Apolipoprotein E Variants, Plasma Lipids, Lipoproteins and DysβLipoproteinaemia during pregnancy in Zimbabwean women.

Thesis submitted by Donald Moshen Tanyanyiwa in fulfillment of the requirements of Part 111 of the degree Master of Medicine in Pathology.

DECLARATION

I Donald Moshen Tanyanyiwa, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work or any part of it is being, or is to be submitted for another degree in this or any other university.

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Signed : _____

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Date : _____

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ABBREVIATIONS

AP	Angina pectoris
Apo	Apolipoprotein- (proteins found in association with plasma lipids in lipoproteins)
ApoA-1	Apolipoprotein A-1
ACAT	Acyl-cholesterol acyltransferase
BA	Bile acid
BS	Bile salts
CAD/CHD	Coronary artery disease/Coronary heart disease (IHD)
CDC	Centre for Disease Control
CE	Cholesterol ester (cholesterol esterified to a fatty acid at the alcohol moiety on position 3.
Chol	Cholesterol
CETP	Cholesterol ester transfer protein
CM	Chylomicrons
dysβ	dysbetalipoproteinaemia
EDTA.	Ethylenediaminetetraacetic acid
FC	Free cholesterol
FDB-B100	Familial defective binding apoB100
FFA	Free Fatty Acid
FH	Familial hypercholesterolaemia.
GGE	Gradient Gel Electrophoresis

HDL	High density lipoprotein.
HL	Hepatic Lipase
IDL	Intermediate density lipoprotein.
IHD	Ischaemic Heart Disease
LCAT	Lecithin cholesterol acyl-transferase. .
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LRP	Low density lipoprotein Related Protein
LP	Lipoprotein.
LPL	Lipoprotein lipase.
Lp(a)	Lipoprotein (a) (pronounced lipoprotein little "a")
LpX	Lipoprotein X.
MI	Myocardial Infaction.
NEFA	Non Esterified Fatty Acid
PL	Phospholipids
TG (TAG)	Triglyceride better known as triacylglycerol.
MUFA	Mono-unsaturated fatty acid is a fatty acid with one double bond.
PUFA	Poly-unsaturated fatty acid has more than one double bond.
VLDL	Very low-density lipoprotein
xma	Xanthomas. The abnormal accumulation of lipids in macroscopically recognisable lesions in the skin and tendons characterised by foam cells on histological examination.

CHAPTER 1

1.1 Introduction

Pregnancy, a physiological and temporary phenomenon in women in their reproductive years, induces changes in lipoprotein metabolism. These changes include mild hypertriglyceridaemia, hypercholesterolaemia and alterations in LDL particle size. The overproduction of VLDL by the liver causes the hypertriglyceridaemia and results in remodelling of LDL to smaller, denser particles. When there is a hindrance of clearance of remnants of the TG-rich lipoproteins or of lipolysis by genetic defects in apoE and LPL, dysβ and severe hypertriglyceridaemia may be induced during pregnancy.

Dysβ also known as broad β disease or type III hyperlipidaemia is a highly atherogenic dyslipoproteinaemia. Premature or accelerated atherosclerosis occurs in one third to more than one half of individuals with type III hyperlipoproteinaemia. Peripheral vascular disease involving the lower extremities is almost as common as coronary artery disease in Dysβ. This is different from the distribution of vascular disease seen in familial hypercholesterolaemia in which there is less involvement of the lower extremities. Although the mechanism underlying the predisposition for atherosclerosis of peripheral vessels in type III is unknown, it is worth noting that certain cholesterol-fed animals with high levels of β-VLDL have a higher incidence of peripheral vascular disease than of coronary atherosclerosis. Morganroth *et al,*

1.2 This study of pregnant women in Zimbabwe therefore set itself the following aims:

i. To describe lipid and lipoproteins during and after pregnancy,

ii. To examine the prevalence of apoE variants,

iii. To evaluate dysβlipoproteinaemia in pregnancy,

iv. The correlation between dysβlipoproteinaemia and the apoE genotypes.

This is the first study to systematically examine lipids and lipoproteins during pregnancy in black Africans. The notion that coronary heart disease (CHD) is not at epidemic proportions in developing countries has hampered progress in this area of research [Gomo]. A poorly developed and poorly supported academic infrastructure has also detracted from comprehensive surveys of Dyslipidaemia in Africa.

CHAPTER 2

2.1 Biochemistry of Apolipoprotein E

Apolipoprotein E is a 299 amino acid peptide with a molecular weight of 34 kilo Daltons. The gene for Apo E is found in chromosome 19 and is 3.7-kilo bases in length and contains 4 exons. It is normally present in Serum at approximately 3-5 mg per decilitre. Approximately three quarters of the plasma apoE is synthesized in the liver, in the hepatic parenchymal cells where it is incorporated into VLDL. Other organs, including the brain, lungs, spleen, adrenals, ovaries, kidneys, muscle cells and macrophages also produce small amounts of apoE.

The secondary structure can be divided into three main areas. An amino terminal end made up of 165 residues is highly ordered, the next 35 residues make up a random structure and the carboxyl terminal becomes highly ordered again. The Carboxyl terminal forms the strongest lipid binding area. The majority of the secondary structure, about 62% is formed from alpha helices which are amphipathic and important in lipid binding. While also providing a more hydrophilic aspect to the water environment, the rest of the secondary structure is made up of beta sheets (9%), beta turns (11%) and random structure (18%) [Mahley 1988].

The five arginine and three lysine residues between 140 and 160 are essential for binding to the LDL receptor. This is important for the cellular uptake of lipoproteins.

2.2 Apo E isoforms.

ApoE was first isolated from plasma in 1973 and was originally known as arginine-rich apolipoprotein. As previously stated, there are three common isoforms of apolipoproteinE designated E2, E3 and E4 according to their charges in isoelectric focusing.

The main differences between the isoforms are at the amino acid residues 112 and 158. ApoE2 has cysteine at both of these residues and it has the lowest affinity for the LDLR: with less than 2% of normal receptor binding activity. Apo E3 has a cysteine at residue 112 and arginine at 158, and has a much better receptor binding activity. ApoE4 has arginine at both these residues and shows 100% normal receptor binding activity and hence it has a very rapid clearance from HDL, VLDL and Chylomicrons. [Mahley 1988] Apo E3 and APO E4 both have normal LDLR binding activity as the R at 158 forms a salt bridge with aspartate at 154 leaving the series of positively charged R and K between 130 and 148 often to interact with the negatively charged area on the LDLR.

	E2/2	E3/3	E4/4
Relative Charge	0	+1	+2
Residue 112	Cysteine	Cysteine	Arginine
Residue 158	Cysteine	Arginine	Arginine

Table 2.1 Locations of amino acid residues and their charge on different apolipoproteins.

Studies of apoE and ε allelic frequency among various populations around the world have revealed interesting ethnic differences and demonstrated that the apoE genotype has a major effect on plasma lipid levels and possibly cardiovascular risk. In almost all populations studied, apo-3/3 phenotype is by far the most common (typically 50-70% of the population) and the ε3 allele makes up a large majority of the apoE gene pool (typically 70-80% of the population. The six common genotypes comprise three homozygous states (E-4/4; E-3/3 and E-2/2) and three heterozygous (E-4/3; E-3/2 and E-4/2) states. The minor sialylated isoforms are designated with subscripts for example E-4s or E-3s depending on the isoform that has been modified. The high frequency of ε3 allele lead to the conclusion that it was the wild type, but the occurrence of ε4 allele in nearly all animal species makes it the ancestral allele.

2.3 Apo E nomenclature

Electrophoretic studies of apoE revealed that it is composed of numerous isoprotein components [Utermann; Clinical Genetics 1977, Utermann; Lipid Research 1977, Pagan, Warnick; Clin Chem 1979, p279-284, Weidman]. The complexity of apoE results from both genetic polymorphism and post-translational modifications with carbohydrate chains containing sialic acid. Current genetic studies have shown the existence of three alleles at two loci that specify six different apoE phenotypes [Zannis, Biochemistry 1981]. These phenotypes can be recognized on a two-dimensional gel electrophoresis and one-dimensional isoelectric focusing [Weisgraber, Utermann, Human Genetics 1982].

The original work based on one-dimensional isoelectric focusing resulted in a nomenclature system [Utermann, Clinical Genetics 1977 and Nature 1977] whereas the later work with two-dimensional polyacrylamide electrophoresis resulted in a different nomenclature [Zannis, Biochemistry 1981 and Human Genetics 1981]. In order to avoid confusion a uniform system that describes the apoE isoproteins, alleles, genotypes and phenotypes is currently used. The apoE alleles are ε4, ε3 and ε2 while the major asialo apoE isoproteins seen in plasma by two-dimensional gel electrophoresis are designated apoE4, apoE3 and apoE2 respectively. ApoE4 is the most basic while apoE2 is the most acidic isoprotein. The minor plasma apoE isoproteins that can be eliminated by treatment with neuraminidase are collectively designated apoEs. Thus the sialo apoE isoproteins of apoE4, apoE3 and apoE2 are designated apoE4s, apoE3s and apoE2s respectively. Distinction between sialo apoE isoproteins can be achieved by adding a number after the subscript as apoE3s1, apoE3s2 and apoE3s3.

2.4 Binding Properties

The heparin-binding domain between 131 and 150 amino acids of apoE is responsible for the high affinity binding to the LDL and LRP receptors.

Receptor binding is due to the ionic interaction between basic amino acid residues in this region and acidic amino acid residues of the LDL receptor. The LDL receptor possesses seven repeated segments that include critical acidic amino acids aspartate and glutamate near the N-terminus representing the ligand-binding site. The LRP contains 31 domains, homologous to the ligand binding sites of LDL receptor.

[Brown] Both residues 112 and 158 are outside the binding region of apoE. The binding affinity can not be directly linked to residue 112, but residue 158 can have an influence on the binding affinity depending on the salt bridge it forms with other amino acids. The basic amino acids in the 131 – 150 region are largely solvent exposed extending away from the backbone of the helix, forming a basic field of charge that may be available to interact with the receptor. The backbone structures of ApoE 2 and Apo E 3 are essentially identical; however there are local changes in the region of the residue 158. In Apo E3 there is a salt bridge between arginine 158 and aspartate 154. In Apo E2, which has a neutral amino acid cysteine rather than the arginine residue at 158, that salt bridge cannot form and the aspartate interacts instead with arginine 150, forming a new salt bridge. This interaction swings the side chain of arginine 150 into a new plane outside the receptor-binding region and disrupts receptor binding because arginine is part of the receptor-binding region. Therefore, the substitution at residue 158 of Apo E2 appears to have a secondary effect on the receptor-binding domain of Apo E, affecting binding indirectly. [Lalazar] All autosomal dominant ApoE mutations cause defective LDL receptor binding, but their LDL receptor binding activities are higher than those of autosomal recessive Apo E2 mutations. The Apo E2 variant with <2% of normal receptor binding activity has the most defective LDL receptor binding and is expected to have reduced remnant metabolism but this is not the case as it is the rare ApoE variants which have 20 – 50% of normal receptor binding activity that are paradoxically associated with the dominant mode of inheritance and the invariable

presence of hyperlipidaemia. Which means the LDL receptor binding activity cannot alone explain the mechanism of producing dysβ. (See remnant metabolism)

2.5 Apo E Mutations

The apo E-2/2 genetic status is associated with dysβ. These subjects clear remnants slowly but most persons with impairment of remnant clearance on this basis will not develop hyperlipidaemia because the production rate of remnants and their clearance rates are balanced. In most subjects dysβ is associated with homozygosity for apoE2 as the permissive state for derangement but an additional 'hit'is still required. Increased production rates are typical of diabetes and reduced clearance is typical of hypothyroidism. Other associations between the environmental factors and the recessive mutation include massive dietary fat or alcohol intake, renal disease, menopause and obesity. Certain mutations are known to confer dysβ in an autosomal dominant fashion, albeit with delayed penetrance because additional stresses still seem necessary to confer the phenotype. The autosomal dominant inheritance of dysβ occurs in several variants of apoE (see table below). These mutations are thought to result in the reduction or abolition of the positive charge on the apoE-binding domain leading to the accumulation of remnants due to reduced binding affinity. In the autosomal dominant cases, the presence of a single variant allele is ideally sufficient for phenotypic presentation.

Table 2.2 below shows apolipoprotein E variants associated with Type 111 Hyperlipidaemia and the parameters modulating expression of Hyperlipidaemia

Mutation*	Mode of Inheritance	β-VLDL present	LDL receptor binding•	Heparin binding defect-	Lipase processing Defect
158Arg→Cys	Recessive	Yes	2%	No	Yes (HL)
136Arg→Ser	Unknown	Yes	40%	__	__
142Arg→Cys	Dominant	Yes	20%	Yes	__
145Arg→Cys	Dominant	Yes	45%	Yes	__
146Lys→Gln	Dominant	Yes	40%	__	Yes (LPL)
146Lys→Glu	Dominant	Yes	<5%	Yes	__
7aa**insertion.	Dominant	Yes	25%	Yes	No

Table 2.2 [Mahley, Metabolic and Molecular Basis of Inherited Disease, Seventh Edition]

Key:

****Lists changes compared to Apoe3 structure (eg 158 arginine→cysteine, arginine at residue 158 changed to cysteine at that site.***

****Presented a %age of ApoE3 binding***

*****7 amino acids duplication of residue 121 to 127***

__indicates not yet determined

The mutations affect the metabolic properties of apolipoprotein E differently. Some mutations produce defects in the LDL receptor binding; others in heparin binding and others may also produce a lipase-processing defect.

It has previously been reported from Cape Town that apo E R145C is particularly prevalent amongst subjects with dysβ. The apoE R145C was especially over-represented in the black population that was predominantly of Xhosa descent. Rohlmann *et al* supported by Veniant *et al* demonstrated that LDL receptor

mutation resulting in reduced binding affinity for the remnant does not seem to produce phenotypic dysβ. They further demonstrated that apolipoprotein E deficiency in the presence of LDL receptors produces worse dysβ than in LDL receptor deficiency or absence [Véniant]. This means that null or any mutation in the LDL receptor is not likely to produce any phenotypic dysβ.

2.6 **ApoE and Alzheimer's disease (AD)**

Increasing interest and research in AD and its association with apolipoproteinE warrants a brief discussion of what is currently known. The association between apoE4/4 and Alzheimer's disease has been reported in several studies but the pathophysiology has not been clearly defined. Lahiri *et al* (Neurobiol Aging. 2004 May-Jun; 25(5): 651-60.) reported that ApoE mRNA and protein are found predominantly in astrocytes within the CNS. There is also a high expression of ApoE mRNA in the brains of people with sporadic AD. ApoE acts as a cholesterol transporter in the brain. Cholesterol controls amyloid production and deposition by regulating beta-secretase. Das *et al* Rev Neurosci. 1996 Oct-Dec; 7(4): 277-83 reported that hyperphosphorylation of "tau" has been indicated for the generation of neurofibrillary tangles in the brains of Alzheimer's patients. Since apoE4 does not bind to "tau", apoE4 may contribute to the hyperphosphorylation of "tau" which may cause the formation of neurofibrillary tangles in AD patients. Ohm *et al* Biochem Soc Symp. 2001; (67): 121-9 have reported that apart from age, the APOE epsilon 4 allele represents the most important risk factor in sporadic Alzheimer's disease (AD). Compared to APOE epsilon 3 homozygotes, the histopathological onset of

tau pathology is found 1-2 decades earlier but progresses with the same speed. ApoE dose-dependently and specifically increases free intraneuronal calcium levels in the order ApoE4 > ApoE3 > ApoE2. This effect is amplified in the presence of beta A4-peptide. The ApoE effects on calcium are not affected by the blockade of action potentials with tetrodotoxin, or by inhibition of common ApoE binding sites. The calcium channel involved has been identified as a P/Q-type-like channel. The production of ApoE in astrocytes is controlled by several receptor/effector systems such as adrenoceptors and cAMP. In the presence of beta A4-peptide fragments, astrocytes stop their synthesis of ApoE resulting in a massive reduction in the bioavailability of ApoE. In the periphery, ApoE directs cholesterol transport and thereby influences its cellular concentrations. In neurons, changes in the concentration of cholesterol influence the phosphorylation status of the protein tau.

CHAPTER 3

3.1 General Lipid and Lipoprotein metabolism.

In order to appreciate the remnant metabolism and the effects of pregnancy on lipid and lipoprotein metabolism, general lipoprotein metabolic pathways may be considered in three broad divisions: exogenous lipid metabolism, endogenous lipid metabolism and reverse cholesterol transport. Special consideration will be given to remnant clearance in this discussion.

3.2 Exogenous pathways

This pathway essentially deals with the dietary acquired lipids, which operates in the post absorptive stage lasting from one to five hours after a meal. Dietary fat is mainly TG. Lipases hydrolyse lipids; lipase secreted by the tongue and soft palate and gastric mucosa can hydrolyse up to 30% of fats in the stomach [Henderson]. These may have a significant role when there is pancreatic dysfunction as they do not require bile salts (BS) and are active at gastric pH [Henderson]. The presence of lipids in the small intestines stimulates the release of CCK (Cholecystokinin), an enzyme from the upper part of the duodenum, which activates gall bladder contraction, resulting in the release of bile salts and the simultaneous release of pancreatic digestive enzymes. Secretin, a hormone highly concentrated in the upper duodenum but found throughout the small intestine, causes the release of a secretion that is rich in bicarbonate. This neutralises the acidic contents coming from the stomach [Henderson]. Cholesterol esters are hydrolysed by cholesterol esterase – phospholipids are hydrolysed by phospholipase A2.

The digestive products form micelles with the aid of bile salts. The micelles convey the non-polar lipid molecules from the lumen of the gut to the epithelial cell surface. A high concentration of monoglycerides and fatty acids adjacent to the enterocytes facilitates absorption. Absorption is facilitated by fatty acid binding protein in the cytosol of the cell that has a high affinity for fatty acids. Within the enterocyte, fatty acids are incorporated into TG, which, together with phospholipids, cholesterol and cholesterol esters and specific apolipoproteins (apoB-48 and apoA-1) are assembled into spherical chylomicrons. These are released by exocytosis into the intestinal lacteals. [Holmes] The apolipoprotein B-48 synthesised by the intestinal mucosal cells is the permanent structural protein of a chylomicron. The lacteals drain into the cystern chyli and thence to the thoracic duct. Chylomicrons finally enter the subclavian vein for systemic circulation. Up to this stage no information is available regarding the effects of pregnancy on the handling of lipids. Absorption of dietary fatty acids is usually complete whereas absorption of total gut cholesterol (biliary and dietary) is variable. Once in the systemic circulation, chylomicrons donate A-I to HDL in exchange of apoC-II and apo-E. ApoC-II, now present on the surface of chylomicrons activates lipoprotein lipase situated on the capillary endothelial surfaces. LPL hydrolyses TG to yield monoacylglycerol and free fatty acids. The fatty acids released into circulation associate with albumin to return to the liver if not taken up and can be taken up by organs such as muscle (energy source) or adipocytes (storage). During chylomicron circulation (5 to 30 minutes), they rapidly deliver TG to peripheral tissues. No chylomicrons should be present in

the plasma of a fasting subject six to eight hours after a meal. The metabolism of TG rapidly depletes the core of the chylomicron particle, reducing its size and increasing its density. Cholesterol ester from HDL or LDL may be incorporated into the chylomicrons by the action of CETP (Choleterol Ester Transfer Protein) in exchange for TG. These changes result in loss of affinity for apoC lipoprotein which partitions to HDL. The chylomicron remnant contains less TG and much more cholesterol ester than the original chylomicrons, possibly explaining the highly atherogenic nature of these LP. Only ApoB 48 and Apo E remain on the chylomicron remnants and the latter serves as ligand for the hepatic receptors. The chylomicron remnants are internalised through the LDL and LRP receptors by endocytosis. The components of the chylomicrons are hydrolysed in the lysosomes and the cholesterol released from the hepatic lysosomes can enter pathways for the formation of bile acids, be secreted into the bile as such, be incorporated in nascent lipoproteins or esterified with long fatty acids and can down-regulate HMG CoA-reductase, the rate limiting enzyme of cholesterol biosynthesis.

3.3 Endogenous Pathway

This pathway essentially deals with the export of TG and some cholesterol from the liver. Hepatocytes have the ability to synthesise TG from carbohydrates and fatty acids. The hepatocyte also regulates the de novo synthesis of cholesterol, when dietary cholesterol acquired from the receptor-mediated uptake of chylomicron remnants is insufficient, hepatocytes increase the activity of HMG CoA reductase together with LDLR. Cholesterol and TG are packaged into secretory vesicles in the

Golgi apparatus, transported by exocytosis into the extracellular space and introduced into the circulation through the fenestrae of the hepatic sinusoidal endothelium in the form of nascent VLDL. The composition is 60% TG, 20% cholesterol and 5% protein. The proteins comprise apoB 100, apoA, apoCII and apoE. Additionally apoCII is transferred after secretion, from circulating HDL. As in chylomicron metabolism, the apoCII on VLDL activates LPL during its circulation and LPL metabolises TG. The released free fatty acids bind to albumin and are delivered to peripheral tissues. The apoCII is transferred back to HDL. The VLDL particles are thus converted to VLDL remnants, some of which are taken up by the liver. These remnants may become smaller and denser particles that may now have the density of IDL. The rate of hydrolysis of VLDL TG is slower than that of chylomicron TG, possibly due to the smaller size of the average VLDL particle, which can bind fewer lipoprotein lipase molecules than the larger chylomicron particle. The normal residence time for chylomicrons in blood is 5 to 10 minutes whereas for VLDL TG it is 15 to 60 minutes. Lipoproteins containing apoE (such as chylomicron and VLDL remnants) and apoB (such as LDL) are cleared by the LDLR. Additionally, LRP can clear apoE-containing lipoproteins. However the apoE-containing lipoproteins are cleared more efficiently than apoB-containing lipoproteins. For this reason, chylomicron and VLDL remnants (IDL) are not measurable in normal individuals because of their rapid clearance. Apo-B 100 in VLDL is similar to that for LDL but cannot bind LDLR owing to inappropriate

conformation for receptors. VLDL, like LDL relies on the apo-E to for its clearance [Rifai].

