AN INVESTIGATION INTO
THE AETIOLOGY OF ACUTE
Fatty Liver of
Pregnancy

Thesis presented for the degree of Doctor of Philosophy
at the Institute for Women’s Health, University College
London

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SIGNED DECLARATION

I, Mandeep Kaur Kaler confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.
ACKNOWLEDGEMENTS

I would firstly like to thank my supervisor Dr. David Williams, for the opportunity to accomplish this exciting PhD. Under his guidance I have developed both as an individual and clinical academic. I am extremely grateful for all of his encouragement and support throughout the PhD.

My work would not have been possible without the help and guidance of several people. I would like to thank Dr. Phil Hennis at the ISEH who helped develop my exercise protocol and supported me with all of my studies as well the analysis. I would also like to thank Dr. Hywel Williams who made it possible to perform and analyze next generation sequencing of the families affected by AFLP in my study.

Most importantly I would like to thank all of my research participants including pregnant patients and families affected by AFLP. I must also thank Dr. Sara Hillman who has not only helped me recruit to the study but has been a much-appreciated mentor.

Finally, I would like to thank my family and friends who have been with me every step of the way. I could not have done this PhD without all of your continuous love and support. Thank you. X
ABSTRACT

Acute fatty liver of pregnancy (AFLP) is a rare, but devastating gestational syndrome. Women with AFLP present in the third trimester with the clinical and biochemical characteristics of a defect in energy metabolism. A minority of AFLP cases are associated with a defect in mitochondrial fatty acid oxidation (LCHAD deficiency). The main aim of my thesis is to discover whether women who have had AFLP, but who do not have LCHAD deficiency, have an alternative subclinical defect in fat metabolism.

I firstly studied the clinical records of 33 women who had AFLP. None of the women (n=28) nor 5 offspring of the remaining 5 women had the common LCHAD gene variant. Only 19 of these women had urinalysis at the time of presentation and despite prolonged starvation, none of them had ketonuria. This observation supported my hypothesis that AFLP is associated with a defect in fatty acid oxidation (FAO).

Having confirmed that fasting during the third trimester of healthy pregnancy leads to accelerated ketosis, I tested the hypothesis that non-pregnant women who had AFLP have a sub-clinical defect in fat metabolism. Following a 24-hour fast and fat-burning exercise, women who had AFLP (n=13) generated ketones at a similar rate to 23 women who had not had AFLP. Furthermore, a proteomic analysis showed no difference in 1300 serum proteins between women who had AFLP and those who had a normal pregnancy.

The whole exome sequence (3 AFLP families) and whole genome sequence (4 AFLP families), did not identify any novel gene variants associated with defective energy metabolism.

