	

URATE	

Full Blood Count

Haematocrit	
Haemoglobin	
RBC	
WBC	
Platelets	

Lipids

Total Plasma Triglyceride	
Total Plasma Cholesterol	
Apo E Phenotype	

PREGLIP STUDY : INCLUSION/EXCLUSION CRITERIA

INCLUSION CRITERIA

	YES	NO
Gestational age between 8-12 weeks	☐	☐
Aged between 18 and 35 years	☐	☐
Previous uncomplicated full-term pregnancy (ie, multigravida)	☐	☐
No significant risk factors for current pregnancy as determined by detailed obstetric assessment	☐	☐
Non-smoker	☐	☐
Singleton pregnancy	☐	☐
Fasting plasma triglyceride < 2 mmol/l and fasting plasma cholesterol between 3 and 6.5 at booking visit	☐	☐
Patients who have given their written informed consent to participate in the study	☐	☐

All answers must be 'YES' for the patient to be eligible for study entry

EXCLUSION CRITERIA

	YES	NO
Patients with significant renal disease as evidenced by serum urea's and serum creatinine levels greater than twice the upper limits of normal	☐	☐
Patients with significant hepatic disease as evidenced by serum transaminases or serum bilirubin greater than twice the upper limit of normal	☐	☐
Patients with endocrine disease which may influence lipid metabolism, ie thyroid disease diabetes mellitus (defined as fasting blood glucose > 8 mmol/l)	☐	☐
Patients with hypertension (diastolic BP > 90 mmHg).	☐	☐
ApoE phenotype E_2/E_2	☐	☐
Previous gestational diabetes or pregnancy-induced hypertension	☐	☐
Patients taking any medication known to interfere with lipoprotein levels	☐	☐

All the answers must be 'NO' for the patient to be eligible for the study

PREGLIP STUDY : SAMPLING PROTOCOL

FOR EACH DATE:

Fasting sample (25 ml) should be taken into **2 x 10 ml** K_2 EDTA tubes (20 ml) and 1 x 5 ml Li/Hep (3 ml) and 1 x 5 ml plain tube (3 ml):

PREGLIP STUDY - LSC
Name of patient and date

Note: The 5 ml Li/Hep sample must be placed on ice immediately.

The sample should be accompanied by a form marked:

PREGLIP STUDY - LSC
FOR THE ATTENTION OF DR NAVEED SATTAR
DEPT OF BIOCHEMISTRY, GLASGOW ROYAL INFIRMARY

and sent to the Department of Biochemistry, GRI

Gestational Age	Time Window = EDD±1 Weeks	Date Taken	Actual Gest. Age	Tick	Name of Sender
10 Weeks					
14 Weeks					
18 Weeks					
22 Weeks					
26 Weeks					
30 Weeks					
38 Weeks					
36 Weeks					
EDD					

PREGLIP STUDY : RESULTS

10 WEEK GESTATION **Time Window for Sample using EDD:** _____

DATE OF SAMPLE: _____

Obstetric Progress:

NAME OF PATIENT: _____

RESULTS

TG	
Chol	
VLDL	
LDL	
HDL	

HORMONES

Oestradiol	
Progesterone	
ß-HCG	
SHBG	
Glucose	

SUBFRACTIONS

VLDL-I	
VLDL-II	
IDL	
LDL	

LDL	
LDL-I	
LDL-II	
LDL-III	

HDL_2	
HDL_3	

$\mu mol\ FA\ Min^{-1}\ Hr^{-1}$

Hepatic Lipase	
Lipoprotein Lipase	
Lp(a)	
CETP	
LCAT	

INSULIN	

Appendix 3

PREGLIP STUDY

PRE-ECLAMPSIA

RECORD FILE FOR

Patient's Name: _____

Tick when completed:

Demographics	☐
Consent Form	☐
Screening Results	☐
Inclusion Criteria	☐
Exclusion Criteria	☐
Sampling Protocol	☐
Results	☐

PREGLIP STUDY : DEMOGRAPHIC DETAILS

Date of Assessment: _____

Patient's Name:

Forename: _____

Surname: _____

Date of Birth: _____

Address: _____

_____

Post Code: _____

Telephone No: _____

General Practitioner: _____

Consultant: _____

Booking Weight: _____ kg **Height (without shoes):** _____

Estimated Date of Delivery: _____

Outcome: _____

Fetal Weight: _____

PREGLIP STUDY : CONSENT FORM

GREATER GLASGOW HEALTH BOARD

GLASGOW ROYAL INFIRMARY UNIT - RESEARCH ETHICS COMMITTEE

FORM OF CONSENT FOR PATIENTS IN CLINICAL RESEARCH PROJECT

Brief Title of Project:

Assessment of Lipoprotein Metabolism in Normal Pregnancy and Pre-Eclampsia (Pre-eclampsia)

Patient's Summary (Purpose of study, nature of procedure, discomfort and possible risks in terms which the patient can understand):

I understand that Professors Greer and Shepherd and their colleagues are investigating in detail blood fat level changes that may be linked to the onset of high blood pressure in pregnancy. My participation would involve the donation of a blood sample. Giving a blood sample can be associated with minor discomfort.