3.4 Reverse Pathway

This deals with the transport of cholesterol in cells from the peripheral tissues to the liver. HDL is produced in the liver, the gut and from the chylomicron surface membrane liberated during chylomicron metabolism. Nascent HDL is a phospholipid disc with no lipid core but during its circulation, it acquires cholesterol either from VLDL and LDL. LCAT, which esterifies the surface free cholesterol provides neutral lipid that forms the core. When the surface cholesterol is esterified, it enters the nonpolar core of HDL thus, leaving the surface of HDL free to acquire more cholesterol from lipoproteins and cell membranes. The size of HDL particles increases as cholesterol esters accumulate (HDL2). The liver, through a putative HDL receptor delivering cholesterol, clears some of the HDL2. The exact mechanism for HDL up-take from circulation to the liver is not clear. However some of the cholesterol esters are transferred from HDL2 by CETP resulting in the formation of the smaller HDL (HDL3) and some is transferred to chylomicrons and VLDL. McCarthy JJ *et al* Journal of Medical Genetics 2003; 40:453-458 reported that the scavenger receptor class B type 1(SR-B1), highly expressed in the liver is a key component in the reverse cholesterol transport pathway.

3.5 Remnant Lipoprotein Clearance/Metabolism Pathways

Until the early work carried out by Hui and associates, and supported by Hers *et al* EMBO J; 1988: (7) 4119-4127, the LDL receptor was thought to be the only one

responsible for the Lipoprotein remnants clearance. Apo-E is postulated to be important not only for remnant metabolism, but could also be rate limiting for remnant clearance. Plasma clearance of intestinally derived remnant lipoprotein by the liver involves several steps that can be grouped into; sequestration, processing and internalisation [Mahley 1991].

Sequestration

Chylomicron and VLDL remnants pass through the fenestrae of hepatic sinusoidal endothelial cells into the space of Disse. The fenestration acts as a dynamic bio-filter that restricts the entry of large chylomicrons while allowing the smaller remnants to enter [Fraser]. The size of the fenestrae can change with age, possibly contributing to the emergence of dysβ in pre-disposed individuals. Although there is no specific information on the effect of ageing on the fenestration in women, it is likely to remain normal in reproductive years. The space of Disse contains heparan sulfate proteoglycans (HSPG) on the microvillous projections of the hepatocytes. There is also an abundance of apoE and HL, which are synthesised and secreted by hepatocytes. LPL is also present presumably carried into the space of Disse on the remnants [Hamilton, Stow, Sanan]. Molecules taken up by the liver and secreted from the hepatocytes traverse this space. The initial step in the clearance of remnants from plasma is their sequestration into the space of Disse where they bind to proteoglycans as well as lipoprotein receptors such as LRP. The heparin binding domains of apoE are important for this step.

Processing

The processing of remnants will result in their uptake by one of the three-uptake pathways. Apo-E binds avidly to heparin and specifically to heparan sulfate of hepatic origin [Mahley 1991] and its abundance suggests that apoE enrichment of remnant particles occurring within the liver be of physiological importance in the clearance of remnants. This step also involves lipases on the sinusoidal endothelial cells or within the space of Disse. Hepatic lipase is a heparin binding lipase produced by the liver. Treatment of chylomicrons with hepatic lipase results in an accelerated rate of clearance of the remnants by the rat liver. Sultan et al demonstrated that inhibition of hepatic lipase by intravenous injection of specific antiserum retards the clearance of chylomicron remnants in rats. LPL which normally resides in the extrahepatic endothelial surfaces attached to cell-surface proteoglycans, appears to attach to the chylomicrons and to be transported into the liver, where it enhances the binding of the remnants to the LRP. In-vitro studies have shown that the lipases serve as ligands for the HSPG-LRP pathway. [Hui]

Internalisation/Uptake

Three major pathways are thought to be important in remnant lipoprotein uptake by hepatocytes. Firstly LDL receptors can mediate the direct uptake of remnant lipoproteins. [Choi, Ishibashi] Secondly, the HSPG-LRP pathway can mediate uptake either by transfer of the remnant from the HSPG to the LRP for internalisation or by binding of the remnant lipoproteins to HSPG forming a tertiary complex with the LRP that is then internalised. The HSPG are critical for the

HSPG-LRP pathway because in their absence, the remnants do not bind and are not taken up to a major extent by the LRP on the hepatocytes. Thirdly, the HSPG alone can mediate remnant lipoprotein uptake [Mahley 1994, Mahley 1996 and Ji, 1993]. Among others, in-vitro laboratory studies by Robert Mahley and Zhong-Sheng revealed that HSPG participates in the binding and uptake of apoE-enriched β-VLDL. Treatment of a variety of cells with heparinase, removing the sulfated glycosaminoglycan side chains from the proteoglycans, significantly inhibits the binding and uptake of the apoE enriched remnant lipoproteins. Heparinase decreased remnant binding by 80% in normal human fibroblasts, and by 90% in FH fibroblasts with the LDL receptor null mutation [Ji, 1993]. These findings were confirmed when hemizygous and homozygous LRP-null fibroblasts from fetal mice were examined. [Ji, 1997 and Herz 1995]

The concept that has evolved is a secretion-capture model: that apoE secreted into the space of Disse from hepatocytes becomes associated with the remnant lipoproteins and thus the apo-E enriched remnants are sequestrated into the space of Disse by interacting with HSPG. This interaction directs the lipoproteins to the LRP for internalisation. LPL on the surface of the remnants may also direct the remnants to the LRP and mediate their interaction with the receptor. The LDL receptor remnant uptake is independent from both the HSPG and the LRP. The decline in the activity of both LPL and HL during gestation [Kinnunen, Sattar 1999] will have a direct effect on remnant metabolism, resulting in the dysbetalipoproteinaemic picture due to the reduced remnant clearance in a predisposed individual. After

remnants are endocytosed, further catabolism occurs in the lysosomes with the various remnant components entering various metabolic pathways.

3.6a Lipid Metabolism in Pregnancy

During Pregnancy maternal metabolism must satisfy not only the usual demands of the mother but also the adaptive changes of gestation as well the demands of the developing foetus. Early pregnancy is considered the anabolic phase, characterised by increased hepatic production of TG and enhanced removal of TG from the circulation, resulting in an increased deposition of fat in maternal adipose tissue. In contrast, late pregnancy is referred to as the catabolic phase; the release of free fatty acids from adipocytes is enhanced due to both relative insulin resistance and stimulation of hormone-sensitive lipase by placental hormones. These metabolic changes allow the metabolism of the gravid female to store energy in early pregnancy to meet the energy requirements of late gestation. [Stock]

As a consequence, the maternal lipid metabolism is specifically altered during pregnancy. Plasma cholesterol and phospholipids increase moderately, whereas plasma TG levels rise markedly [Boyd, Peters]. Increased amounts of TG are not only found in the very low-density lipoprotein (VLDL) fraction, but in all lipoprotein fractions (LDL and HDL) during late gestation [Montelongo, Knopp]. Two mechanisms specific for pregnancy seem to be responsible for this phenomenon. First, elevated oestrogen levels during gestation result in increased hepatic synthesis of TG-rich VLDL [Julius, Walsh]. Secondly, removal of lipoprotein TG is reduced due to low activities of lipoprotein lipase (LPL) and

hepatic TG lipase (HL), the effect being more striking for HL than for LPL [Kinnunen, Sattar]. The abundance of VLDL-TG drives an accelerated transfer of TG to lipoproteins of higher density by the cholesteryl ester transfer protein (CETP). Thus the reduced HL activity appears to be responsible for the shift of HDL subclasses toward larger, TG rich and more buoyant species in late gestation. During gestation, LDL particles become enriched in TG as well, but in contrast to HDL particles, LDL particles have been reported to become smaller and denser. [Silliman 1994, Hubel] LDL particles are heterogeneous with regard to their chemical and physical properties. Using non-naturing gradient gel electrophoresis, Austin *et al,* demonstrated that two patterns of LDL subclass distribution, A and B, could be distinguished. Pattern A is characterised by a predominance of LDL particles that are large and buoyant, and pattern B is characterised by a predominance of small, dense LDL particles. The larger, more buoyant subclasses of LDL predominate in healthy females of reproductive age, whereas smaller, denser LDL often occurs after menopause [MacNamara]. Compared with large and buoyant LDL, small dense LDL particles are more susceptible to oxidation, show increased binding to proteoglycans of the vessel wall, and exhibit reduced uptake by the LDL receptor. [Abner]. In men and non-pregnant women, plasma TG account for 40 – 60% of the variability in small, dense LDL concentrations. Several studies have shown an association between elevated plasma TG concentrations, small dense LDL (pattern B) and decreased HDL cholesterol, in particular HDL2 cholesterol [Austin 1990]. This metabolic situation is referred to as the atherogenic lipoprotein

phenotype. Thus elevated TG and the accumulation of small, dense LDL during pregnancy are thought to increase the risk of endothelial damage despite the fact that there is a preponderance of large buoyant HDL in late gestation [Alvarez, Silliman]. Elevated TG present in the first trimester may be responsible for the increase in the LDL seen in the early stages of pregnancy. However with advancing gestation, there is no further increase in the dense LDL, but there is an increase in buoyant lipoproteins VLDL, IDL and

LDL-1 a pattern resembling HL deficiency. HL is reported to be responsible for the conversion of IDL to LDL [Demant] in chylomicron remnant catabolism [Daggy, Shafi] and in HDL metabolism [Kuusi, Blades]. Impaired HL activity results in elevated HDL2-cholesterol and enrichment of LDL and HDL particles with TG [Ishibashi]. The prolonged circulation of TG-rich LDL is probably responsible for the smaller size despite the decrease in HL and LPL activity. Alvarez *et al* demonstrated a progressive decrease in post heparin HL activity after the first trimester throughout pregnancy. This decrease was significantly correlated with the changes in the HDL subclasses. [Alvarez] In addition HL activity was negatively correlated with oestradiol levels as demonstrated in other studies where oestrogen concentrations are associated with decreased HL activity [Applebaum-Bowden]. The significant increase in apoC-III, an inhibitor of LPL may also contribute to the impairment of maternal lipolysis, thereby increasing the residence time of TG-rich lipoproteins. The prolonged exposure of these lipoproteins to CETP rather than elevated activity of CETP is therefore is responsible for the observed

compositional changes when VLDL and IDL become enriched in CE, whereas LDL and HDL particles become enriched in TG. Sattar *et al* found that, in mothers with intrauterine growth retardation, the appropriate synthesis of LDL precursors, namely VLDL and IDL, fails to occur in the third trimester, suggesting that in late gestation the welfare of the foetus depends on an adequate supply of lipids. [Sattar 1997] Free fatty acids are transferred across the placenta by simple diffusion, but the capacity of this transport is limited. Human placenta expresses lipoprotein receptors in high amounts, with the binding of VLDL to placental membrane exceeding that of LDL, suggesting that the placenta is primarily endowed with receptors that preferentially bind to VLDL [Naoum]. Northern blot analysis of the placenta revealed a 2.6 fold increase in VLDL/apoE receptor messenger RNA between the first trimester and delivery [Wittmaack]. Thus, in the catabolic phase of pregnancy, the effect of placental hormones is to enhance VLDL production and decrease HL activity. Coupled with the increased expression of the VLDL/apoE receptor in the placenta, this may result in a coordinated re-routing of the TG-rich lipoproteins from the mother to the feto-placental unit. In conclusion, current evidence does not support the idea that the same mechanisms as those described for the atherogenic lipoprotein phenotype govern lipid metabolism in late pregnancy.

3.6b Lipoprotein Lipase Activity in Pregnancy

Lipoprotein lipase, gastric lipase, hormone sensitive lipase, acid lipase and pancreatic lipase are members of a multigene family. LPL is synthesised in parenchyma cells in a variety of tissues, especially striated muscle. From these cells,

it is transported to the endothelial surface of blood capillaries where it is bound to the heparan sulphate. Heparan sulphate is a glycosaminoglycan, which is degraded by lysosomal enzymes. LPL is required for hydrolysis of TG in chylomicrons and VLDL and the conversion of chylomicrons to chylomicron remnants with apoC-II as a cofactor. During gestation, the removal of lipoprotein TG is reduced resulting in the increase in VLDL which is coincidental with a decrease in the LPL activity and hepatic TG lipase (HL), the effect being more striking for HL than LPL [Kinnunen, Sattar 1999] It is the LPL activity and variability in different tissues that presents an interesting discussion. The plasma enzyme activity in late gestation was measured in pregnant women 10 minutes after administration of 50IU/kg intravenous heparin. There was a decrease in activity, which is much more than just a dilutional effect.

Table 3.1 below shows plasma VLDL lipids and post heparin LPL activity in pregnant women during the first and third trimester.

	First Trimester	Third Trimester	P value
VLDL-TG (mg/dl)	31.0 ± 5.1	112.8 ±16.5	<0.001
VLDL-cholesterol (mg/dl)	5.22 ± 0.85	21.92 ± 3.13	<0.001
VLDL-Trig/VLDL-Chol (mg/dl)	5.83 ± 0.23	5.28 ± 0.35	NS
Post heparin LPL activity (nkat/ml)	424 ± 51	71 ± 16	<0.001

Table 3.1 N=12 women per group. P= significance of the difference between third and first trimesters of gestation. [Herrera]

The activity of LPL has been shown to be variable (decreases in the adipose tissue and the liver but increased in the mammary and placental tissues).

This plays an important role in the fate of circulating TG, which is diverted from uptake by adipose tissue to uptake by the mammary glands for milk synthesis and also hydrolysis by the placental LPL to release and transfer non-esterified fatty acids to the foetus. The high level of LPL activity in the adipose tissue compared to other tissues under non-pregnant conditions, and the reduction in activity during pregnancy must produce a diminished clearance of circulating TG-rich lipoproteins. The above variables were demonstrated by measuring the post heparin enzyme activity in different pregnant rat tissues that were rapidly excised and stored in liquid nitrogen. [Nilsson-Ehle]

3.6c Literature review on lipid metabolism in pregnancy

Considerable research has been undertaken to investigate lipids and lipoprotein changes in pregnancy. These studies dating as far back as 1912 by Herman and Neumann are still continuing with improved technology and better understanding [Gardner JA, Warth]. More detailed studies have unveiled the changes in Lipoproteins during the different trimesters. Whilst the current study was primarily for dysβ during pregnancy, these changes need to be viewed in the context of the other reported lipoprotein changes during pregnancy.

Peters, Heinemann and Maine in 1950, looked at the serum lipids in pregnancy. They concluded that the serum lipids of 34 normal women and women with medically treated hyperthyroidism, may decline slightly in the early weeks of pregnancy. After twelve weeks serum lipids rise progressively, until delivery. Thereafter, they decline at variable rate. Total and free cholesterol and

phospholipids, participate proportionally in the hyperlipidaemia of pregnancy, maintaining their normal relations to one another. Neutral fat however rises proportionally far more than the other lipid fractions and declines more rapidly after delivery [Peters]. Mazurkiewick *et al* investigated the effects of pregnancy on serum lipid, lipoprotein and apolipoprotein concentrations. A comparison of women in the second trimester to their age matched non-pregnant counterparts revealed that there were significant increases in total cholesterol, TG, LDL cholesterol, HDL cholesterol and apolipoproteins AI, AII and B during the second half of pregnancy. Contrary to the longitudinal study by Desoye *et al* no significant differences were noted in apolipoprotein (a). Desoye *et al* reported a positive correlation between changes in the lipid and lipoprotein concentrations and the changes in the concentrations of the pregnancy hormones oestradiol, progesterone and human placental lactogen. However TG was also positively correlated with increasing concentrations of insulin in the second half of gestation [Desoye]

Knopp *et al*, Bergelin, Wahl, and Walden, examined lipoprotein lipid concentrations and lipoprotein electrophoresis in 553 Caucasian women at 36 weeks gestation, 64 of these at six weeks post partum, and groups of comparably- aged non-pregnant women. They reported that TG increases in the lipoprotein fraction are proportional to the total TG rise. The majority of the cholesterol rise occurred in low density lipoprotein (LDL) which are increased by 49% in pregnant women, 26% in postpartum women and 7% in hormone treated women compared to control subjects. Median high-density lipoprotein (HDL) cholesterol is elevated by 23% in

pregnancy and 12% postpartum but was 45% lower in hormone users, compared to control subjects. With respect to electrophoretic characteristics, chylomicronaeamia is slightly more prevalent in pregnant and postpartum women by 2.4% and 1.6% respectively, compared to either hormone users or non-users. Floating beta bands were also found more frequently in pregnant women by 1.1% and postpartum women by 1.6%, compared to either hormone users or non-users. Sinking-pre beta lipoprotein (lipoprotein (a)) is less common in pregnant women (4.9%) than in postpartum women (12.5%), hormone users (13.9%) and non-hormone users (18%) [Knopp].

Desoyer *et al* obtained data concerning plasma lipids, apolipoproteins and lipoproteins measured longitudinally during pregnancy and postpartum. They analyzed hormonal influences using pregnancy as a model of altered lipid metabolism. [Schwertner] The subjects were 42 pregnant women and 24 non-pregnant women as controls. The hormone studied was chosen according to their established effects on plasma lipid levels oestradiol (E2), progesterone (PG), insulin and due to their specific occurrence during pregnancy (hCG and human placental lactogen (hPL). They concluded that by time series analysis, hPL, E2 and PG affect lipid concentrations during gestation. Most of the existing data regarding the effects of hormones on lipid metabolism was derived from the effects of oral contraceptives and exogenous steroid hormone administration in postmenopausal women. Oestrogen replacement therapy was believed to be associated with low rates of coronary heart disease in postmenopausal women [Psaty, Grodstein]. Campos *et al*

studied the effect of 17β-oestradiol on apolipoprotein B-100 metabolism in eight healthy postmenopausal women. After an overnight fast, apoB in VLDL, IDL and LDL subclasses was endogenously labeled with a non-radioactive isotope D3L-Leucine. Lipoprotein measurements were done on all the samples. For light LDL, oestrogen increased the mean fractional catabolic rate by 63%, whereas the production rate increased by a lesser amount (42%). These metabolic changes reduced light LDL cholesterol and apoB concentrations by 26% and 19% respectively. In contrast the dense LDL cholesterol and apoB concentrations were unchanged by the intervention as both the apoB fractional catabolic rate and production rate were significantly increased by similar amounts 39% and 38% respectively. Oestrogen increased the production rate of light VLDL by 64%. It exclusively affected the production rate of the fast compartment of light VLDL particles, representing 76%-78% of light VLDL particle mass, while the production rate of slow, light VLDL was unchanged. In contrast the IDL production was not significantly affected. Pregnancy can therefore be accepted to have at least part of its hypertriglyceridaemia explained by overproduction of TG-rich VLDL. van Stiphout *et al* investigated the association between pregnancy and serum lipids in a cohort of 831 Dutch women initially aged 5 to 19 years whom they followed up yearly from 1975 to 1985. At the final examination, women who had been pregnant showed lower HDL cholesterol levels than those who had never been pregnant. The difference was most marked in users of oral contraceptives. These observations suggested that serum total and HDL cholesterol levels were elevated during

pregnancy probably because of hormonal changes. In addition it raised the probability of a lowering effect of parity on HDL cholesterol. This finding may help to explain the reported positive association between parity and the occurrence of cardiovascular disease. [van Stiphout]

Lars Fahraeus et al carried out a prospective study in 19 healthy women starting before conception and ending up to eight weeks after delivery. Plasma lipoprotein fractions were determined before conception and every six to eight weeks during pregnancy and eight weeks after delivery [Fahraeus 1982]. The elevation of serum lipids during human pregnancy has been documented in numerous reports, [Fahraeus, Knopp 1981] but few longitudinal studies of the alterations of the different lipoprotein fractions during various stages of gestation have been carried out. In the study LDL concentration was decreased in the eighth week, which was in agreement with the reduced cholesterol and phospholipids found in early pregnancy [Knopp 1981, Darmady]. Oestrogen might be responsible for the reduced LDL, as exogenous oestrogen has been shown to reduce LDL and as oestrogen deficiency in menopause has been associated with a rise in LDL. [Fahraeus 1985] LDL started to increase in mid and late pregnancy. HDL was elevated in the 14th week and showed maximum rise of 41% in the 28th week of pregnancy that was due to an increase of HDL2. VLDL-TG showed a continuous increase from week 14, reaching three times pre-pregnancy levels in week 36. During lactation, eight weeks after delivery, the LDL remained elevated whereas the other lipoproteins had returned to pre-pregnancy levels. Siliman *et al* carried out a study to determine

whether the hyperlipidaemia associated with late pregnancy was associated with changes in HDL subclass distribution and neither observed changes could be correlated with plasma lipid concentrations and/ or other factors that play a role in HDL metabolism. They looked at a group of 36 women at 35 to 36 weeks of gestation and again at 6 weeks postpartum. HDL levels were increased in the presence of hypertriglyceridaemia, unusual in that triglyceridaemia is usually associated with decreases in HDL levels. Non-denaturing gradient gel electrophoresis (GGE) demonstrated the existence of two subclasses for HDL2 fractions (HDL2b, 9.7 to 12.9nm and HDL2a, 8.8 to 9.7nm) and three subclasses for HDL3 (HDL3a, 8.2 to 8.8nm HDL3b, 7.8 to 8.2 and HDL3c, 7.2 to 7.8nm). Late pregnancy is accompanied by significant increases in TG and cholesterol. [Desoye, Williams] Their analysis showed that at 35 to 36 weeks of gestation, 86% of the subjects had substantial increases of the most buoyant and largest of the HDL species, HDL2b, while postpartum and non-pregnant HDL subclass distribution was characterized by the predominance of HDL3a. There were significant elevations in the concentrations of cholesteryl ester transfer protein (CETP) and oestrogen during late pregnancy.