My results suggest AFLP is a pregnancy-specific defect in maternal fatty acid oxidation, which has no latent impact on maternal FAO. The study of women with AFLP during pregnancy is necessary to identify altered concentrations of a pregnancy-specific factor that inhibits maternal FAO.
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<tr>
<td>5HB</td>
<td>5-β-hydroxybutyrate</td>
</tr>
<tr>
<td>A2M</td>
<td>Alpha-2-macroglobulin</td>
</tr>
<tr>
<td>ACC</td>
<td>Acetyl coenzyme A carboxylase (ACC)</td>
</tr>
<tr>
<td>Acetyl COA</td>
<td>Acetyl Coenzyme A</td>
</tr>
<tr>
<td>AcAc</td>
<td>Acetoacetate</td>
</tr>
<tr>
<td>Ac-CoA</td>
<td>Acetyl CoA</td>
</tr>
<tr>
<td>AcAc-CoA</td>
<td>Aceto-acetyl CoA</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>AFLP</td>
<td>Acute Fatty Liver of Pregnancy</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine transaminase</td>
</tr>
<tr>
<td>AMPK</td>
<td>Activated protein kinase (AMPK)</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate transaminase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>CAT</td>
<td>Carnitine/acylcarnitine translocase</td>
</tr>
<tr>
<td>CETP</td>
<td>Cholesterol ester transfer protein activity</td>
</tr>
<tr>
<td>CK</td>
<td>Creatinine Kinase</td>
</tr>
<tr>
<td>CPET</td>
<td>Cardiopulmonary exercise testing</td>
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CPT1  Carnitine Palmitoyltransferase 1
CPT2  Carnitine Palmitoyltransferase 2
cAMP  Cyclic Adenosine Monophosphate
CT   Computed Tomography
DLK1  Delta-like homolog 1
DMT1  Divalent metal transporter 1
DNA  Deoxyribonucleic Acid
e⁻   Electron carriers
EFA  Essential fatty acids
ELISA  Enzyme-linked immunosorbent assay
ETC Chain  Electron transport chain
FABPpm  Plasma Membrane bound Fatty acid binding protein
FACS  Fatty acyl CoA Synthase
FAD  Flavin Adenine Dinucleotide
FAO  Fatty Acid Oxidation
FAOD  Fatty Acid Oxidation Disorder
FAT  Fatty acid translocase
FATP  Fatty acid transport proteins
FGF21  Fibroblast growth factor 21
GDP  Guanosine Diphosphate
GGT  g-glutamyl transferase
<table>
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<tr>
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<tr>
<td>GLP-1</td>
<td>Glucagon Like peptide-1</td>
</tr>
<tr>
<td>GPC2</td>
<td>Glypican-2</td>
</tr>
<tr>
<td>HELLP</td>
<td>Haemolysis, elevated liver enzymes and low platelets</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia inducible factor</td>
</tr>
<tr>
<td>HMG-CoA</td>
<td>3-hydroxy3-methylglutaryl-CoA</td>
</tr>
<tr>
<td>HMGCS2</td>
<td>3-hydroxy-3-methylglutaryl-CoA synthase</td>
</tr>
<tr>
<td>HRS</td>
<td>Hepato-renal syndrome</td>
</tr>
<tr>
<td>HSL</td>
<td>Hormone Sensitive Lipase</td>
</tr>
<tr>
<td>hPGH</td>
<td>Human placental growth hormone</td>
</tr>
<tr>
<td>HDL</td>
<td>High density Lipoprotein</td>
</tr>
<tr>
<td>HL</td>
<td>HMG-CoA Lyase</td>
</tr>
<tr>
<td>HPL</td>
<td>Human placental lactogen</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
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<td>IGFBP-1</td>
<td>Insulin-like growth factor binding protein-1</td>
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<tr>
<td>ISEH</td>
<td>Institute of Sport, Exercise and Health</td>
</tr>
<tr>
<td>INR</td>
<td>International normalized ratio</td>
</tr>
<tr>
<td>LCHAD</td>
<td>Long Chain 3-hydroxyacyl-CoA Dehydrogenase</td>
</tr>
<tr>
<td>LCPUFA</td>
<td>Long chain polyunsaturated fatty acids</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density Lipoprotein</td>
</tr>
<tr>
<td>LHYD</td>
<td>Long Chain 2,3-enoyl-CoA</td>
</tr>
<tr>
<td>LKAT</td>
<td>Long Chain 3-ketoacyl-CoA thiolase</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>MAP</td>
<td>Mean arterial blood pressure</td>
</tr>
<tr>
<td>MCAD</td>
<td>Medium Chain acyl-CoA Dehydrogenase</td>
</tr>
<tr>
<td>MCT</td>
<td>Medium Chain triglycerides</td>
</tr>
<tr>
<td>mHS</td>
<td>Mitochondrial HMG-CoA synthase</td>
</tr>
<tr>
<td>MFO</td>
<td>Maximal fat oxidation</td>
</tr>
<tr>
<td>MRS</td>
<td>Magnetic Resonance Spectroscopy</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
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<tr>
<td>MTP</td>
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<td>Nicotinamide Adenine Dinucleotide (Reduced)</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NAFLD</td>
<td>Non-alcoholic fatty liver disease</td>
</tr>
<tr>
<td>NICE</td>
<td>National Institute for Health and Care Excellence</td>
</tr>
<tr>
<td>NEFA</td>
<td>Non-esterified fatty acids</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>PCr</td>
<td>Phosphocreatinine</td>
</tr>
<tr>
<td>PDE</td>
<td>Phosphodiesters</td>
</tr>
<tr>
<td>PEP</td>
<td>Phosphoenolpyruvate</td>
</tr>
<tr>
<td>Pi</td>
<td>Inorganic Phosphate</td>
</tr>
<tr>
<td>PME</td>
<td>Phosphomonoesters</td>
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<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator activated receptor</td>
</tr>
<tr>
<td>PYY</td>
<td>Peptide YY</td>
</tr>
<tr>
<td>RER</td>
<td>Respiratory exchange ratio</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RQ</td>
<td>Respiratory quotient</td>
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<tr>
<td>SCAD</td>
<td>Short Chain acyl-CoA Dehydrogenase</td>
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<tr>
<td>SCOT</td>
<td>Succinyl-CoA:3-oxoacid CoA transferase</td>
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<tr>
<td>T2</td>
<td>Acetoacetyl-CoA thiolase</td>
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<td>TAG</td>
<td>Triacylglycerols</td>
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<tr>
<td>TCA Cycle</td>
<td>Tricarboxylic Acid Cycle</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour Necrosis Factor-α</td>
</tr>
<tr>
<td>UDPG</td>
<td>Uridine diphosphoglucone</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low-density lipoprotein</td>
</tr>
<tr>
<td>VLCAD</td>
<td>Very long-chain acyl-CoA dehydrogenase</td>
</tr>
<tr>
<td>VO₂</td>
<td>Volume of oxygen</td>
</tr>
<tr>
<td>VCO₂</td>
<td>Volume of carbon dioxide</td>
</tr>
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</table>
CHAPTER 1