The study may be of little or no benefit to me directly but the results may be of help to others in the future.

Participants can withdraw from the project at any time and this will not affect my regular care and treatment.

CONSENT

I, (Name) _____

of (Address) _____

_____

agree to take part in the Research Project Study Programme described above.

Dr/Mr _____ **has explained to me what I have to do, how it might affect me and the purpose of the Research Project/Study Programme.**

Signed: _____ **Date:** _____

Witness: _____ **Date:** _____

PREGLIP STUDY : SCREENING RESULTS

BP

	Date 1	Date2
Systolic		
Diastolic		

Urine Protein

Dipstix	
24 hour	

Urea and Electrolyte

Na^+	
K^+	
Cl^-	
HCO_3	
Urea	
Cr	

Liver Function

Bil	
ALB	
AST	
ALT	
LDH	
CPK	

Blood Glucose

GLU	

URATE	

Full Blood Count

Haematocrit	
Haemoglobin	
RBC	
WBC	
Platelets	

Lipids

Total Plasma Triglyceride	
Total Plasma Cholesterol	
Apo E Phenotype	

Drug History

Drug Prescribed	**Dose**	**No. Taken**

PREGLIP STUDY : INCLUSION/EXCLUSION CRITERIA

INCLUSION

	YES	NO
Age between 18-35 years	☐	☐
Diastolic BP > 110 mmHg on one reading or exceeding 90 mmHg on repeated readings	☐	☐
Proteinuria > 0.3 g/24 hours or ≥ 2+ on dipstick testing in absence of infection or renal disease	☐	☐
Pre-pregnancy weight between 55-70 Kg	☐	☐
Non-smoker	☐	☐
Singleton pregnancy	☐	☐
Healthy before onset of pre-eclampsia (ie, normal obstetric and medical history)	☐	☐
Have given their written informed consent to participate in the study	☐	☐

All answers must be 'YES' for the patient to be eligible for study entry

EXCLUSION CRITERIA

	YES	NO
Patients with significant renal disease as evidenced by serum ureas and serum creatinine levels greater than twice the upper limits of normal	☐	☐
Patients with significant hepatic disease as evidenced by serum transaminases or serum bilirubin greater than twice the upper limit of normal	☐	☐
Patients with endocrine disease which may influence lipid metabolism, ie thyroid disease diabetes mellitus (defined as fasting blood glucose > 8 mmol/l)	☐	☐
ApoE phenytope E_2/E2	☐	☐
Patients taking any medication known to interfere with lipoprotein levels for longer than 48 hours	☐	☐
Evidence of gestational diabetes/impaired glucose tolerance (fasting glucose > 8.0 mmol/l)	☐	☐

All the answers must be 'NO' for the patient to be eligible for the study

PREGLIP STUDY : SAMPLING PROTOCOL

PRE-ECLAMPSIA

NAME OF PATIENT: _____

Ideally following the day of admission, a 25 ml blood sample taken after an overnight fast into:-

2 x 10 ml K_2 EDTA - 20 ml
1 x 5 ml K_2 EDTA - 2 ml
1 x 5 ml Li/Hep - 3 ml

Note: The two 5 ml tubes must be placed on ice immediately

and labelled:

PREGLIP STUDY - PET
NAME OF PATIENT AND DATE

The sample should be accompanied by a form marked:-

PREGLIP STUDY - PET
FOR THE ATTENTION OF DR NAVEED SATTAR
DEPARTMENT OF BIOCHEMISTRY, GLASGOW ROYAL INFIRMARY

and sent to the Department of Biochemistry, GRI, promptly

Note: All samples require separation and storage within two hours of collection

Sample	Date Taken	Tick	By Whom

PREGLIP STUDY : RESULTS

PRE-ECLAMPSIA

DATE OF SAMPLE: _____

NAME OF PATIENT: _____

TG	
CHOL	
VLDL	
LDL	
HDL	

	Concn	Composition				
		Free Cholesterol	Cholesterol Ester	Trigs	Phos	Protein
$VLDL_1$						
$VLDL_2$						

LDL	
LDL-I	
LDL-II	
LDL-III	

Susceptibility to Copper Oxidation

Inhibition Period	
FDR-IP	
FDR-PP	

	Plasma	**Total LDL**
Baseline MDA		

	Pre Incubation	**Post Incubation**
NEFA's		

HL	
LPL	
CETP	
LCAT	

LP(a)	
INSULIN	

Lipid Perox (Dumfries)	

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