Within each subject, oestrogen was significantly linked to changes in HDL2b; for every 1 ng/ml increase in oestrogen level during late pregnancy, there was 0,94 % increase in HDL2b mass. They speculated that the shift in HDL subclass distribution during the late pregnancy might be accounted for by an increase in CETP activity

with a parallel decrease in hepatic lipase activity. The major modulator for both of these activities is likely to be oestrogen. [Silliman 1993]

They further investigated if the LDL subclasses change during the hyperlipidaemia state of pregnancy and whether such changes were related to plasma TG and apolipoproteins. [Silliman 1994] LDL particles are heterogeneous in their chemical and physical properties [Shen, Krauss] exhibiting two major particle sizes, with subclass A being larger (>25.5 nm diameter) and more buoyant whereas subclass B is smaller (≤ 25.5 nm diameter) and denser and is associated with increased risk of myocardial infarction. [Silliman 1994] Of the thirty-six Hispanic subjects at 35 to 36 weeks gestation, 97% were categorized as LDL subclass patterns B or I, indicating that the smaller and dense LDL particles were predominant. The predominance of the smaller and dense LDL was associated with plasma TG concentration, showing a significant inverse relationship between the LDL peak particle diameter and plasma TG. Sattar *et al* carried out a longitudinal examination of plasma lipoprotein sub fractions concentrations and compositions during pregnancy. During the course of normal pregnancy, plasma TG and cholesterol concentrations rise by 200 – 400% and 25 – 50% respectively. Although the rudiments of this process have been the subject of many studies [Piechota, Ordovas, Montelongo], 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 homogenous but contain discrete sub-fractions differing in structure, physicochemical properties, kinetic behaviour, and

LDL-peak particle diameter (LDL-PPD) is significantly decreased in preeclampsia relatively to normal pregnancy. It would be important to measure not only LDL-PPD (predominant LDL size at maximum optical density) but also LDL – mean particle diameter (LDL – MPD), a reliable indicator of the size of the entire LDL population. The evaluation of LDL size is important because it has implications for atherosclerosis. Smaller, denser subpopulations of LDL are more susceptible to oxidation and may also penetrate tissue better. Once oxidised, LDL is believed to have enhanced atherogenic potential, promoting foam cell formation and initiating endothelial dysfunction. Whereas, there is a strong inverse relationship between HDL-cholesterol (HDL-C) concentration and atherogenic situations, no agreement exists in literature about changes in HDL-C in preeclamptic pregnancies.

Prevalence of apoE Isoforms in different populations

Zekraoui *et al* 1997and Lucotte *et al* 1997 both analysed the frequency of ApoE 4 allele and pattern of apoE4 allele in Africa and Europe respectively. The highest frequency of apoE4 was observed in Aka Pygmies (40.7%). There is some disagreement in the literature concerning the identity of the ancestral allele at the apoE locus. ApoE3 is the most prevalent allele in most human populations and has been considered the original allele accordingly [Mahley 1988]. At the protein level both ApoE2 and ApoE4 can be derived from the apoE3 gene sequence by single mutational events, whereas two successive mutations would be needed to derive apoE2 from apoE4. However analysis of the apoE protein of apes by electrophoresis gave signals at a similar position to the human apoE2, suggesting

[Zannis 1985] that apoE2 may be the ancestral allele (the analysis depends on a protein's molecular weight and charge and is therefore not a reliable indicator for sequence comparisons between species). At the genomic level, for both positions 112 and 158, there is a change of the nucleotide sequence from CGC to TGC, resulting in a change from arginine to cysteine in the protein; because the likely direction of mutation is C to G and so Larsen *et al* 1993 suggested that the apoE4 allele is the ancestral one. This was supported by the sequenced apoE genes of baboons by Hixson *et al* 1988, cynomolgus monkeys by Marotti *et al* 1989 and Lucotte *et al* in 1997.

Table 3.2 below shows Apo E Gene Frequencies in 30 European Populations according to latitude (from north to south) A study was selected if it included at least 100 subjects.

			APOE Allele frequency(%)			
Population	*Latitude (Degrees north)*	*Sample Size*	**2*	**3*	**4*	*Reference*
Iceland	64	185	6.8	76.8	16.5	Hallman et al (1991)
Finland						
Kuopio	64	729	3.5	78.1	18.4	Lehtimaki et al. (1990)
Tampere	61	698	5.2	74.2	20.5	Salo et al. (1993)
Helsinki	60	615	4.1	73.3	22.7	Ehnholm et al (1986)
Oslo, Norway	60	156	8.3	76.0	15.7	Pedersen and Berg (1989)
Linkoping, Sweden	58	279	11.9	67.5	20.6	Gerdes et al (1992)
Grampian region, Scotland	57	400	8.3	77.0	14.8	Cumming & Robertson (1984)
Belfast Ireland	55	175	10.3	75.4	14.3	Luc et al (1994)
London, England	51	159	9.0	77.31	13.7	Gerdes et al (1992)
Aarhus, Denmark	56	466	8.5	74.1	17.4	Gerdes et al (1992)
Amsterdam, Netherlands	52	2318	8.2	75.0	16.7	Smit et al (1988)
Germany						
Munster	52	1557	8.2	78.2	13.6	Assman et al (1984)
Marburg	51	1031	7.7	77.3	15.0	Uterman et al (1984)
France						
Lille	50	178	11.9	74.1	14.0	Luc et al (1994)
Strasbourg	49	159	8.5	80.8	10.7	Luc et al (1994)
Nancy	49	303	12.0	76.4	11.6	Gueguen et al (1989)
Reims	49	249	8.1	80.0	11.9	Lucotte et al (1995)
Paris	49	249	7.9	80.1	12.0	Bailleul et al (1993)
Toulouse	44	171	5.6	86.3	8.2	Luc et al (1994)
Hungary	48	202	6.4	80.7	12.9	Hallman et al (1991
Tyrol, Austria	47	469	9.0	79.2	11.8	Hallman et al (1991)
Geneva, Switzerland	46	173	7.2	87.1	10.7	James et al (1987)
Italy						
Padua	45	352	4.4	85.7	9.8	Gerdes et al (1992)
Central Italy	42	365	7.2	84.7	8.1	James et al (1993)
Rome	42	195	6.2	86.7	7.2	Xu et al (1990)
Southern Italy and Sicily	40	1696	6.0	85.2	8.8	Corbo et al (1995)
Sardinia	41	280	5.0	89.8	5.2	Corbo et al (1995)
Spain						
Barcelona	41	100	7.5	82.5	10.0	Gerdes et al (1992)
Madrid	40	186	5.7	88.2	6.1	Ibarreta et al (1995)
Istanbul, Turkey	41	8366	7.9	86.0	6.1	Mahley et al (1995)

Table 3.2

Table 3.3 below shows the frequencies of the Apoε alleles (%) in the various Non-Pygmy ethnic groups.

Ethnic Group (Countries)	*Number of Alleles*	*Allele Frequency*		
		Apoε*2	Apoε*3	Apoε*4
Afar and Issas	34	2.9	85.3	11.8	<0.20
Arabs (Mauritian)	20	7.5	82.5	11.8	NS
Bambara (Mali)	16	6.2	71.9	21.9	NS
Bamileke (Cameroon)	18	11.1	66.7	22.2	NS
Djerna (Niger)	16	6.2	81.2	12.5	NS
Ewe (Togo)	19	31.6	47.4	21.0	<0.20
Fang (Gabon)	25	12.0	68.0	20.0	NS
Fon (Benin)	17	0.0	70.6	29.4	<0.20
Haoussa (Niger)	37	2.7	78.4	18.9	NS
Hutu (Rwanda)	21	9.5	66.7	23.8	NS
Malinke (Guinea)	30	23.3	60.0	16.7	<0.20
Merina (Madgascar)	22	22.7	59.1	18.2	NS
Mossi (Burkina Faso)	20	37.5	50.0	12.5	0.01
Wolof (Senegal)	33	3.0	93.9	3.0	<0.5
Peul (Senegal)	45	7.8	67.8	24.4	NS
Songhai (Mali)	17	20.6	73.5	5.9	NS
Chadians	22	4.5	68.2	27.3	NS
Toucouleur (Senegal)	17	11.8	70.6	17.6	NS
Tutsi (Burundi)	13	0.0	61.5	8.5	<0.10
Zairians	24	4.2	62.5	33.3	<0.10

NS, nonsignificant

Table 3.3 Zekraoui *et al* 1997
In some ethnic groups allele frequencies are statistically different from the whole group of sub-sahara non-Pygmy Africans considered

Table 3.4 below shows the frequency of apoE genotype and apoE alleles in pygmies from Central African Republic compared to other Africans from West, Central and East Africa.

Genotype or Allele	**Pygmies**	**Africans**
Genotype		
ApoE2/2	0	2.3
ApoE2/3	7.2	14.3
ApoE2/4	4.3	4.3
ApoE3/3	27.1	52.1
ApoE3/4	45.7	22.8
ApoE4/4	15.7	4.3
Allele		
Apoε2	5.7	11.6
Apoε3	53.6	70.6
Apoε4	40.7	17.8

Table 3.4 Zekraoui *et al* 1997. Table shows that apoε3 is the most predominant allele in the two groups, but apoε4 is very high in the Pygmies.

Lipoprotein Reference Range During Pregnancy

Despite documented differences between pregnant and non-pregnant women's lipid profiles, the reference ranges used for pregnant subjects is the same as for the general population. Difficulties have been encountered in setting up credible reference ranges for the lipids and lipoproteins variations for the three trimesters. According to the ideal guidelines for the establishment of reference ranges, precision of the percentiles increases with an increasing number of observations as shown by the narrowing of their confidence intervals. The International Federation of Clinical Chemistry Expert Panel on Theory of Reference Values has recommended a sample size of at least 120 reference values in setting up limits for the reference ranges. Most studies have not been able been able to fulfill these criteria. Several factors like parity, age, weight and consent from patients with normal pregnancy contribute to the intra-racial difficulties that are encountered in such an exercise. Racial and ethnic differences in the lipid and lipoprotein metabolism during pregnancy have been reported with blacks displaying lower values than their white counterparts. To investigate if this difference was reflected in the babies, using cord blood, Glueck *et al* 1977 reported, in a comparison of black and white neonates, that there were no differences in total cholesterol, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol. However cord blood TG were slightly higher in black neonates ($p = 0.02$). Several other factors have been reported and these include a report by Sirikulchayanonta *et al* 2000 that serum TC and LDL-C levels increased with parity. There was a significant

difference between maternal TC and parity ($F = 4.702$, $p = 0.01$) as well as LDL-C and parity ($F = 4.883$, $p < 0.01$), especially P1 and P3. There was no significant difference between maternal TG and parity as to HDL-C and parity ($p > 0.05$). In 1987, Jarnfelt-Samsioe *et al* examined serum lipid and lipoprotein concentrations in 98 healthy pregnant women in early and late pregnancy. Sixty of these women complained of emesis gravidarum. Compared to non-pregnant controls the pregnancy values of serum cholesterol, TG and phospholipids were elevated in all subjects due to an increase in all lipoprotein classes. In addition, low-density lipoproteins (LDL) and high-density lipoproteins (HDL) were enriched in TG relative to other components. Differences in serum lipids and lipoproteins between the emetic and non-emetic subjects were found. The lipid contents of LDL and HDL were significantly higher and lower, respectively, in the emetic women in early pregnancy. During late pregnancy the total lipid content in all fractions was higher in previously emetic subjects. Thus, a metabolic difference between the groups persisted throughout pregnancy. Despite these difficulties, several studies have been conducted in an effort to establish lipoprotein (a) levels during and after pregnancy. [Zechner] Manten *et al* 2003 constructed a curve for plasma lipoprotein (a) that may serve as the standard reference for changes in pregnancy.

The curve defined by the formula:

Lp(a) (mg/l) = exp [4.789 + (0.05215 x GA) + (-0.0007371 x GA2)]

where GA = gestational age in weeks. It is helpful in predicting changes of gestational age-dependent changes of lipoprotein (a) in normal pregnancy. In a

population-based study Knopp *et al* 1982, compared lipoprotein lipid reference values for pregnant women to non-pregnant women some of whom used sex hormones. The study was on 553 Caucasian women at 36 weeks gestation, thus fulfillilling the minimum number criterion. 64 of the women were studied 6 weeks postpartum.

Table 3.5 below shows the plasma lipoprotein lipid distribution for 100 women not using hormones, 79 women using oral contraceptive hormones, 553 women at 36 weeks gestation and 64 women at 6 weeks post partum.

Parameter	Mean	Std Dev	Range	Percentile				
				5th	10th	50th	90th	95th
Total TG								
Total plasma								
Hormone non users	0.72	0.31	0.31-2.10	0.38	0.42	0.66	1.07	1.16
Hormone users	1.20	0.48	0.50-3.80	0.59	0.69	1.16	1.63	1.91
36 weeks gestation	2.57	0.95	0.93-9.09	1.50	1.65	2.36	3.85	4.37
6 weeks post partum	1.06	0.74	0.40-6.10	0.54	0.61	0.87	1.55	1.77
VLDL								
Hormone non users	0.43	0.27	0-1.56	0.10	0.15	0.36	0.70	0.86
Hormone users	0.72	0.43	0.16-3.37	0.24	0.31	0.68	1.11	1.31
36 weeks gestation	1.38	0.74	0.19-7.25	0.54	0.66	1.19	2.26	2.78
6 weeks post partum	0.64	0.69	0.14-5.41	0.18	0.23	0.46	1.07	1.38
LDL								
Hormone non users	0.17	0.10	0-0.51	0.01	0.05	0.15	0.28	0.38
Hormone users	0.28	0.11	0.05-0.57	0.15	0.16	0.26	0.43	0.46
36 weeks gestation	0.33	0.26	0.23-1.92	0.43	0.49	0.72	1.10	1.28
6 weeks post partum	0.28	0.10	0-0.57	0.08	0.14	0.28	0.38	0.41
HDL								
Hormone non users	0.12	0.06	0-0.36	0.05	0.06	0.11	0.19	0.21
Hormone users	0.19	0.08	0.09-0.38	0.09	0.10	0.19	0.31	0.33
36 weeks gestation	0.40	0.11	0.15-0.88	0.24	0.26	0.37	0.53	0.60
6 weeks post partum	0.14	0.06	0.05-0.37	0.05	0.06	0.12	0.18	0.21
Cholesterol								
Total Plasma								
Hormone non users	4.39	0.75	2.94-6.76	3.30	3.43	4.32	5.51	5.59
Hormone users	4.71	0.82	3.17-7.54	3.41	3.82	4.60	5.82	6.40
36 weeks gestation	6.42	1.09	3.77-11.05	4.81	5.10	6.32	7.77	8.27
6 weeks post partum	5.36	0.86	3.41-3.41	3.95	4.19	5.20	6.60	6.89
VLDL								
Hormone non users	0.34	0.21	0-1.20	0.03	0.13	0.29	0.62	0.68
Hormone users	0.44	0.23	0.05-1.14	0.08	0.13	0.42	0.73	0.78
36 weeks gestation	0.75	0.44	0-4.21	0.21	0.31	0.68	1.22	1.53
6 weeks post partum	0.36	0.34	0.08-2.65	0.13	0.13	0.29	0.65	0.75
LDL								
Hormone non users	2.65	0.69	1.38-4.68	1.66	1.79	2.57	3.61	3.93
Hormone users	2.86	0.78	1.38-5.49	1.61	1.82	5.36	3.77	4.21
36 weeks gestation	3.95	1.02	1.20-9.02	2.31	2.76	3.85	5.20	5.67
6 weeks post partum	3.38	0.82	1.56-5.75	2.05	2.39	3.25	4.26	4.60
HDL		0.00						
Hormone non users	1.38	0.29	0.83-2.29	0.91	0.99	1.35	1.74	1.79
Hormone users	1.40	0.40	0.83-2.60	0.88	0.96	1.30	1.98	2.11
36 weeks gestation	1.72	0.40	0.55-3.25	1.09	1.20	1.66	2.24	2.42
6 weeks post partum	1.61	0.34	0.68-2.73	1.14	1.20	1.51	2.05	2.29

Table 3.5 [Knopp et al]

The results in the table above were in mg/dl but were converted to mmol/l using the conversion factors 1) for cholesterol is mg/dl x 0.026 = mmol/l

2) for TG is mg/dl x 0.0113 =mmol/l.

Our patient with severe hypertriglyceridaemia was hospitalised but the details surrounding the illness have not yet been established. However Qin Hua *et al* 2004 reported 3 cases of hyperlipidaemic pancreatitis. According to Nies et al 1990 hyperlipidaemic pancreatitis is rare, accounting for only 4%-6% of acute pancreatitis during pregnancy. It usually occurs in the second and third trimesters of primapara women who have hyperlipoproteinaemia. This condition has been reported to lead to a high rate of fetal mortality (up to 37%), primarily due to premature birth. Tonolo *et al,* after examining 16 normal women between 25 and 36 years with normal menstrual cycles, concluded that the physiological variation of sex hormones during the menstrual cycle in normolipidaemic subjects influences the plasma levels of lipids and lipoproteins, indicating ovarian hormones s the major modulator of lipoprotein metabolism. Despite extensive research and reports of dyslipidaemia in pregnancy, this study is the first to investigate dysβ in pregnancy. Glueck *et al,* Am.J. Obstet. Gynecol 1980, March 15 reported an asymptomatic subject with TG levels greater than (5000mg/dl) 56.5mmol/l during pregnancy and subsequent studies revealed familial dysβ.

CHAPTER 4

METHODS /MATERIALS

Both the University of Cape Ethics Review Committee and the Medical Research Council of Zimbabwe approved the study. Informed consent was obtained during the first antenatal clinic visit. After clinical data was documented, venesection was performed for the tests outlined below.

4.1 Study population

The study subjects were recruited at two major Polyclinics in Harare Zimbabwe. Mbare Polyclinic is located in the oldest high-density suburb Southwest of the City of Harare. It comprises a highly mobile community serving all the Zimbabwe rural areas and Southern African Development Countries (SADC) countries by buses and mini buses. Inevitably the antenatal clinic deals with some clients from outside its boundaries. Its catchment area includes all the Mbare wards and nearby suburbs like Sunningdale, Waterfalls and some city dwellers (Avenues). The good antenatal service offered has attracted some white clients who cannot afford the private hospital fees. Mabvuku Polyclinic is located in a high-density suburb East of Harare. The clients are from the nearby rural areas (Chishawasha and Ruwa), semi-formal centres (Epworth), and adjacent high and low-density suburbs (Tafara, Portland and Greendale).

4.2 Recruitment Stages/Phases

690 normo-tensive subjects aged between 16 and 42 years attending antenatal consultation at two major polyclinics in Harare took part after giving informed consent. The recruitment was divided into four stages:

Stage 1

The first stage was the explanation of the protocol to all those who had come for the initial antenatal booking. Participants were Shona women without any known medical conditions. Those who were willing to participate were requested to return the following day after an overnight fast. Travelling allowance was paid after realising that the prevailing harsh economic conditions prevented most subjects from participating because the overnight fast meant an unbudgeted trip to the clinic. The travelling allowance increased every week because of the fuel situation in the country.

Fig 4.1 Mabvuku Polyclinic. Patient going through the initial stages of registration and this formed part of stage one.

Stage 2

The second stage was individual interviews for those who came back in order to get a detailed history and exclude any conditions that could influence lipid metabolism. In addition to the study subjects' normal demography, the original home area and maiden name were requested in order to confirm Shona grouping. The past medical and drug history focused on the causes of secondary dyslipidaemia like (malaria, leprosy, tuberculosis, renal, thyroid disease and alcohol consumption). Anti-retroviral drugs were not available in the public health institutions and none of the participating subjects could afford these drugs in the private sector. Despite the availability of the HIV status from another project that was also underway, the HIV status was not requested because it was not in the present in our proposal.

Fig 4.2 Only patients selected from stage one came to this stage where the Sister in Charge SRN Jenami explained the detailed criterion for the project.

Fig 4.3 SRN Jenami taking history at Mabvuku Clinic

Stage 3

The third stage involved the usual physical examination, including height, weight, and blood pressure and urinalysis. If no obvious pathology was detected, signing of the consent form, which was written in both Shona and English, (see appendix 1 and 2) followed and then venesection was done.

Fig 4.4 Part of clinical examination

Fig 4.5 Venesection after consent was given. SRN Maravanyika of Mbare Polycline (Edith Opperman Maternity Hospital) preparing for venesection. In difficult times with scarce resources they improvised to get the work done.

Fig 4.6 Happy young and proud mothers at the post-partum Clinic at Mabvuku Poly Clinic

Stage 4

The final phase obtained information and blood samples after the pregnancy in 545 subjects (79%). One death (M177) was recorded at Mabvuku Polyclinic but no detailed account of this event was available.

Sample collection and preparations

4,5mls of blood was collected into EDTA tubes. Samples were stored at 4C° in the clinic and then delivered to Chemical Pathology laboratory for further preparation on the same day. The samples were spun at 3000 RPM and plastic Pasteur pipettes were used for the separation of plasma, which was aliquoted into two microreaction vials, to be used for lipid profile and GGE. The buffy coat (layer between plasma and red cells) was harvested and dispensed into 2ml cryovials and mixed with same volume of freeze mix (10% glycerol and sodium azide). Glycerol is a very viscous trihydric alcohol (propane-1, 2,3-triol). The high viscosity prevents cell from

adhering and clumping and thus facilitates DNA extraction. The plasma and the buffy coat were stored at –80C° until exported to South Africa. The samples were imported to South Africa in terms of Regulation 2 and 3 of GN (Government Notice) 2306 of 21 December 1920 and our Reference J1/11/2/3/2. To ensure that the samples remained frozen during the four-hour transportation period, on the day of export the samples were packaged into a cooler bag containing freezer "bricks" containing ethylene glycol. The samples were still frozen on arrival and they were stored at -20C°. The GGE were carried out within one week of arrival and the results were reported and recorded. DNA extraction was done. A limited lipid profile was done last.

4.4 Analytical Methods

An array of Biochemical tests is required in order to confirm the diagnosis of dysB because there isn't a single accepted standard test [Blom]. Genotyping and ultracentrifugation offers confirmatory diagnostic tests, but besides the prohibitively high costs, they both demand a high level of expertise and only a limited number of samples can be run at time.

Gradient Gel Electrophoresis

Assay Principle

Pre-stained lipoproteins in plasma are run at 4C in a gel sandwiched between two glass plates. The lipoproteins are separated according to size, in a gel in which the concentration of acrylamide rises progressively. The gel is designed to separate all

apoB-containing lipoproteins. The small particles like LDL-B migrate the furthest in the gel because the size of pores decrease with an increase in acrylamide content.

Procedure

1) The frozen samples were thawed at 4C over night.

2) Lipoproteins in plasma were stained with Sudan black by mixing 100µl of plasma with 50µl of Sudan Black stain. The 100µl and 50µl in the sample preparation results in plasma constituting 2/3 of the mixture. The Sudan Black Stain is prepared by dissolving 1g in 100ml ethylene glycol .The mixture was mixed on a vortex and left to incubate at 4C for a minimum of one hour.

Lipids in plasma and in tissues are normally demonstrated by Sudan dyes. Not all lipids are stained; free fatty acids, phosphoglycerides and free cholesterol stain less intensely. Oil-soluble dyes stain lipids because they are more soluble in the lipids than in their solvent. Most of these are not dyes in a sense, i.e. they do not contain auxochromic groups but are chromogens. An exception to this is Sudan Black, which contains the amino group auxochrome. This accounts for the more sensitive fat staining occurring with this dye, which is particularly apparent during staining of phospholipid-rich tissues, where some degree of salt binding occurs. Oil-soluble dyes are virtually insoluble in water and dissolve to a variable degree in alcohol.

3) Spin sample and Sudan Black mixture at 10000 RPM for 20minutes in order to sediment the granules of Sudan black that may have formed.

4) Pipette 50µl of the top layer (supernatant), which represents 33.3% of plasma and mix with 50µl of saturated sucrose. This is done to ensure that the sample mixed

with the dense sucrose will settle at the bottom of the well. The 100µl mixture contains 1/3 of plasma sample.

5) A worksheet was prepared and 12µl of the stained sample and sucrose mixture were loaded into the appropriate well on the labelled gel. Since the final mixture contains 1/3 of initial plasma sample, 12µl represents 4µl of the initial plasma sample.

Gel Preparations

Two separate solutions (A) and (B) were freshly made according to the tables below and then mixed to produce a gradient gel, in which the concentration of acrylamide rises progressively.

2% Gel Preparation (A solution)

Water	2.40 ml
Buffer B (No glycerol)	1.34 ml
30 % Acrylamide	0.27 ml
AMPS (Ammoniumpersulphate)	30µl
TEMED (1:10 solution)	15 µl

Table 4.1

This solution was dispensed into the left side of the gel maker

7% Gel Preparation (B solution)

Water	1.73 ml
Buffer A (Contains glycerol)	1.34 ml
30% Acrylamide	0.93 ml
AMPS (Ammoniumpersulphate)	30µl
TEMED (1:10 solution)	15µl

Table 4.2

This solution was dispensed into the right (outlet) side of gel maker

Calculation

IF 4 ml of a 5% gel solution is desired it is calculated as follows.

Desired concentration obtained by 5/30 X 4/x

1/5 X 4/x

x = 4/5

x = 0.80ml.

Therefore 0.80ml of the 30% acrylamide stock solution is required. So 0.80ml acrylamide + 1.34ml (buffer) + 1.66ml (water)=4ml

3% Stacking Gel (Volumes for only one pair of gel)

Stacking Buffer	4.50 ml
30 % Acrylamide	0.50 ml
AMPS (Ammoniumpersulphate)	200µl
TEMED (1:10 solution)	40 µl

Table 4.3

Tank buffer was prepared by adding 600mls of water to 150 ml of stock buffer.

Polyacrylamide mini-gels (6cm long) were freshly made. A 30% stock solution of acrylamide with 2.7% bisacrylamide was used to prepare the 8% (B) and 2% (A) solution of acrylamide in a buffer containing 13.6g% Tris and 30%(v/v) glycerol at pH8.8

2) Gel polymerisation for both solution A and B was done separately by adding

i) 30µl of a 100mg/ml solution of (AMPS) ammoniumpersulphate (prepared by weighing 100mg or 0.1g of AMPS and dissolved in 1 ml of deionised water)

(ii) 15µl of TEMED (tetramethylethylenediamine)

Acrylamide is polymerised in the presence of free radical generators and a cross-linker enhances the formation of a mesh.

3) The activated solutions were dispensed into A and B chambers of the gradient gel marker respectively. The 8% gel was dispensed into the B chamber, nearer the outlet and the 2% gel into the A chamber on the left. So the 2% gel has to flow into the B chamber where it is mixed with the 8%. The mixture is achieved by means of the small magnets in each chamber. As the 8% gel flows out from chamber B, there is a reduction in volume in chamber B and so the 2% gel solution from chamber A, flows in to maintain the volume equilibrium between the two chambers since they have a communicating channel between them. As the 2% solution flows in, it mixes with the 8% solution at the base resulting in a less dense gel, which ensures it will overlie the denser solution allow at least one hour for complete polymerisation to take place.

4) Once the separation gel is set, adding 100µl of ammoniumpersulphate and 40µl of tetramethylethylenediamine (TEMED) sets up the polymerisation action of the stacking gel containing 30% of stock acrylamide in 1.68g% Tris at pH6.8. This allows a firmer gel that will permit the formation of loading wells.

5) The sample holding wells were created using a 15-lane comb placed into the space above the separating gel and then the stacking gel is dispensed using a glass Pasteur pipette. After setting, the combs are removed and the gels are transferred to gel holder (one pair of gels per holder). Care is taken to have ≤1mm of stacking gel above the separation gel.

Sample Loading and Running the Gel

1) The gel holders are positioned inside the gel tank.

A 1:5 running/tank buffer reconstituted by making up 150mls of Tris glycine buffer pH 8.3 to 750mls with deionised water is poured into the space formed between the gels to check for leaks. And if there are no leaks detected, the rest of the tank buffer is dispensed in the tank.

2) Samples are then loaded into the corresponding wells and known LDL controls are loaded into the position 7 (LDL-A the larger species) and 8 (LDL-B the smaller species) of all the gels. The same set of standards is used on successive gels as a quality control measure.

3) Place the tank lid onto the tank making sure that the red goes to the red connector and the black to the black connector.

4) The tank is then transferred to a 4C environment where the gels are run for 12-18 hours at 120V at 4C in Tris Glycine buffer pH 8.3)

Gel Reports and Records

The gels are removed at the end of the run and excess fluid/buffer is blotted from the glass slides and the gels are photocopied onto a white paper for reporting and record keeping. Direct gel interpretation was made by mostly by Prof AD Marais and sometimes by Dr D Blom, both of whom have used the method for several years. Mrs P Byrnes recorded the report onto the appropriate/corresponding number on the gel copy. The GGE were done before any other tests were run and without knowledge of the patient's identity or clinical data.

The gel terminology was derived to describe the lipoproteins corresponding to the particles separated and calibrated by ultracentrifugation. The origin of the separation gel represents **O** and the migration of small dense represents 1,0 and all lipoproteins in this gel system were calibrated by ultra centrifugally prepared samples. Chylomicrons appear at the origin, Rf 0 - 0.15 termed as **O**. VLDL 1 appears between 0.15 and is refrred to as Me. Whilst VLDL2 is between – and -, referred to as **MI**. IDL is between Rf 0-085 and in this system includes Lp(a). This range is called pre A. The LDL fraction ranges from Rf 0.85- 1.0 displaying for most subjects a single or highly dorminant discrete band. The largest is designated **A** and the smallest is **B**. An **I** species is recognised in the middle and further categories of **AI** and **IB** are recognised. The general categorical classification is used in the

laboratory, as measuring the distance of migration is tedious. After reporting, the glass slides were removed and the gels dried using the BIO-RAD Gel Air Drying Frame Assembly procedure. The dried gel and the copied gel constitute the hard copies for permanent storage. Over 15 years the Lipid Laboratory has built up experience that permits other diagnoses from the same electrophoretic system depicted in figure 4.7. Of interest is that dysβ can be identified with high sensitivity and specificity [Blom] and this pattern is abbreviated MI in the laboratory shorthand.

Position of Lipoproteins on GGE

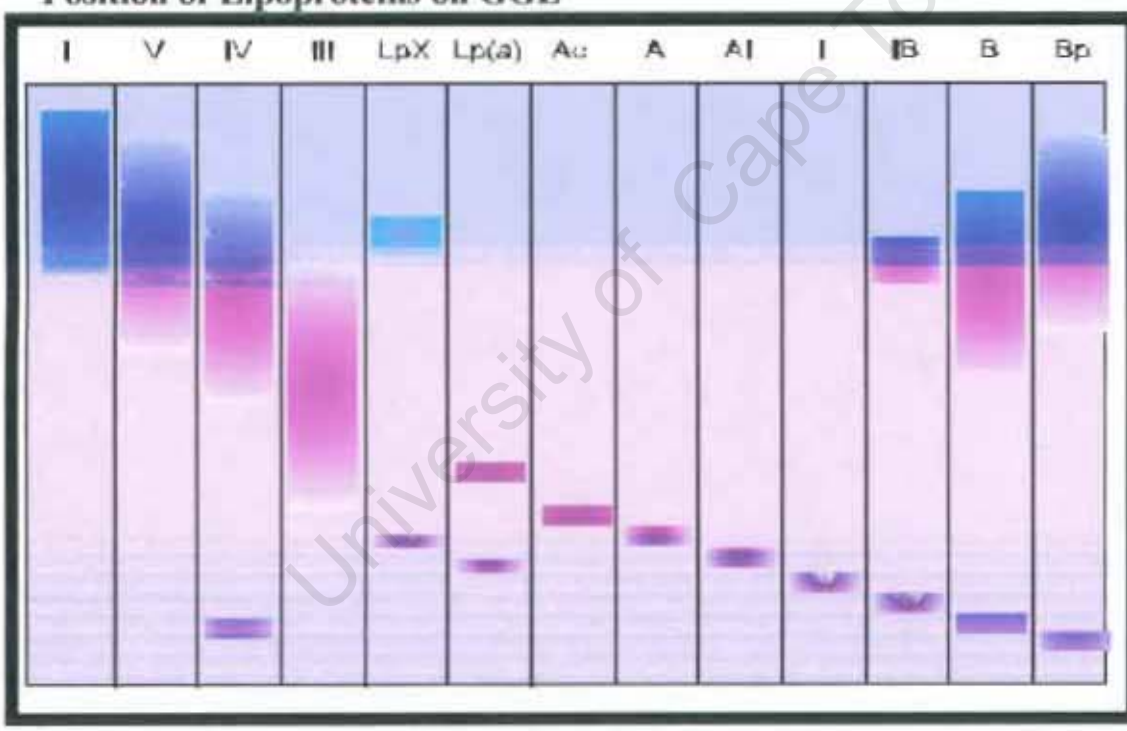


Fig 4.7 A schematic diagram illustrating the ideal migration positions for the different lipoproteins. The first four lanes on the left indicate how the typical Fredrickson Types I, V, IV and III would appear.

Representative GGE on videodensitometer

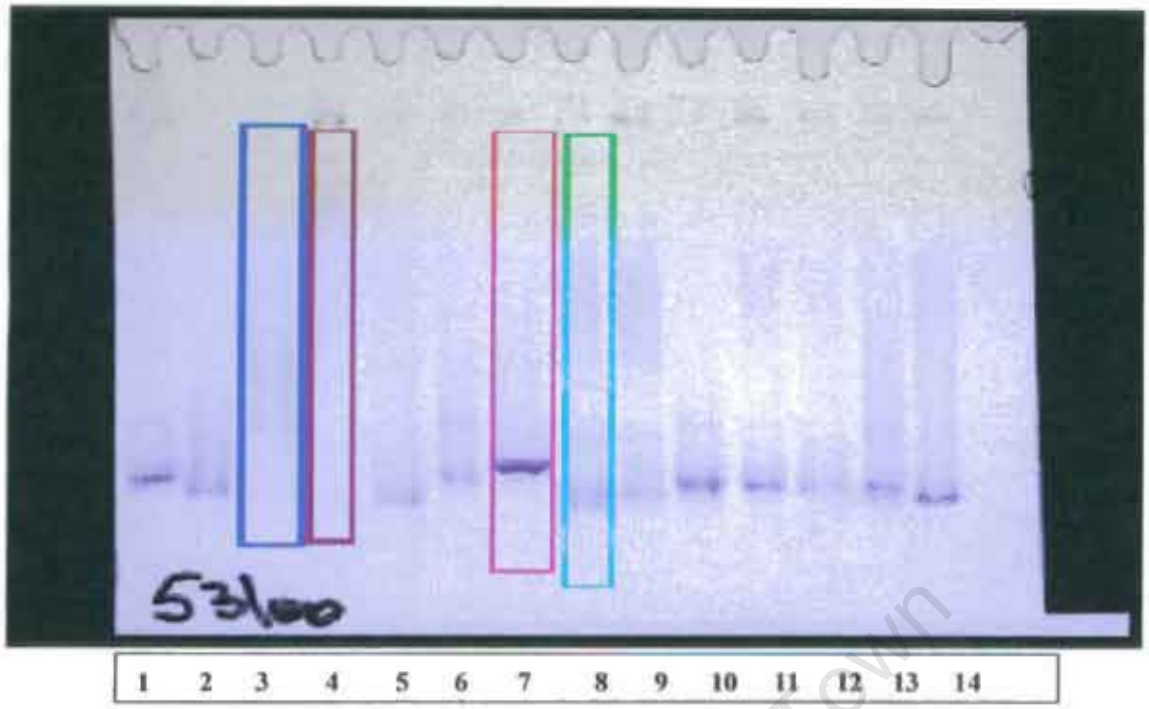


Fig 4.8 This is a picture of the gel that is used during the reporting session.

KEY

1) The black columns on either side of the gel are the gel spacers

2) 53/00 is the gel laboratory identity number

3) Lane 3 (blue) is a Ml band with no LDL species (Type III)

4) Lane 4 (brown) shows the origin in a chylomicronaemic subject.

5) Lane 7 (red) LDL-A species

6) Lane 8 (green) LDL-IB + B species

4.6 LIPID PROFILE

Lipid profile included TC, TG, and HDL-C. LDL-C was calculated by the Friedewald formula.

Total Cholesterol

Brief Review

Liebermann first reported cholesterol analysis in 1885 followed by Burchard in 1889. In the Liebermann-Burchard reaction, cholesterol forms a blue-green dye from polymeric unsaturated carbohydrates in an acetic acid/acetic anhydride/concentrated sulphuric acid medium.

The current fully automated enzymatic methods are a result of several modifications dating back to 1944 when GE Turfitt reported the oxidation of cholesterol by proactinomyces species (Nocardia) [Turfitt]. The method in use on the Roche-Hitachi Modular Analytics is based on the first fully enzymatic work done in 1974 [Allain].

After enzymatic cleavage of the cholesterol ester by cholesterol esterase, the conversion of cholesterol to cholest-4-en-3-one and hydrogen peroxide is catalyzed by cholesterol oxidase. The hydrogen peroxide forms the quinonemine dye in the Trinder reaction. Other assays like those utilized on some Beckman Instruments do not incorporate the Trinder reaction step, they determine cholesterol concentration by amperometric measurement of the rate of oxygen consumption.

Test Principle

1) Cholesterol Esters + H_2O → Cholesterol + Fatty Acids

Cholesterol esters are cleaved by the action of cholesterol esterase to yield free cholesterol and free fatty acids.

2) Cholesterol +Oxygen → Cholest-4-en-3-one + H_2O_2

Cholesterol is converted by oxygen with the aid of cholesterol oxidase to cholest-4-en-3-one and hydrogen peroxide

3) $2H_2O_2$ +4-aminophenozone + phenol →

4-p-benzoquinone-monoimino)- phenazone + $4H_2$

Hydrogen peroxide forms a red colour after reacting with 4-aminophenazone and phenol (Trinder reaction). The colour intensity is directly proportional to the concentration of cholesterol.

Assay Specifications

Serum, heparinised or EDTA plasma can be used.

Stability 5-7 days at 4°C or 3 months at -20°C

Interferences

Icterus: No significant interference up to a concentration of 426µmol/l conjugated bilirubin and 171umo/l unconjugated bilirubin.

Haemolysis: No significant interference up to a concentration of 700mg/dl of haemoglobin.

Lipaemia: No significant intereference up to TG concentration of 28.5mmol/l.

Several polyanion-divalent cation combinations have been used. Heparin sulfate-Mn Cl_2 was widely used, but the introduction of enzymatic cholesterol methods, residual Mn^{+2} was found to interfere giving artifactually high results. This has resulted in the use of other precipitants like phosphotungstate-Mg^{2+} and dextransulfate-Mg^{2+}

The HDL-C method utilized on the modular was developed by Hiroyuki Sugiuchi et al in 1994 Cholesterol esterase (EC 3.1.1.13) and Cholesterol oxidase (EC 1.1.3.6) are both from *Pseudomonas species.* The α, β, and γ cyclodextrins are cyclic oligosaccharides consisting of six, seven, and eight D-glucopyranose units respectively. The number of these units determine the dimension of the torus-shaped hydrophobic cavity, in which guest molecules of suitable size and low polarity can be accommodated and establish an equilibrium.

They have many primary and secondary hydroxyl groups exchangeable for substituents that could extend the physicochemical properties and inclusion capacities of such derivatives. The sulfation of a majority of the hydroxyl groups of cyclodextrins imparts unique biological activities similar or superior to those of heparin [Folkman].

Szejtli et al 1988 successfully utilised cyclodextrins as substrates, stabilisers, solubilizers and scavengers of interfering substances in diagnostic preparations. The modified PEG-modified cholesterol esterase and oxidase exhibit selective catalytic activities towards HDL-cholesterol in serum and α-cyclodextrin sulfate reduces the reactivity towards cholesterol, especially in those lipoproteins with a low protein/lipid ratio in the presence of Mg^{2+}. N-hydroxysuccinimide and

dicyclohexylcardodiimide according to the method described by Leonard at el activate the PEG 6000. The enzymes are then coupled to the activated PEG and any excess PEG is removed by ultrafiltration.

The modular method, utilises PEG (polyethylene glycol) modified enzymes and sulfated dextran. PEG only modified cholesterol esterase and cholesterol oxidase showed selective catalytic activities towards lipoprotein fractions, with the reactivity increasing in the order LDL< VLDL< Chy< HDL. In the presence of magnesium ions, α-cyclodextrin sulfate reduced the reactivity of cholesterol especially chylomicrons, and VLDL without the need for precipitation of these lipoprotein fractions.

General Principles

Polyanions react with positively charged groups on lipoproteins; their action is facilitated by the presence of divalent cations, which interact with negatively charged groups. When added to an aliquot of plasma or serum, an immediate heavy precipitate is formed. The HDL-C is determined in the same way as total cholesterol. The CDC reference method uses a combination of ultracentrifugation and polyanion precipitation to prepare the HDL containing fraction.

TG

Brief Review

Until 1957 TG were estimated by the subtraction method:

TG = Total lipids – (Cholesterol +Phospholipids), when Van Handel and Zilversmit published a direct manual method in which the phospholipids were removed from

the lipid extract by an adsorbent and the TG were determined by measurement of the amount of glycerol released by saponification with potassium hydroxide (KOH) [Russell]. This method has been widely adopted and modified. Lofland established a semi-automated adaptation of this method on the Technicon AutoAnalyser-I. In all the current methods, the first step is the lipase-catalysed hydrolysis of TG to gylcerol and fatty acids [Rifai]. The glycerol portion of the TG molecules after hydrolysis is used to determine the TG concentration.

Test Principle

1) TG + 3 H_2O $\rightarrow$ glycerol + 3 Fatty acids (RCOOH)

2) Glycerol + ATP $\rightarrow$ glycerol-3-phosphate + ADP

3) Glycerol-3-phosphate + O_2 dihyroxyacetone phosphate + H_2O_2

4) H_2O_2 + 4-aminophenazone + 4-chlorophenol

4-(p-benzoquinone-monoimino)-phenazone (colour) + 2 H_2O_2 + HCl

Assay Specifications

Serum, heparinised or EDTA plasma can be used.

Stability 5-7 days at 4°C.

3 months at -20°C

Interference

Icterus: No significant interference up to a concentration of 426:mol/l conjugated bilirubin and 171umo/l unconjugated bilirubin.

Haemolysis: No significant interference up to a concentration of 600mg/dl of haemoglobin

Lipaemia: Lipaemic samples should be diluted with 0.9% NaCl and the result corrected with the dilution factor.

Reportable Range

0.08 –20.7 mmol/l

Calculation

System automatically calculates the TG concentration of each sample.

Conversion factors: mg/dl x 0.0113 = mmo/l

mmol/l x 87.5 = mg/dl

The CDC reference method covered in these references [Rifai]. The other commonly used method by Bucolo and David [Russell] is based on the decrease in absorbance of NADH (Nicotinamide adenine dinucleotide).

In this method, TG is hydrolysed by lipase to release glycerol and fatty acids. The glycerol is reacted with adenosine triphosphate (ATP) in a reaction catalysed by glycerol kinase with magnesium as a co-factor to produce glycerol-3-phosphate and adenosine diphosphate (ADP). The ADP is reacted with phosphoenolpyruvate in a reaction catalysed by pyruvate kinase to produce ATP and pyruvate. The pyruvate then reduces NADH to NAD + lactate in a reaction catalysed by lactate dehydrogenase. The decrease in absorbance of NADH as it disappears is measured at 340nm.

Low Density Lipoprotein (LDL)

LDL methods assume that total cholesterol is composed primarily of cholesterol in the VLDL, LDL and HDL.

Total Cholesterol = VLDL-C + LDL-C +HDL-C

It can be measured using both direct and indirect methods. The indirect method utilizes the Friedewald Equation. Cholesterol, TG and HDL-C are measured and LDL-C is calculated using the empirical equation of Friedwald et al.

(LDL-C) = (TC) – (HDL-C) – (TG/5) where all concentrations are in milligrams per decilitre but TG/2.22 is used when the units are expressed in millimoles per litre. The factor TG/5 is an estimate of VLDL-C concentration and is based on the average ratio of TG to cholesterol in VLDL. Many other workers have come up with other factors but NCEP recommends the use of the original factor.

At high TG levels, the factor TG/5 cannot be applied because such samples can also contain chylomicrons, which all have higher TG/cholesterol ratios than normal. The use of the factor TG/5 would overestimate VLDL-C and therefore underestimate LDL-C. The opposite error occurs if the factor TG/5 is used in Type III hypercholesterolaemia. VLDL found in type III hypercholesterolaemia has a TG/cholesterol ratio in the order of 3:1 and application of the factor would underestimate VLDL-C resulting in the overestimation of LDL. So the LDL calculated for dys2 must be interpreted with caution.

CDC is currently trying to develop a reference method for LDL cholesterol.

Quality Control

Internal and external quality-control schemes are used to ensure high accuracy and precision in these determinations. For internal quality control, Precinorm and Precipath control sera were used and results of the tests were only accepted when the results for control sera were within the manufacture's given range. For external quality control, unknown concentration control sera from the BIORAD cycle quality control program were assayed and the results were within 2SD during the period our samples were run. Shaina et al 1997 reported that the Friedewald equation and the direct LDL C assay correlated well with beta quantification

($r = 0.969$ and $r = 0.971$ respectively) for LDL-C determination in diabetic patients. The Friedelwald equation in comparison with beta quantification, underestimated (8%) LDL cholesterol values in diabetic patients, the direct LDL cholesterol assay had a mean bias of less than 1%.

4.7 GENOTYPING

DNA Extraction

The rapid method for the isolation of DNA from whole blood was used in preparation for the (polymerase chain reaction) PCR. The frozen mixture of buffy coat and freeze mix is defrosted and mixed thoroughly; 100:l of the defrosted blood is transferred into 1.5ml mix reaction vials.

400:l of freshly made ammonium chloride is added and mixed by inversion and left at room temperature for 20 minutes or more for the precipitation of DNA. The

mixture is then spun for 30 seconds in a microfuge and the supernatant is carefully discarded without dislodging the pellet of white cells at the base of the tube.

The pellet is re-suspended in 400:l of cold saline to wash/clean the cells and mixed well by vortexing and then spun again in a microfuge. This is repeated three times until the pellet is clear.

After the final wash the pellet is re-suspended in 200:l of 0.05M or 0.2g/dl of sodium hydroxide (NaOH) and boiled for 10 minutes, then cooled in ice before being neutralised with 25:l of TRIS buffer. The DNA can then be stored until the (polymerase chain reaction) PCR is done.

The apoE genotyping was according to Hixson and Vernier method. [Hixson] The method employs the restriction enzyme *Hha*1 for the identification of the common apolipoprotein E isoforms (E2, E3, and E4). The apoE gene fragment containing amino acid arginine on position 112 and 158 is amplified. The PCR amplified products are digested with *Hha*1 and the digested fragments are separated by electrophoresis on polyacrylamide gels. The nucleotide substitutions that result from the arginine–cystine interchanges at position 112 and 158 alter *Hha*1 cleavage sites, enabling each genotype to be identified by unique combinations of the *Hha*1 fragment sizes in all combinations. The apoE 2/2 samples contain 91 and 83 base-pair (bp) *Hha*1 fragments, reflecting the absence of sites at 112 cystine and 158 cystine. The apoE3/3 sample contains the 91bp fragment as well as 48 and 35 bp fragments from cleavage at the *Hha*1 site at 158 arginine. The apoE4/4 sample also contained these 48 and 35 bp fragments (158 arginine) as well as a unique 72bp

fragment from the cleavage of at 112 arginine (the 19bp fragment is too small for detection) Each of the samples from heterozygotic combinations contains both sets of fragments from each apoE allele.

PCR Master mix for apoE2, 3 and 4

Premix (50uls)	x1 assay (*Boehringer)	x1 assay (*Promega)
Water	32.8:ls	29.8:lss
10x Buffer	5.0:ls	5.0:ls
DNTPs (2mM)	2.5:ls (100 :M)	2.5:ls
G23 (20 :M)	2.5:ls (1 :M)	2.5:ls
G24 (20 :M)	2.5:ls (1 :M)	2.5:ls
DMSO	2.5:ls (5%)	0.2:ls
Taq polymerase (5unit/ul)	0.2uls(1 unit)	0.2:ls
DNA (denatured)	2.0:ls	2.0:ls
$MgCl_2$ 25mM/L	0	3.0:ls (final 1.5mM/L)

Table 4.1

Note that the Boehringer buffer contains Mg^{2+} while the Promega buffer does not and thus needs Mg supplement in assay. Run positive controls of apo E2, E3 and E4 homozygotes.

Overlay with 2 drops of mineral oil to prevent evaporation because the lid does not heat up. PCR cycles: 1 cycle 95 for 5 minutes, for 35 cycles: 94,5 for 45 seconds, 60 for 1 min.

Hha 1 digestion

Run 10µl of PCR production on 1% agarose gel to check whether PCR worked.

Transfer 30µl of product to Eppendof tube.

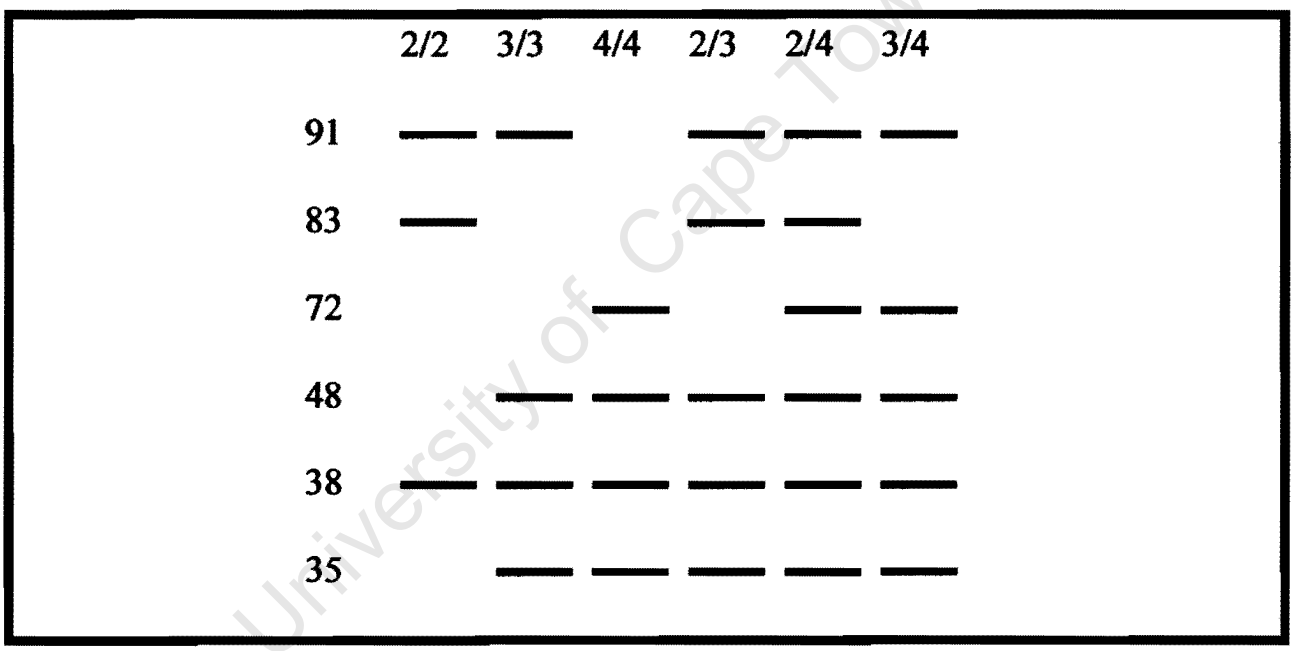
Add 1µls (10 units) Promega Hha1 enzyme to tube and incubate in 37C water bath overnight.

Spin in microfuge 2 seconds and add 4µls sucrose loading buffer.

Run 12% polyacrylamide gel.

Stain with ethidium bromide for 10 mins. Photograph with Polaroid camera.

The nucleotide substitutions that result in arg-cys interchanges at positions 112 and 158 also alter Hha1 cleavage sites. Unique combinations of *Hha*1 fragment sizes in all homozygous and heterozygous combinations can distinguish each genotype. The 7aa repeated by insertion of 122-129 at 122 in apoE yields a larger fragment than normal.



The possible apoE fragments obtained with *Hha*1 restriction

***Hha*1**

Restriction enzymes, also referred to as restriction endonucleases, are enzymes, which recognize short, specific (often palindrominc) DNA seqeuences. They cleave double-stranded DNA (dsDNA) at specific sites within or adjacent to their recognition sequences. Most restriction enzymes (RE) will not cut DNA that is

methylated on one or both strands of their **recognition site**, although some require substrate methylation.

Each restriction enzyme has specific requirements to achieve optimal activity. Ideal storage and assay conditions favour the most activity and highest fidelity in a particular enzyme's function. Conditions such as temperature, pH, enzyme cofactor(s), salt composition and ionic strength affect enzyme activity and stability. Two buffers usually accompany each Promega's restriction enzymes. One buffer is the optimal reaction buffer, which may be from the **4-CORE®**

DNA Substrate Considerations

DNA substrates commonly used for restriction enzyme digestion include DNA from bacteriophage lambda, bacterial plasmid DNA and genomic DNA. Lambda DNA is a linear DNA form that is an industry standard for measuring and expressing unit activity for many restriction enzymes. Compared to linear DNA, intact supercoiled plasmid DNA (and DNA with a large number of the target restriction site) requires more units of enzyme (2-to-10-fold) per microgram than the DNA used in the enzyme's activity assay.

PCR products and oligonucleotides are relatively small compared with DNA used for defining RE units. Therefore, when using these substrates in a restriction digest, it is essential to take into consideration the molar concentration of enzyme recognition sites and not just the mass of DNA with an RE site near its end. When PCR cloning strategies include the use of primers containing a RE site, care is necessary in designing the primer with adequate DNA surrounding the core RE

recognition sequence. In addition to the form and original source of the DNA, the purity is another factor that must be considered. Depending on the purification method and the handling of the DNA, it may contain varying amounts of contaminants that affect restriction enzyme digestion and analysis. Contaminants may include other types of DNA, nucleases, salts and inhibitors of restriction enzymes. The effect of a contaminant on a RE digest is generally dose-dependant: i.e., the inhibitory effects will increase with the volume of DNA added to the restriction enzyme reaction. Relatively pure DNA is required for the efficient restriction enzyme digestion. Contaminating nucleases are usually activated only after the addition of salts (e.g., restriction enzyme buffer) to the DNA solution. Therefore, appropriate control reactions should always be run in parallel with the restriction digest. Buffers solutions containing EDTA in low concentration91mM) are often used to protect DNA from nucleases degradation during storage, but the EDTA can interfere with restriction enzymes digestion if the final concentration of EDTA in the reaction too high. This situation usually results when the concentration of the substrate DNA is low and it is necessary to use a large volume of DNA in the digest. In such cases, it is best to concentrate the DNA (e.g., by ethanol precipitation). The organic solvents, salts, detergents and chelating agents that are sometimes used during the purification of DNA can also interfere with restriction enzyme activity if they carry over into the final DNA solution. Dialysis and/or ethanol precipitation with 2.5M-ammonium acetate (final concentration before adding ethanol) followed by drying and resuspension can remove many of these

substances. While relatively pure DNA is required for efficient restriction enzyme digestion, addition of acetylated BSA to a final concentration of 0.1mg/ml can sometimes improve the quality and efficiency of enzyme assays containing impure DNA and we recommend that it be included in all digests.

Setting up a Restriction Enzyme Digest

An analytical scale restriction enzyme digest is usually performed in a volume of 20µl on 0.2-1.5µg of substrate DNA, using a 2-to-10-fold excess of enzyme over DNA. If an unusually large volume of DNA or enzyme is used, aberrant results may occur and may or may not be readily recognized. The following is an example of a typical RE digest. In sterile tubes, assemble in order:

Sterile, deionized water	16.3µl
RE 10X Buffer	2µl
Acetylated BSA, 10µg/µl	0.2µl
DNA, 1µg/µl	1µl

Mix by pipetting, and then add:

Restriction Enzyme, 10u/µl	0.5µl
Final volume	20µl

Mix gently by pipetting; close the tube and centrifuge for a few seconds in a microcentrifuge. Incubate at the optimum temperature for 1-4 hours.

Add 4µl of 6X loading buffer and proceed to gel analysis. Note that overnight digest is usually unnecessary and may result in degradation of the DNA.

Procedure For ApoE R145C

From the PCR product 10µl of the sample is run on a 1% agarose gel to check whether the PCR worked. 30µl of product is transferred to an eppendorf tube. Add 1µl (10 units) Promega to Hha1 enzyme to tube and incubate at 37degrees Celsius overnight. Spin in microfuge for 2 seconds and add 4-µl sucrose loading buffer and run on 1% polyacrylamide gel. It is stained with ethidium bromide for 10 minutes and photographed with a Polaroid camera under UV viewing box. The nucleotide substitution that results in arg-cys interchanges at positions 112 and 158 alter the Hha 1 cleavage sites. Each genotype can be distinguished by a unique combination of the Hha1 fragment sizes in all homozygous and heterozygous combinations. The 7aa repeated by insertion of 122-129 at 122 in ApoE yields a larger fragment than normal. The Bbv1 cuts at GCAGC yielding from 5-prime to 3-prime fragments 25,108,33,78 bases in the wild type and in R145C the fragments are 25,108,111 from 5-prime to 3-prime. The latter process gives a clearer distinction between heterozygous and normal types. Prepare the same amplified fragments as above. The procedure of choice is to use the enzyme Bbv1 for which the buffer is the same as Taq polymarase. Transfer 30µl of the product to eppendorf tubes and dry by ultracentrigation under vacuum (about 3 hrs). To remove dimethylsulphoxide which can impair enzyme activity and reconstitute the pellet with 30µl pure water. Cut with 2 units of enzyme overnight at 37 degrees Celsius and run on 12% PAGE for 4 hours at 110 vaults and document the gel as above.

DATA ANALYSIS

All statistical analyses were performed using commercially available software packages. Data is presented as mean ±SD and analysed by unpaired or paired *t* test when appropriate. Categorical variables were analysed with the chi-square test (χ^2) A p-value less than 0.05 taken as statistically significant.

CHAPTER 5

RESULTS

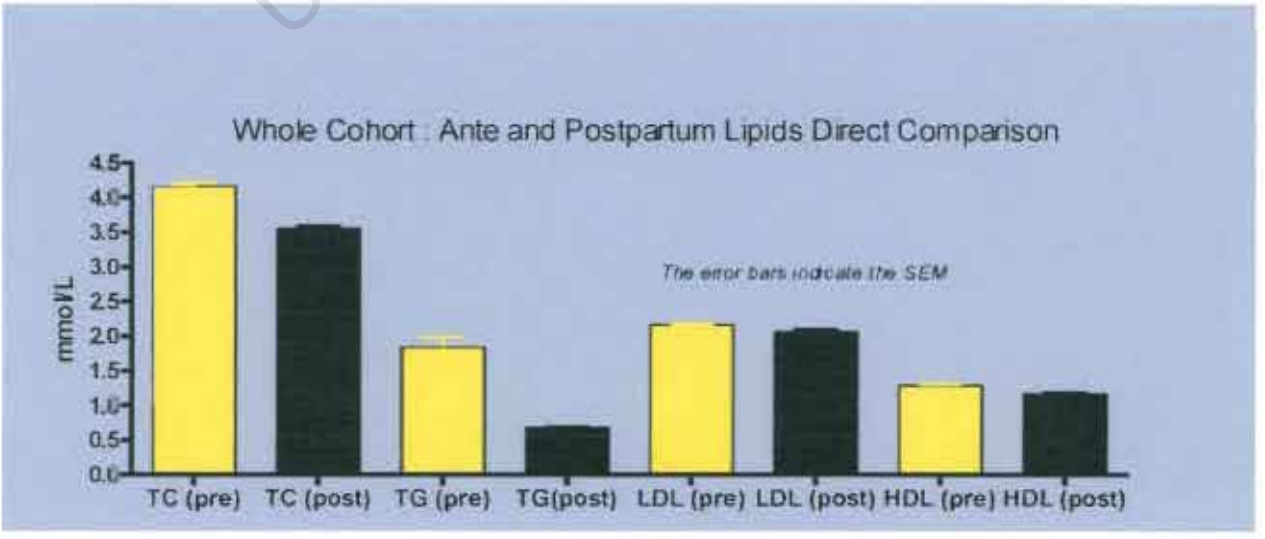
5.1 Description of cohort and lipoproteins

690 Shona subjects were recruited, 323 (47%) of them were primiparous. Of the 483 (70%) who participated in the postnatal (minimum six weeks postpartum) follow-up, only those whose samples were adequate for both antenatal and postnatal full investigations had paired analysis done. 30% of the subjects were excluded from the postpartum stage because of their refusal to participate or inability to schedule a postpartum meeting within 15 weeks of delivery. In order to keep within 15 weeks after delivery after hour's home visits were arranged and other nurses were recruited in order not to over burden the Clinic nurses. Those who refused to participate despite home visits requested financial incentives that could not be satisfied.

The table 5.1 below shows whole cohort baseline clinical characteristics, the antenatal and postnatal laboratory results for the whole cohort. The *p* value compares the data from whole cohort ante partum and postpartum.

Variable	Participants (n)	Mean, Std Deviation and Range	P value
Age (years)	682	24.41 ± 5.293 (16 – 42)	
Gestation (weeks)	690	29.17 ± 3.966 (13 – 38)	
BMI (kg/m^2)	690	24.29 ± 3.55 (16.7 – 45.2)	
Bp (systolic)	688	112.8 ± 10.54 (80.0 – 190.0)	
Bp (diastolic)	688	70.84 ± 7.45 (40 – 110)	
		Antenatal	
Total Cholesterol	686	4.17 ± 0.89 (2.00 – 7.70)	
TG	686	1.84 ± 3.53 (0.40 – 79.1)	
LDL-Cholesterol	669	2.17 ± 0.72 (0.40 – 5.0)	
HDL-Cholesterol	678	1.29 ± 0.38 (0.20 – 2.6)	
		Postnatal	
Total Cholesterol	485	3.56 ± 0.77 (1.0 – 6.10)	p0.0181
TG	488	0.69 ± 0.26 (0.10 – 1.90)	p<0.0001
LDL-Cholesterol	484	2.07 ± 0.65 (0.40 – 4.4)	P0.0012
HDL-Cholesterol	483	1.18 ± 0.35 (0.10 – 2.3)	p<0.0001

Table 5.1



Graph 5.1

5.2 Direct Comparison of Ante partum and Postpartum Lipoproteins

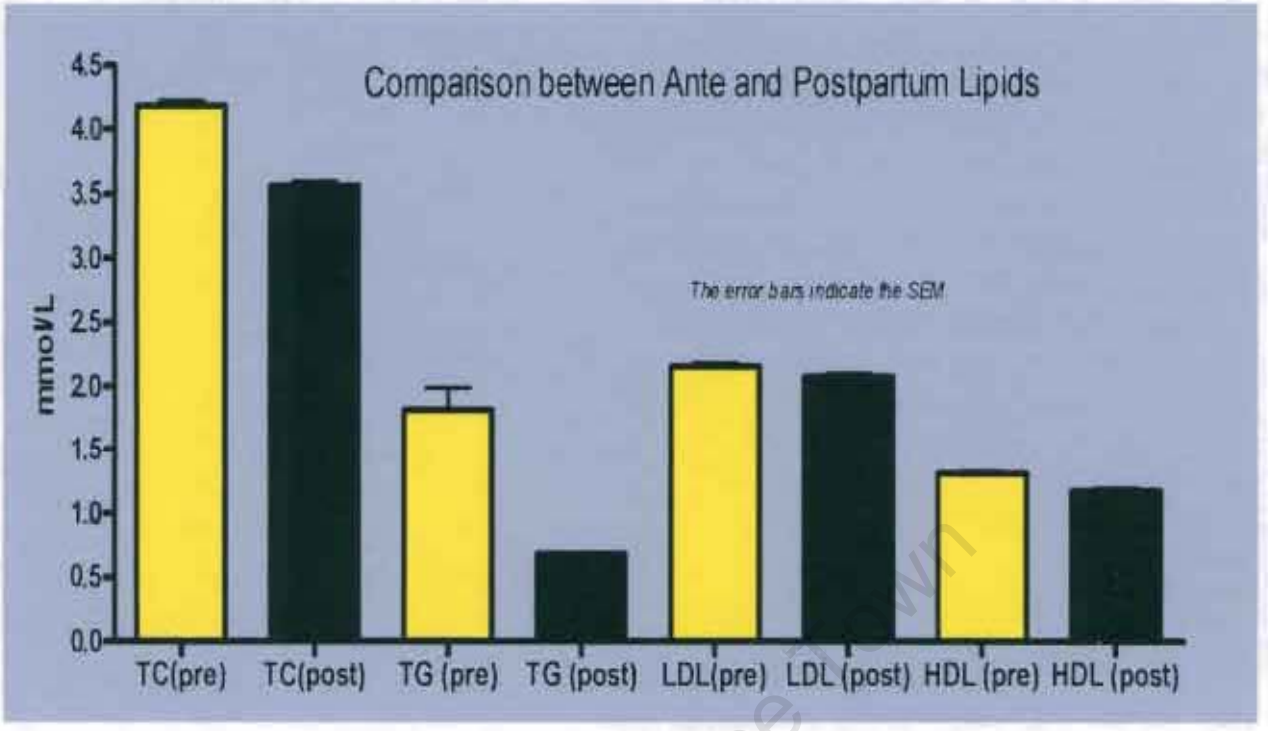
Variable	Participants (n)	Mean, Std Deviation and Range	P value
Age (years)	477	24.58 ± 5.30 (16 – 41)	
Gestation (weeks)	483	29.18 ± 3.89 (13 – 38)	
BMI (kg/m^2)	483	24.34 ± 3.52 (17.0 – 39.3)	
Bp (systolic)	481	112.9 ± 10.50 (80 – 190)	
Bp (diastolic)	481	70.95 ± 7.4 (40 – 110)	
		Antenatal	
Total Cholesterol	483	4.20 ± 0.91 (2.00 – 7.70)	
TG	483	1.83 ± 3.70 (0.40 – 79.10)	
LDL-Cholesterol	472	2.17 ± 0.72 (0.40 – 5.0)	
HDL-Cholesterol	477	1.31 ± 0.38 (0.20 – 2.60)	
		Postnatal	
Total Cholesterol	483	3.56 ± 0.77 (1.00 – 6.10)	p0.0167
TG	483	0.69 ± 0.26 (0.10 – 1.90)	p<0.0001
LDL-Cholesterol	482	2.07 ± 0.65 (0.40 – 4.40)	p0.0013
HDL-Cholesterol	481	1.18 ± 0.35 (0.10 – 2.30)	p0.0001

Table 5.2: Values given as mean ± SD: P value is difference between the minimum and maximum value

Antepartum versus Postpartum Lipid Profiles					
n = 472		TC	TG	LDL-C	HDL-C
Antepartum	Median	4.1	1.5	2.0	1.3
	95% CI	4.1- 4.3	1.5 - 2.2	2.0 – 2.1	1.28 – 1.47
Postpartum	Median	3.6	0.6	2.0	1.1
	95%CI	3.5 - 3.6	0.6 – 0.7	2.0 – 2.1	1.14 – 1.21
Mann Whitney		P<0.001	<0.001	0.16	<0.001

Table 5.3 only the difference between the antepartum and postpartum LDL-Cholesterol is not significant.

These subjects show very desirable lipid profiles, associated with low Coronary Artery Disease.



Graph 5.2

In both whole cohort (Table 5.1 and Graph 5.1) and the paired direct comparison (Table 5.1 and Graph 5.2). For all the clinical characteristics, the *p* value was significant, but even though significant; the BMI value shows a higher *p* value. The study was conducted during a very difficult economic period for the population with most of them struggling to get even one meal a day. The hard economic conditions made it difficult for the subjects to book early and maintain the required antenatal consultations as shown by the differences in the gestation at the time of booking. The economic hardship also impacted on the patients' body weight, but those patients who had come from the nearby affluent areas/suburbs improved the statistical significance.

5.3 Dyslipidaemia

Lipoprotein	Criterion	Antenatal	Postnatal	Antenatal %	Postnatal (%)
TG	>5mmol/l	5	0	0.7	0
	>15mmol/l	4	0	0.6	0
LDL-Cholesterol	>5mmol/l	0	0	0	0
	<1.0mmol/l	18	15	2.6	3.1
HDL-Cholesterol	>2.5mmol/l	1	0	0.1	0
	<0.6mmol/l	8	8	1.2	1.6
Dysβ	GGE	16	1	3	0.2

Table 5.3 Tabulation of dsylipidaemic results revealing that only one subject with postpartum dys2 electrophoretic pattern on GGE. The subject has R145Cys mutation.

The dyslipidaemia observed includes an increase in plasma TG, Total Cholesterol and HDL-Cholesterol. LDL-Cholesterol levels did not show any significant difference but the LDL size was altered as discussed below. These findings are in agreement with numerous other investigators and therefore, may be applied more widely. Severe hypertriglyceridaemias with significant risk of pancreatitis accepting that hypertriglyceridaemia of >15mmol/l is regarded as a risk factor for pancreatitis, severe hypertriglyceridaemia, accepting that values >75mmol/l are indicative of significant metabolic abnormalities in the fasting state were encountered. Only one subject would be classified as FH if a cut-off value of >5mmol/l for LDLC is taken into account. Accepting a cut off value of 1.5 mmol/l, decreased levels of LDLC were observed in 2.6% and 3.1% of the subjects

antenatally and postnatally respectively. These observations could possibly be related to either disease/nutrition or hypobetalipoproteinaemia. Dysβ pattern observed on Gradient Gel Electrophoresis (GGE) during antepartum was observed in only one postpartum sample for a subject with R145Cys mutation, confirming reports that the metabolic changes during pregnancy may stress lipid metabolism.

CHANGES IN LDL SPECIES

Normal	Σ	+2	+1	0	-1	-2	-3	-4	-5
A	221	0	0	4	29	69	85	22	12
%				2	13	31	36	10	5
AI	120	0	2	18	48	37	11	9	0
%			2	11	40	31	9	8	
I	118	0	6	33	34	30	15	0	0
%			5	28	29	25	13		
IB	10	1	5	7	4	2	0	0	0
%		32	37	21	11				
B	2	0	0	0	0	0	0	0	0

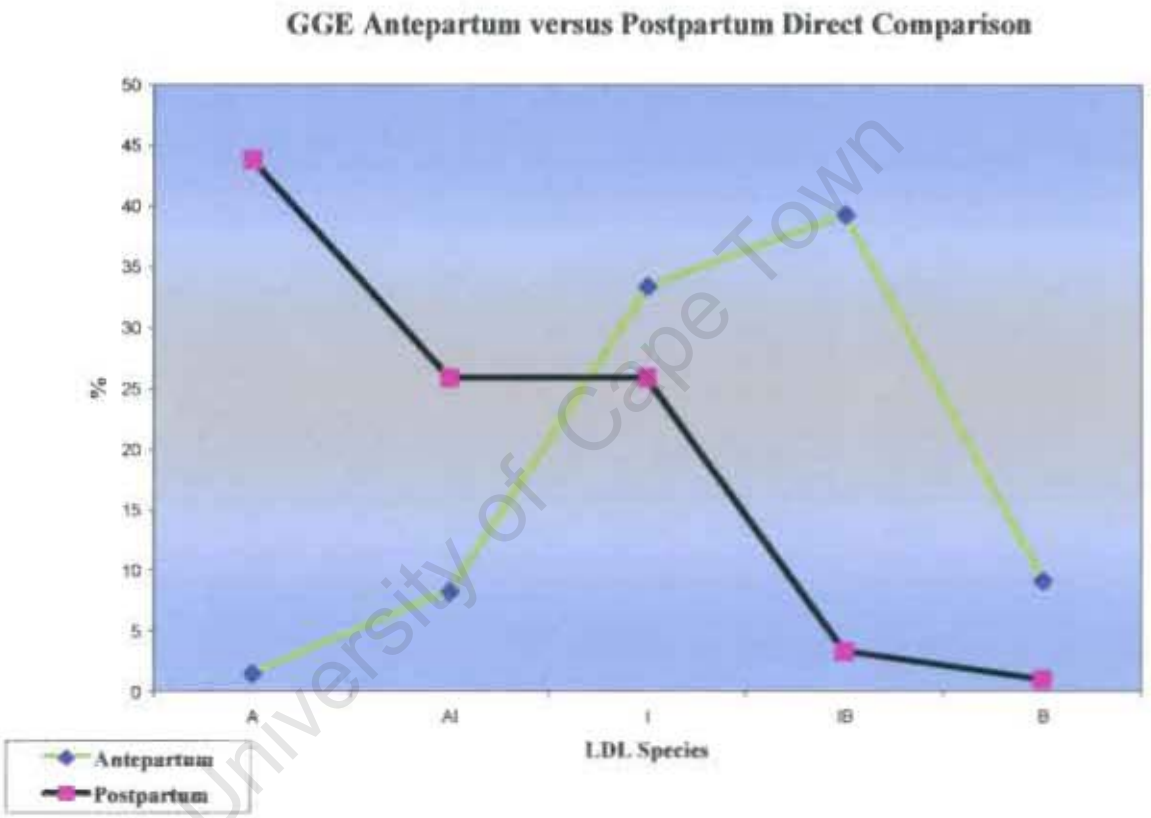
(negative) denotes reduction in size, 0 = No change + (positive) denotes increase in size in categories relative to the antepartum state.

Table 5.4

Excluded from table 4 are 30 subjects and 1 subject that had no LDL species during antepartum and post partum respectively. Also excluded are 26 and 56 subjects who displayed dual LDL species during ante partum and post partum respectively as shown in the summary table 5.5 below.

	A	AI	I	IB	B	Bp	M	Dual	Total
Antepartum	11	78	207	246	61	3	30	26	660
Postpartum	172	101	93	13	0	0	1	56	450

Table 5.5

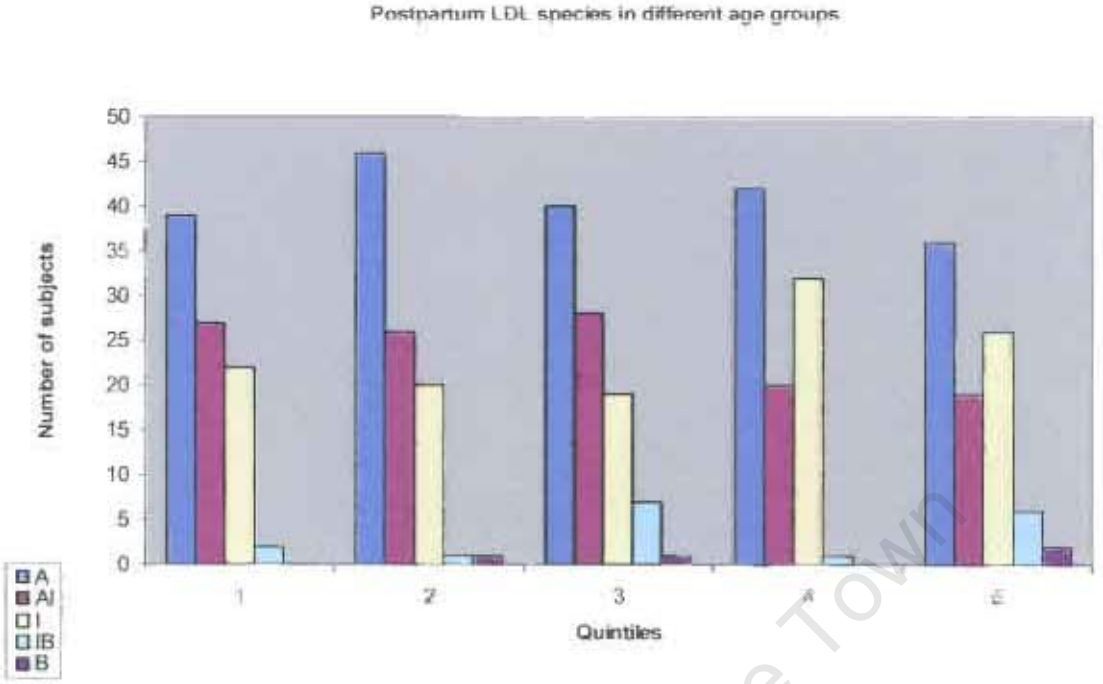


Graph 5.3

Since there were differences in the LDL size when comparing the ante partum and postpartum states, an attempt was made to determine whether the particle size influenced the change. Taking postpartum as the norm, comparing the categorical change in LDL species in pregnancy, the change can range through A, AI, I, IB and B as well as loss of LDL species. Analysis of 480 subjects revealed that 14 (2.9%)

subjects had LDL-C species changing from smaller to larger species larger to smaller species, 59 (12.3%) remained the same while 407 (84.4%) had LDL species changing to smaller. The analysis of the LDL species becoming smaller was significant with χ^2 analysis for the whole cohort p <0.0001 and χ analysis of A, AI and I p <0.0001. The magnitude of change analyzed as the categorical change product/n showed A = 2.6, AI =1.8, I = 1.8, IB = 0.6 and B =0 with an average change of 2.2. This reveals a significant trend for larger species of LDL having a greater change in the category than mid or smaller LDL. It is likely that more CE in the larger species is exchanged for TG by CETP. The limited HL and LPL activity may still suffice to remodel the LDL species and the absolute change in particle size is determined by the size of the original species before pregnancy. It is also possible that a more TG-rich LDL is directly secreted from the liver in pregnancy, yielding the same changes as outlined above.

The influence of age on LDL species was examined in the post partum state.



Graph 5.4

The subjects were divided into five quintiles according to their ages with

1= Quintile 1 (16 – 20 years)

2= Quintile 2 (20 – 22 years)

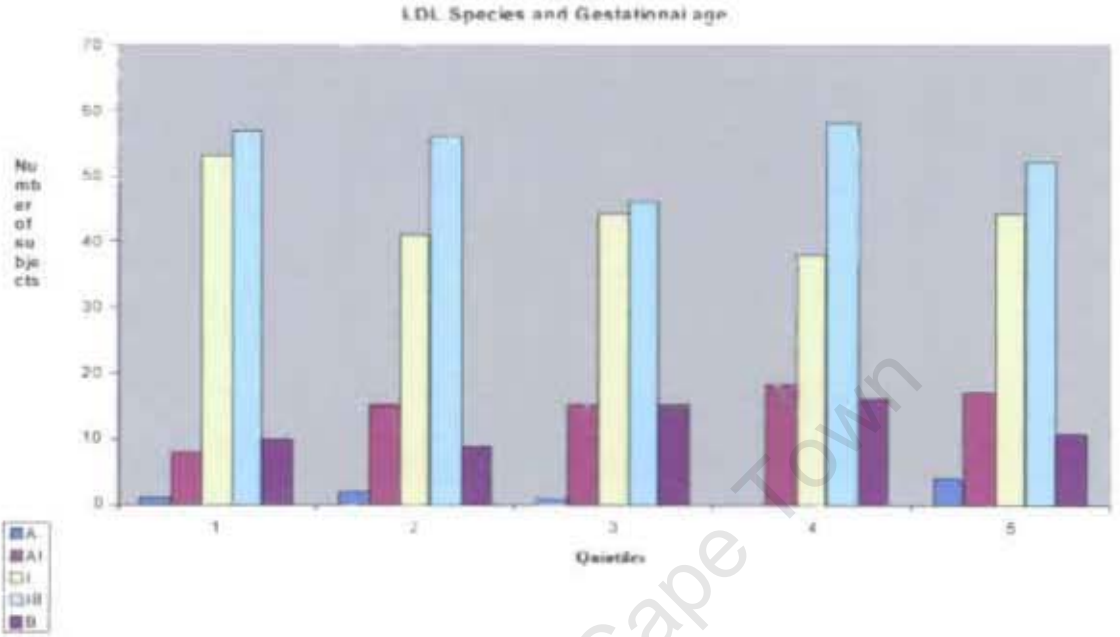
3= Quintile 3 (22 – 25 years)

4= Quintile 4 (25 – 28 years)

5= Quintile 5 (28 – 42 years)

The graph shows quintile 5 subjects as having the highest number with the small dense (B) LDL-Cholesterol species but this was not statistically analysed in this study. There is no influence of age on the LDL species during pregnancy. However in the general population several studies have found that males and older women have higher proportions of the small dense low-density lipoprotein. Results from the Framingham Offspring study 1992 reported a small (<255Å) dense low-density

lipoprotein prevalence of 30% in males. 5% premenopausal women and 14% postmenopausal women



Graph 5.5

The subjects were divided into five quintiles according to gestational ages

1= Quintile 1 (13 – 26 weeks)	Quintile 1 versus Quintile 3	p= 0.24
2= Quintile 2 (26 – 28 weeks)	Quintile 1 versus Quintile 4	p= 0.074
3= Quintile 3 (28 – 30 weeks)	Quintile 1versus Quintile 5	p= 0.056
4= Quintile 4 (30 – 32weeks)		
5= Quintile 5 (32 – 38 weeks)		

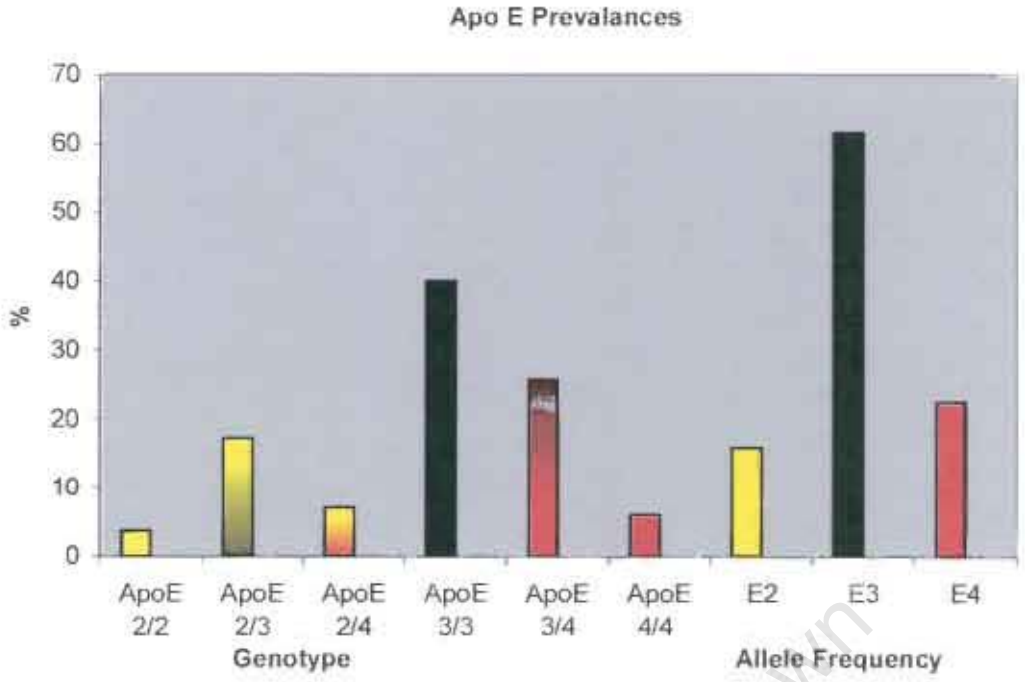
Low-density lipoprotein distribution appeared to favour smaller species with advancing gestation. evident by 28–30 weeks relative to 13–26 weeks. This did not reach statistical significance but a p value of <0.10 suggests that this may be a trend. Owing to limited equipment and time, the LDL particle size could not be derived as a continuous parameter to improve the power of this analysis. A greater sample size

could resolve the issue or a longitudinal study may also improve the resolution. Carl et al 1988 noted the same decrease by 16 – 20 relative to 5 – 12 weeks. They also reported that the average diameter decrease from early to late gestation was 13 Å. Other researchers have reported differences in the period during which the decrease was observed.

Number of subjects (% 678)

Genotype	Number of Subjects	Percentage
E2/2	25	3.7
E2/3	118	17.4
E2/4	48	7.1
E3/3	271	40.0
E3/4	175	25.8
E4/4	41	6.1
Allele	Number of Subjects	Percentage
ε2	216	15.90
ε3	835	61.60
ε4	305	22.50

Table 5.6 Tabulation of the apo E genotypes in the whole cohort



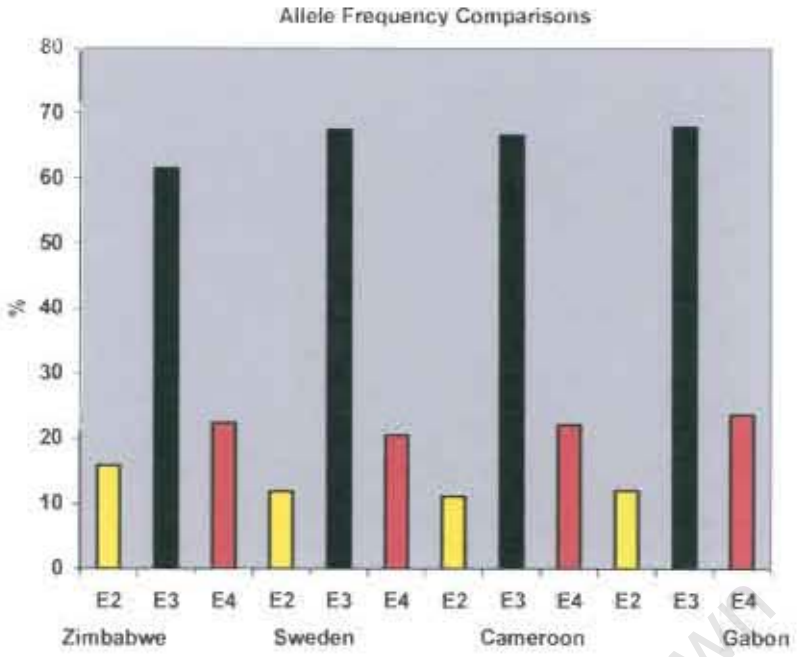
Graph 5.6

The Apolipoprotein E genotype percentage and Apolipoprotein E allele frequency in this study closely resembles the study from Central African Republic and other Africans from West, Central Africa and East Africa grouped together.

ApoE gene frequency reported in some countries. Countries have been arranged alphabetically.

Country	ε2	ε3	ε4
Cameroon	11.1	66.7	22.2
Gabon	12.0	68.0	20.0
Sweden	11.9	67.5	20.6
Zimbabwe	15.9	62.6	22.5

Table 5.7 These tabulated results are graphically represented below.



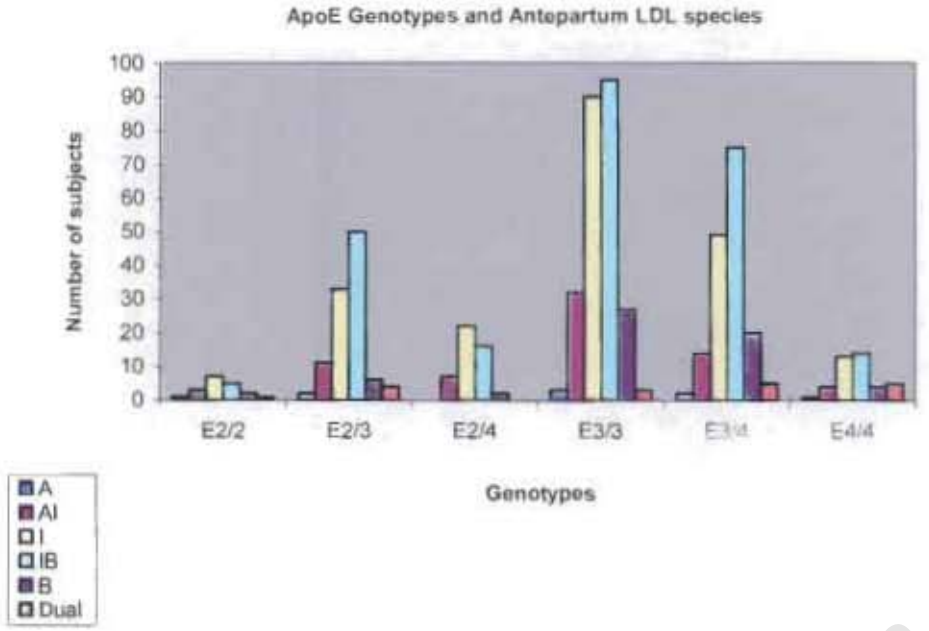
Graph 5.7

The comparisons shown in graph 7 were done by different investigators but reported in combined reports. Shona (Zimbabwe), Linkoping (Sweden) Gerdes et al 1994, Bamileke (Cameroon) and Fang (Gabon) Zekraoui et al 1997. The graph clearly confirms the already established fact that apoε3 is the most prevalent followed by apoε4 with apoε2 being the least.

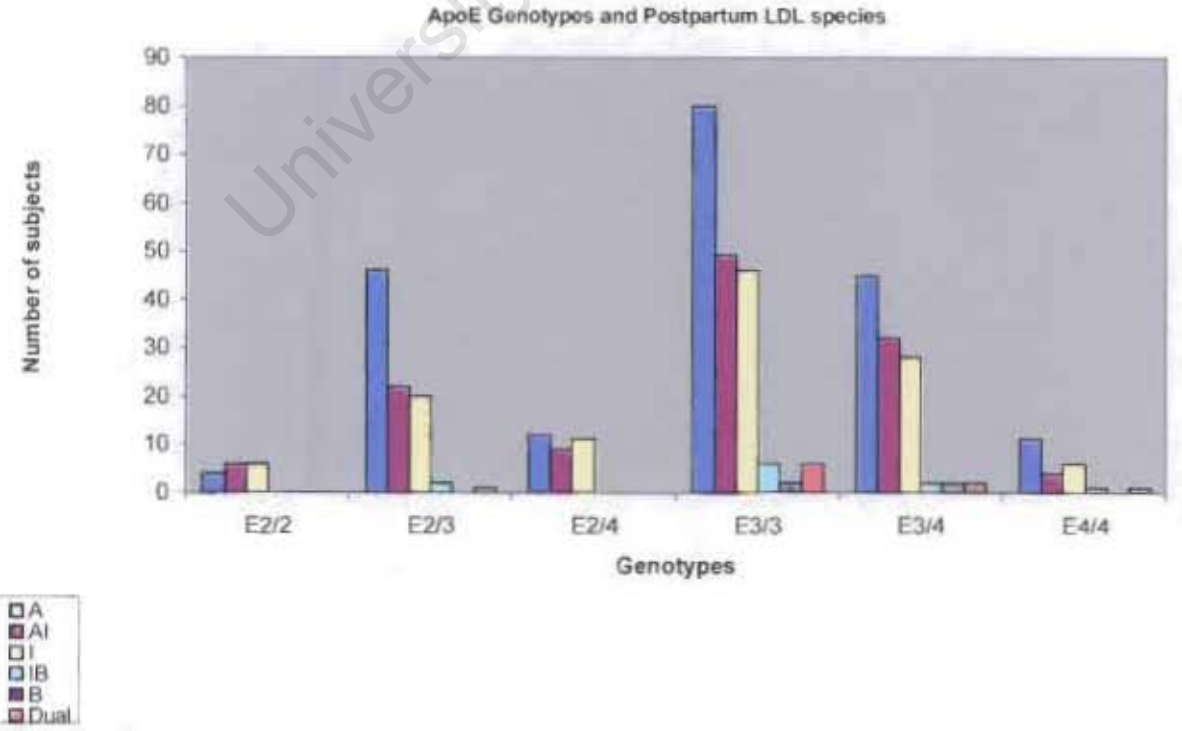
Table 5.8 below shows lipids and lipoprotein profiles in each of the six possible genotype combinations. Mean ±SD values for Ante partum and post partum lipids and lipoproteins in mmol/l.

Ante Partum						
	E2/2	E2/3	E2/4	E3/3	E3/4	E4/4
TC	3.85 ± 0.71	4.08 ±0.90	4.22 ±0.88	4.25 ±0.88	4.14 ±0.91	4.27 ±0.87
TG	2.12 ±1.67	1.77 ±1.77	1.57 ±0.80	1.60 ±0.83	2.31 ±6.49	2.14 ±3.80
LDLC	1.78 ±0.68	1.98 ±0.59	2.22 ±0.73	2.24 ±0.74	2.22 ±0.73	2.28 ±0.84
HDLC	1.26 0.44±	1.39 ±0.42	1.30 ±0.36	1.31 ±0.36	1.21 ±0.38	1.28 ±0.31
Post Partum						
	E2/2	E2/3	E2/4	E3/3	E3/4	E4/4
TC	3.48 ±0.89	3.43 ±0.77	3.59 ±0.74	3.58 ±0.73	3.60 ±0.84	3.63 ±0.60
TG	0.69 ±0.27	0.68 ±0.25	0.68 ±0.27	0.68 ±0.27	0.69 ±0.26	0.74 ±0.31
LDLC	1.84 ±0.83	1.92 ±0.60	2.10 ±0.63	2.09 ±0.63	2.14 ±0.67	2.14 ±0.60
HDLC	1.31 ±0.40	1.21 ±0.39	1.17 ±0.33	1.18 ±0.34	1.14 ±0.37	1.15 ±

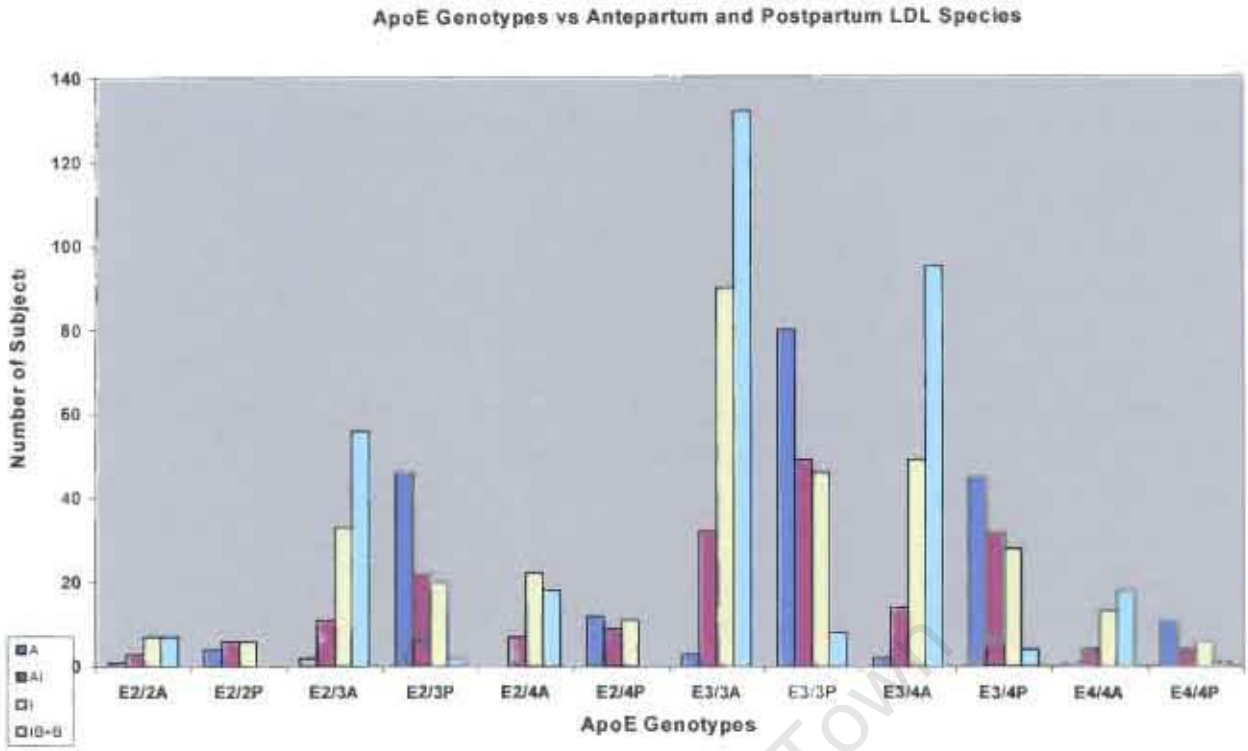
Table 5.8 There was no apparent influence of apoE isoforms on the ante partum and post partum lipids.



Graph 5.8 The smaller LDL species are predominant antepartum.



Graph 5.9 The graph shows that the larger LDL species are predominant postpartum.



Graph 5.10 This graph combines the two previous graphs 5.8 and 5.9.

This is probably one of the very few if not the first study in which the behaviour/changes of the LDL species was investigated in relation to the ApoE genotype. In order that larger numbers could be obtained for analysis, (A and AI), I and (IB and B) were compared.

Analysis revealed that the ApoE genotype did not influence LDL species distribution. The contingency table analysis of the distribution of LDL species revealed the following;

Ante partum "A"/ "I"/ "B" χ^2 $p = 0.33$ for E2/2 versus E3/3 versus E4/4

Postpartum "A"/ "I"/ "B" χ^2 $p = 0.53$ for the same subjects as analysed ante partum. The LDL species changed significantly with pregnancy in all the apoE genotypes "A"/ "I"/ "B" χ^2 $p = \leq 0.01$.

An examination was done to establish whether apoE isoforms ε2/X, ε3/3, ε4/X influence the LDL species changes, according to the LDL species that pertained in the postpartum state. χ^2 analysis of the change revealed that LDL (A) was not significant in all genotypes with a $p = 0.66$, LDL (AI) with $p = 0.09$ revealed that ε2 seems to be resistant or changed the least. LDL (I) with $p = 0.006$, ε2 changes the least but ε4 changes the most and for LDL IB and B with $p = 0.04$ ε4 changes the most. This provides evidence that apoE influences the susceptibility of LDL species size to change, with ε4 being most susceptible to change.

These categories, depicted in table 5.10, were derived from the data displayed in table 5.9.

	A		**AI**		**I**		**IB**		**B**	
	Ante	Post	Ante	Post	Ante	Post	Ante	Post	Ante	Post
E2/2	1	4	3	6	7	6	5	0	2	0
E2/3	2	46	11	22	33	20	50	2	6	0
E2/4	0	12	7	9	22	11	16	0	2	0
E3/3	3	80	32	49	90	46	95	6	27	2
E3/4	2	45	14	32	49	28	75	2	20	2
E4/4	1	11	4	4	13	6	14	1	4	0

Table 5.9 Tabulation of the numbers of subjects with various categories of LDL species according to apoE isoforms.

	A		AI		I		IB		B	
	Ante	Post	Ante	Post	Ante	Post	Ante	Post	Ante	Post
E2/X	3	62	21	37	62	37	71	2	10	0
E3/3	3	80	32	49	90	46	95	6	27	2
E4/X	3	56	18	36	62	34	89	3	24	2

Table 5.10 Tabulation of 3 variant causes of apoE according to LDL species categorization antepartum and postpartum for the analysis

Results of Genotyping for R145C

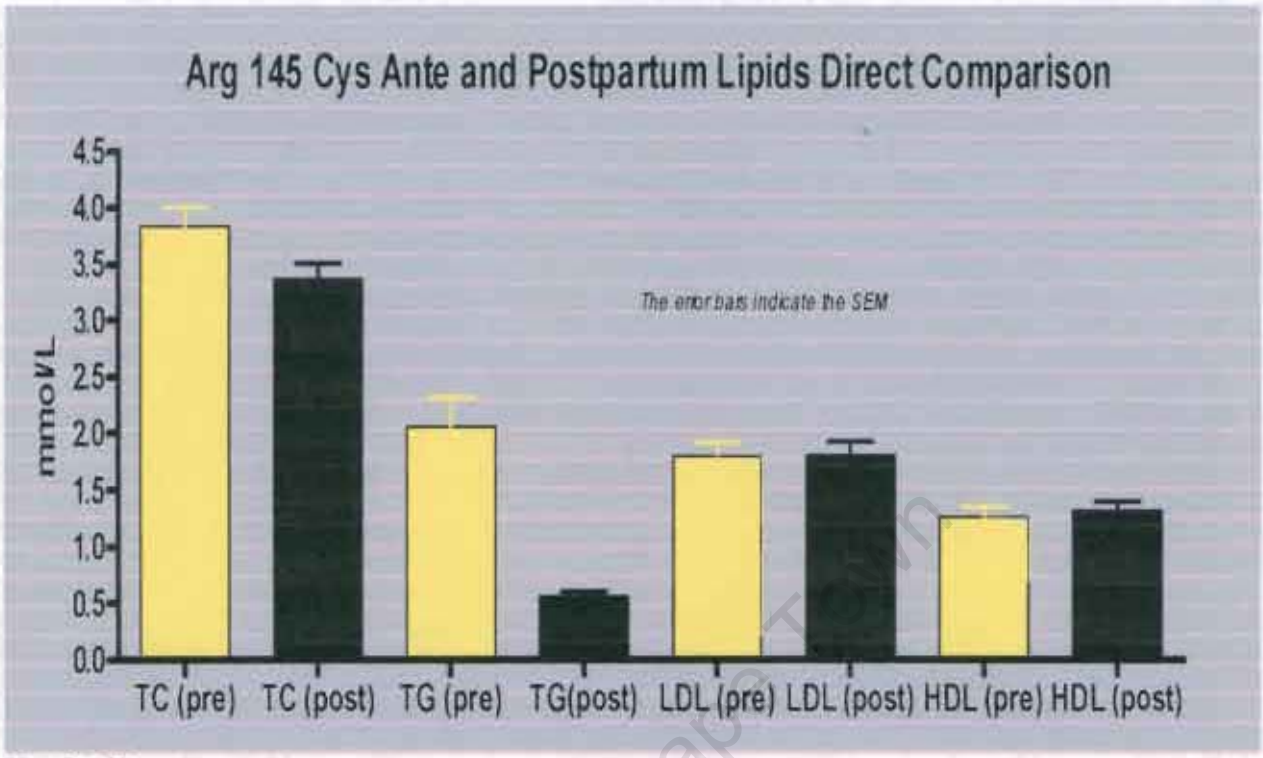
564 subjects with apoE3 background were genotyped for R145C mutation, revealing the mutation in 31 (5%) with 8 (26%) of them displaying a dysβ pattern on GGE. In this cohort 24 (77%) were apoE3/3, 5 (16%) were apoE3/4 and 2 (7%) were apoE2/3. There were 2 (7%) homozygous for R145C.

Arg 145 Cys Antepartum and Postpartum Lipid Profiles

		TC	TG	LDL-C	HDL-C
Antepartum	Median	3.8	2.1	1.8	1.3
	95%CI	3.5 - 4.2	1.5 - 2.6	1.6 -2.0	1.1-1.4
Postpartum	Median	3.4	0.57	1.8	1.3
	95%CI	3.1 - 3.7	0.49 - 0.64	1.6 – 2.1	1.2 - 1.5

Table5.11 These results are graphically represented in graph 5.12 below

Poor nutritional status and possible underlying disease could be the reason why lipid levels in this cohort is much lower than reported in other studies.



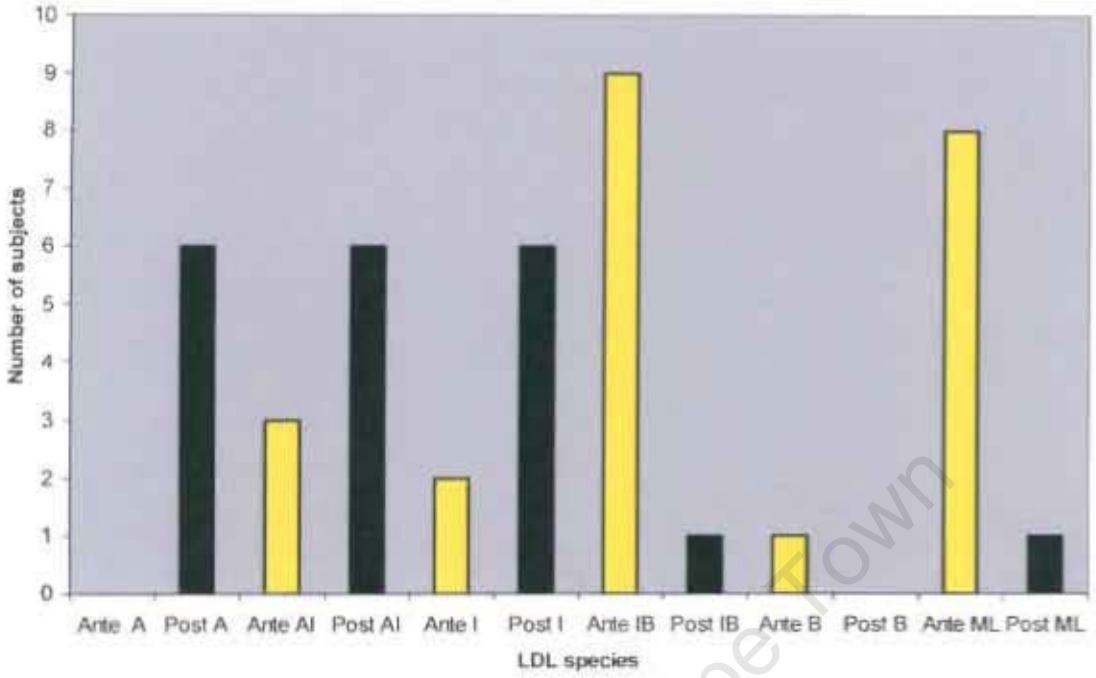
Graph 5.11

The Arg145Cys ante partum and postpartum lipids show concentrations are similar to the other ApoE genotypes.

LDL species		A	AI	I	IB	B	MI
Ante partum	%	0	3	2	9	1	8
Post partum	%	6	6	6	1	0	1

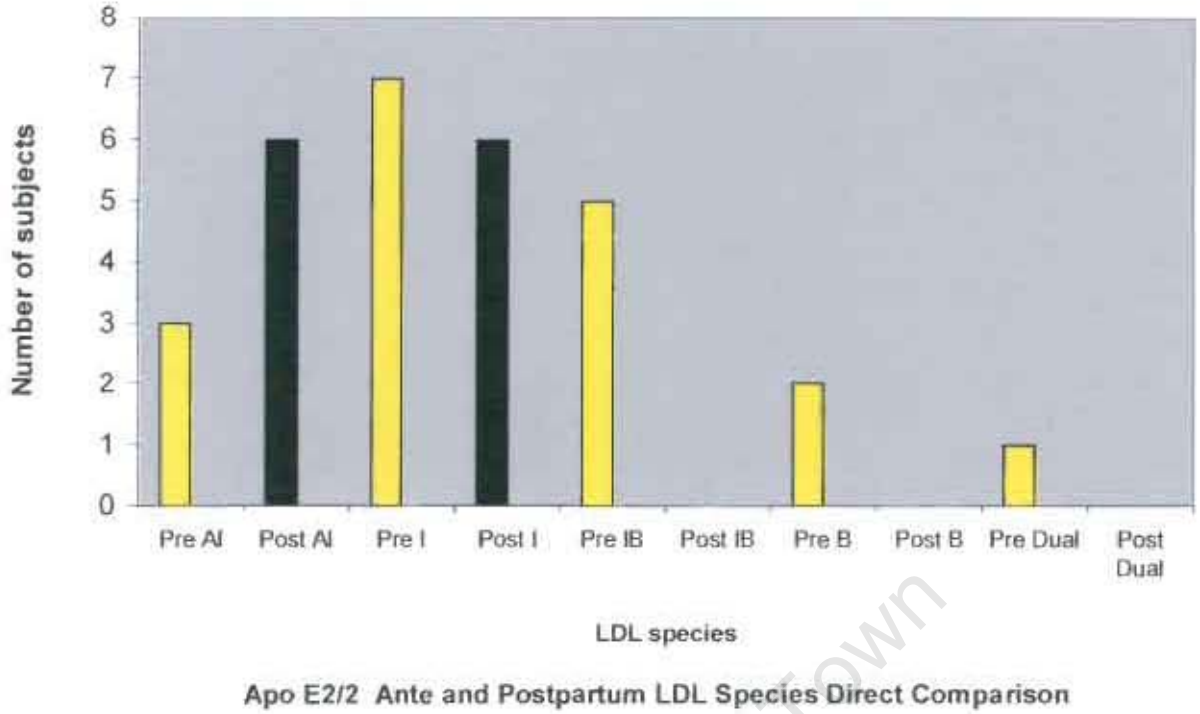
Table 5.12

The influence of apoE R145C on LDL species was examined, and the results in table 5.11 show that smaller LDL species are predominant during antepartum. 8 (26%) in this cohort clearly displayed a dysβ electrophoretic pattern on GGE.



Graph 5.12

Arg145Cys Antepartum and Postpartum LDL species also display a similar pattern with the smaller species predominant during antepartum and reverting to the larger species postpartum. The large (A) species is absent antenatally while the smaller (B) is absent postnatally. The ML = Dysβ pattern on GGE constitutes 25% of all the Arg145Cys subjects patients during pregnancy and there is a reversion from dysβ pattern in 7/8 of these subjects post partum. The same phenomenon was examined for apoE2/2.



Apo E2/2 Ante and Postpartum LDL Species Direct Comparison

Graph 5.13

25 (4% of whole cohort) subjects who were homozygous for apoE2/2. Of this cohort, 6 (24%) displayed a classical MI characteristic on GGE during antepartum. Only 1(4% of apoE2/2) displayed a classical MI pattern post partum. From these findings it is clear that pregnancy presents an adequate metabolic stress for converting susceptible genotypes to dys2

CHAPTER SIX

6.1 Limitations of Study

The study was conducted during an economic depression in Zimbabwe and limited budgets and equipment in South Africa. In Zimbabwe this posed problems because it was a period when no budget could be followed because costs were going up every 24 hours at rates as high as 500%. The incentives given to both patients and staff needed to be reviewed daily. Secondly the acute shortage of food and general commodities resulted in severe staff absenteeism from work in search of these commodities. For example sessions would be abruptly abandoned if word got round that there some 'bread' had just arrived at a nearby shop as the subjects and staff chose to go and queue for what ever commodity that would have arrived. The economic state might have affected the subjects since most of them could not afford a single meal a day. The ante partum period faced imminent collapse because the few who initially came, did so without fasting and were requested to return the following day after an overnight fast. Their return was not guaranteed, as the lack of price control would see transport costs fluctuating. This was made worse by the lack of support from any institution including the University as no one had any spare funds in their budgets. The second reason also related to the economic situation was that most of them with healthy babies did not see the need to come back as transport re-imbursement offered earlier during the antenatal period was no longer enough even for a single trip to the clinic (the transport costs had risen by 500-1000 %). The third problem was that some subjects came from other parts of Harare to live

with relatives in order to attend these clinics because of their good antenatal care record. Other subjects came from their own newly acquired homes in and around Harare to live with their parents in order to give birth in or around their parents' home (maiden home) according to African culture. In order to overcome these problems home visits were scheduled and more appropriate transport incentives were made. Those who had gone back to other parts of Harare were contacted and separate arrangements were made to either meet them at the nearest clinic or to have venesection done at home. The remaining 20% had either gone back to their rural homes >50-kms from Harare or were untraceable. It was very sad to see people abandoning their professional post in search of day-to-day living commodities, things that are taken for granted in South Africa. The proportions of degeneracy would have been difficult for an individual to present without the reports in the newspapers. Family financial and assets sacrifices, whose effects will persist for a very long time, overcame this and kept the project running. The Lipid Research Fund without which this project would not have taken place was also limited in that not all the assays were done especially lipoprotein (a).

6.2 Lipids and Lipoprotein Values

The study confirmed several other reports that normal human pregnancy is characterized by rises in serum TG and cholesterol, with reversal postpartum. The study was limited by a lack of standardization of meal content and the interval between last meal and venepuncture, introducing a potential source of error to analyses involving TG. However, recent prospective studies of myocardial

infarction [Stampfer] and coronary artery disease [Gardner CD] reveal strong inverse correlations between nonfasting TG and LDL diameter; the latter study pointed out the nonfasting TG may be more indicative of 24-hour average TG levels [Gardner CD]. Nevertheless, the relationship between TG and LDL size in our study might not be equivalent to the fasted state in all respects. There is no significant effect of prandial status on LDL particle diameter. [Gardner CD, MacNamara] During the first half of normal pregnancy, increased maternal adipose fat accumulation sets the stage for the physiologic hyperlipidaemia of late gestation. [Herrera] Serum lipid changes seen in this study, documented previously, [Potter, Alvarez] include increases in TG and total cholesterol concentrations, with TG increasing more than the other lipids. Interestingly, in this study LDLC did not change significantly. This may be as a result of a low baseline concentration and needs further investigations. Despite this, LDL species changed between ante partum and post partum. The physiological mechanisms involved in the gestational increases in TG include increased adipocyte lipolytic activity resulting from the insulin-resistant condition of late gestation. [Alvarez] The resultant increased release of free fatty acid and glycerol into the circulation increases substrate for hepatic TG (VLDL) synthesis. Oestrogen-induced increases in hepatic output of VLDL also likely occur. [Knopp 1992] In addition, both adipose tissue lipoprotein lipase and hepatic lipase activities decrease during normal pregnancy (effects reportedly related to insulin resistance and increased oestrogen, respectively), whereas placental lipoprotein lipase increases as term approaches. These changes probably

impair removal of TG-rich lipoproteins from the circulation [Alvarez] and boost transfer of maternal essential fatty acids to the growing fetus. [Knopp 1992, Coleman] the decrease in diameter of the predominant LDL subclass during pregnancy could reflect increased production and/or decreased clearance of small dense LDL particles. [Silliman 1994] Increased transfer of TG from VLDL to LDL coupled with hydrolysis of TG in LDL, even with lower gestational activity of hepatic lipase, may increase production of small, dense LDL particles during pregnancy. [Sattar 1999] Additionally, more TG-enriched VLDL may be metabolized to smaller LDL by virtue of its composition.

6.3 LDL Species

This study confirmed other reports of a decrease in diameter of the predominant LDL subclass during pregnancy. This change and the accompanying rise in TG are transient because LDL reverts to larger particles and TG concentrations decrease postpartum. Blood lipoproteins exert a variety of cellular effects in addition to lipid transport, and many of these effects are modulated by size and density distribution. Small, dense LDL particles are intrinsically less resistant to oxidation; and thus may contribute to oxidative stress and later vascular endothelial cell dysfunction in the grandmultiparous subjects. However despite the changes in LDL profile during pregnancy, it was reported that the oxidative resistance of LDL increases with progressing gestation, which could be partially explained by the concomitant increase in plasma vitamin E levels. However, despite the increase in maternal

levels of Vitamin E and, it was reported that other lipid soluble vitamins with antioxidant activity, such as 2-carotene and vitamin A and decrease in plasma throughout gestation. This led to an evaluation of changes in levels of oxidized low-density lipoprotein (Ox-LDL) during pregnancy and how they correlate with changes in LDL size and serum total antioxidant status (TAS). [Belo 2004]

Studies in Zimbabwe, by Gelfand M et al; Clinica Chemica Acta, 1974 (57) 131-134 showed that Africans had lower cholesterol levels than their European counterparts. Adebisi SA et al; showed that pregnant Nigerian women had significantly lower total cholesterol and HDL-Cholesterol than their non-pregnant counterparts, Niger Postgrad Med J 2004 (1) 1-3. This study concurs with the Nigerian study.

6.4 HIV and Dyslipidaemia

The HIV statistics in Zimbabwe as published in two newspapers articles warrant some comments on the dyslipidaemia associated with HIV. Two reports in the National Newspaper 'The Herald' on the 28th October 2004 and 20th December estimated that nearly two million (20%) people in Zimbabwe were HIV positive. Close to a million children in the country have lost one or both parents as a result of HIV and Aids, the United Nations Children's Fund (UNICEF) country representative to Zimbabwe Dr Festo Kavishe has said. In a statement to mark the launch of the State of the World's Children Report for the year 2005, Dr Kavishe said he expected the number of children orphaned by Aids to grow in the next year.

"By 2003 some 2, 1 million children under the age of five were living with HIV and Aids, most of them infected during pregnancy, birth or breastfeeding. "Zimbabwe, with one of the world's highest prevalence rates, registered the largest swells in child death rates from 1990 to 2002," he said. At least 1,8 million people in Zimbabwe are living with HIV and Aids, while more than 2 000 people die each week from Aids-related illnesses; Herald Health Reporter. It can thus be expected that about 1/5 of the subjects in this study was HIV positive. The study did not include the HIV status and the time required for additional ethics approval was likely to be too long and the funding for this additional parameter was lacking. None of the subjects were likely to be on conventional treatment for HIV, even though some would probably be on some traditional treatment. The traditional treatment for HIV still requires an evaluation on their effect on lipids and lipoproteins. Sharon A et al reported in JAMA June 11 2003, Vol 289 No 22 that before the availability of highly active antiretroviral therapy (HAART) studies in persons infected with human immunodeficiency virus (HIV) demonstrated lipid abnormalities, especially hypocholesterolaemia with and without hypertriglyceridaemia. An association between plasma TG levels and circulating interferon K level has been observed in persons with AIDS. A pattern of hyperlipidaemia characterized by elevated total cholesterol, low-density lipoprotein cholesterol, TG and reduced level of high-density lipoprotein cholesterol has been observed in patients treated with protease inhibitors. Ducobu J, et al reported that HIV infection induces an early decrease of cholesterol and a late increase of TG (TG) with a reduction of HDL. These changes

are proportional with the lowering of CD4, which reflects the infection's severity. Both the increase of TG synthesis and the decrease of TG catabolism, in relation with a reduction of lipoprotein lipase activity, are responsible of these changes. Moreover, LDL catabolism is enhanced by macrophage scavenger receptors, due to a high proportion of small, dense LDL, which are more easily oxidized.

An association of hypocholesterolaemia and HIV infection in our subjects can therefore most likely be linked to the observations made before the availability of HAART because none of the subjects could afford them, as they were only available in the private sector during the time of the study. There is currently no published work comparing the lipoprotein patterns in HIV-positive and HIV-negative pregnant subjects. Hyperlipidaemia is frequently associated with antiretroviral treatment caused concern about the possible increased cardiovascular risk in treated HIV-positive patients. It has been shown that hypocholesterolaemia found in HIV-positive patients is frequently associated with the elevation of very low-density lipoprotein (VLDL). Mauss S et al, AIDS: Volume 17(2) 24 January 2003 pp 189-194 used two well-described genetically inherited lipid disorders that involve an increase in VLDL as models to analyse the lipoprotein pattern in HIV-positive patients with elevated VLDL and to estimate their cardiovascular risk. Familial combined hyperlipidaemia is caused by overproduction of small VLDL

particles in the liver, leading to a parallel increase in apolipoprotein B and is associated with increased cardiovascular risk. Familial hypertriglyceridaemia is characterized by production of normal levels of large VLDL particles containing more TG than normal resulting in normal apolipoprotein B levels and without or with only modestly increased cardiovascular risk The lipoprotein patterns in HIV-positive patients with and without antiretroviral treatment, HIV-seronegative patients with familial combined hyperlipidaemia (high cardiovascular risk) or with familial hypertriglyceridaemia (low cardiovascular risk) as well the size of VLDL particles were measured in a subgroup of HIV-positive were analysed. In the HIV-positive patients, the ratio of VLDL-TG to VLDL-apolipoprotein B was 16.2 ± 6.0 (range, 9.4-42.5). This ratio was not different from the ratio found in patients with familial hypertriglyceridaemia, 16.9 ± 6.0 (range, 8.8-24.3; P = 0.61). In contrast, the ratio of VLDL-TG to VLDL-apolipoprotein B in these two groups differed highly from the 10 patients with familial combined hyperlipidaemia, who had a ratio of 6.7 ± 1.0 (range, 5.3-8.5; P < 0.00001). In addition, patients with familial combined hyperlipidaemia showed increased serum apolipoprotein B compared with patients with HIV-associated hyperlipidaemia or familial hypertriglyceridaemia. This analysis of VLDL particle size indicated that the particles in HIV-positive patients were large, similar to those in patients with familial hypertriglyceridaemia. Since this study fulfills the reference range required numbers, the lipid levels can be used to establish the third trimester reference range for a similar population.

6.5 ApoE 2/3/4 and R145C

4% of the subjects have an apoE Arg145Cys mutation further adding credence to a report by de Villiers et al 1997 that this mutation could be prevalent among the African population. However the lipid levels in this cohort is much lower than those reported by de Villiers et al 1997 and Hsia et al 1995. A factor that seems to have affected our subjects is probably nutrition and underlying disease to explain lower post partum levels but pregnancy appeared to trigger dysβ at least temporarily. Dyslipidaemia is unusual in Zimbabwean women during their reproductive years with 1% revealing serious hypertriglyceridaemia, 4% hypobetalipoproteinaemia and hypoalphalipoproteinaemia. Dysβ present during pregnancy reverted after postpartum in most subjects. Pregnancy increases TG, Total Cholesterol, and High Density Lipoprotein-Cholesterol. Low Density Lipoprotein-Cholesterol remained unchanged but the size decreased. Apolipoprotein E genotypes are similar to those reported for other populations. The LDL size distribution is similar in all apolipoprotein E subsets. Apolipoprotein E genotype may influence change in LDL species and this is the first time that evidence has been found that apolipoprotein E influences the susceptibility of LDL to change.

6.2 Future Studies

1) Analysis of modulating factors antepartum and postpartum
2) Analysis of hypoβ to include primary and secondary causes.

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APPENDIX 1

UNIVERSITY OF CAPE TOWN
Health Science Faculty Lipid Laboratory
and
Groote Schuur Hospital Lipid Clinic

UNIVERSITY OF ZIMBABWE
Department of Obs and Gynaecology
and
Harare City Health Department

ZVIRIMAERERANO NEONGORORI INO

Mubasa iri, tichazenge tichiogorora mhando dzemafuta onowanikwa munvanhu, kuparadzana kwawo kana achifambiswa nemagetsi nekuonawo zviripamusoro pemhodzi inoita basa rekugadzirwa kwemafuta anowanikwa muropa. Wongororo yose inoda ropa shomanini (chipunu chimwe ne chidhimbu) chete richatorwa katatu. Mafuta akawanda muropa onogona kukanganisa hutano asiwo mafuta aya, anogona kuwanikwa akawanda panguva yekuzvitakura ndukusaka tichanda rimwe ropa mushure mekusumunguka. Kana pachinge pewanikwa zvingakanganise utano,chiremba wenyu anoziviswa. Kana pane chakaipa chingazowira munhu nekuda kwemafuta, munhu iyeye onobva ataurirwa nenyanzwi yezvemafuti nzira dzekuzvidzivirira nadzo kutiitira kuti aderedze njodzi yekukuvadzwa kwemoyo.

BVUMIDZO YEKUONGORORWA KWEMAFUTA MUNE VAKAZVITAKURA
Ini ndinobvuma kuti ini kana hama dzangu dzitorwe ropa kana zvimwewo kuti zviongororwe nekuti zvizopfibikwa kana zvasara.

Zita.....

Kero Yekumba.....
Nhengo ichange iri : mhodzi dzinobva muropa O,
Mushure mekutsanangurirwa zvakakwana ndinonzwisisa kuti:
*Kutorwa kwembodzi uku, hakuna njodzi yakakura nekuti nzira nezvinhu zvinoshandiswa ndizvo zvirimumuteno.
*Mhodzi idzi nezvichawanikwa mukuongororwa uku, zvichachengetwa zvakatsindidzwa zvakanyanya, zvinoenderana nemiteno yekurapwa kwevarwere uyezve nekutevedzera bumbiro remiteno yetsiksa dzavanhu yakatarirwa vaongorori neUniversity. Mhodzi idzi dzichachengetwa dzichizivikanwa nemucherechedzo we nhamba inenge usina zita rangu. Bvumo yangu yakanyorwa ichazenge ichidiwa kuti zviitiko nezvose zvinoenderana nekuongorora uku zvitaurirwe kana kuratidzwa kune mumwe munhu kana chikwata.
*Chikonzero chechirwere kana chechiitiko chinogona kusabuda pachena nekuda kwekusanzwisisa zvakakwana pamusoro pezvinoitwa nedzinde iri kunyanya kana zvisingakwanisike kuti riongororwe mumhuri kana kuti muhama dzose, uyezve nekuti mamwewo madzinde anogona kupomhodza kana kukuchidzira maitiro idzinde iri. Izvi zvose zvinogona kuti ndizopa dzimwe modzi kana kuti mhuri yengu yozoongororwa mushure mokunge ndatsanangurirwa vamwe vanhu vasati vakumbirwa
*Mhodzi dzinonga dzakapfimbikwa, dzinogona kutorwa muchihwandehwande kuti dzishandiswe mukutsvaka nekutsigira kuburitsa pachena.umbowo utsa.
*Ndinogona kubvisa bvumo yangu muongororo iyi pandinenge ndada uyezve zvisingazoshandura marapirwo angu munaramangawana.

Signature.....Nzvimbo.....

Chapupu (Nyora zvinoonekwa.....Zuva.....

APPENDIX 2

UNIVERSITY OF CAPE TOWN
Health Science Faculty Lipid Laboratory
and
Groote Schuur Hospital Lipid Clinic

UNIVERSITY OF ZIMBABWE
Department of Obs and Gynaecology
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Harare City Health Department

INFORMATION ABOUT THE STUDY

This study will examine the fat in the blood (cholesterol and triglyceride) and how it is distributed into different particles (lipoprotein electrophoresis) and a gene that plays a role in governing the fat concentration in the blood. The study will require only a small amount of blood (1.5 teaspoons) at 3 visits. Excessive fat in the blood can be harmful to health but may only relate to the time that you are pregnant – that is why a sample should be taken after the pregnancy is over. If a problem is found during or after the pregnancy, the result will be made known to your doctor. If a significant lipid problem is discovered, you would be able to undertake preventive measures and lessen the risk of heart disease on the advice of an expert clinic.

CONSENT FOR STUDY OF PLASMA LIPOPROTEINS IN PREGNANCY

NAME.....

ADDRESS.....

Analysis and Storage of Biologic Samples: Plasma and DNA

I hereby consent to the removal, processing, storage and analysis of the above material from my own body for the purpose of diagnosis and research into disorders of lipid and lipoprotein metabolism. After due explanation, I understand that:

(1)Conventional procedures and techniques are employed and that the health risk is minimal,

(2)The material and results of the investigations remain strictly confidential according to medical practice and such ethical guidelines as govern research at the universities. To preserve anonymity, the samples are coded by numbers and my written permission is required for release of identifiable information to another party.

(3)Precise diagnoses may not always be possible because the defect(s) may not yet be known or there is inadequate other information to derive the defect owing to modulatory roles that other genes or the environment may play.

(4) The stored material may be used anonymously in future to derive new information or for research purposes. Such future use may be of no direct benefit to the subject.

(5) Permission to participate in the study may be withdrawn at any time and any stored biological material will also be destroyed. The withdrawal will not affect the subject's future medical care.

Study Subject Name..... Signature.....

Witness Name..... Signature .....

Date..... Place.....