INTRODUCTION
Chapter 1 Introduction

Acute Fatty Liver of pregnancy (AFLP) is a rare complication of pregnancy that affects approximately 1 in 10 000 pregnant women in the UK (1). Although rare, it is a life-threatening complication of late pregnancy. Criteria for the diagnosis of AFLP have been derived from a group in Swansea (2). In the UK, recently reported maternal mortality rates are 2% and fetal mortality 11%, but morbidity rates are much higher although less well documented (3). In particular, fetal morbidity includes lifelong cerebral palsy.

AFLP typically develops in the third trimester of pregnancy with non-specific symptoms such as abdominal pain, nausea, vomiting, lethargy and thirst. AFLP is more common in first-time pregnant mothers, underweight women, pregnancies with male fetuses and mothers of multiple gestations (twin pregnancy). Delivery of the fetus is the only cure (1, 3).

The clinical and biochemical presentation of AFLP suggests strong similarities with inherited disorders of fatty acid oxidation (FAO) (4). See Table 1-1. Defects in FAO result in reduced mitochondrial adenosine triphosphate (ATP) production, reduced ketogenesis and multi-organ dysfunction as manifest in the liver (synthetic dysfunction), kidney (renal failure), brain (encephalopathy), myocardial tissue (cardiomyopathy) and skeletal muscle (weakness). Despite its name, AFLP is therefore a multi-organ disorder affecting the same organs as FAO disorders (4).

There is further evidence to support the idea that AFLP has a genetic basis, which in a vulnerable mother might lead to AFLP. This complication of pregnancy has been associated with deficiencies in four enzymes required for normal FAO, specifically; long chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD), carnitine palmitoyltransferase (CPT1), medium chain acyl-CoA dehydrogenase (MCAD) and short chain acyl-CoA dehydrogenase (SCAD) (5-8). Fetal expression of an autosomal recessive mutation (G1528C) of the LCHAD enzyme has been reported in heterozygous mothers’ who developed AFLP (4, 5, 9, 10).
Table 1-1 Clinical features of patients with a disorder of Fatty Acid Oxidation and AFLP

<table>
<thead>
<tr>
<th>FEATURE</th>
<th>FAOD</th>
<th>AFLP</th>
</tr>
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<tbody>
<tr>
<td>Nausea and Vomiting</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Lack of energy</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Jaundice</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Hepatic encephalopathy</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Seizures/Coma/Death</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Liver Failure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Hypoglycaemia</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>- Hypoketotic</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Renal Failure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Elevated Creatinine</td>
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</tr>
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</table>

The exact mechanism of how this enzyme deficiency causes AFLP has yet to be determined. It is possible that as the mutation causing the enzyme deficiency is recessive, if both parents are heterozygous for this abnormality and these mutations are acquired by the fetus (obligate homozygote), the fetus will not be able to oxidize fatty acids, which would then return to the mother’s circulation via the placenta. These excess fatty acids may overwhelm the heterozygous mother’s ability to metabolize fatty acids leading to a fatty liver and elevated hepatic enzyme levels in her circulation (4). However, enzyme deficiencies in fatty acid oxidation have only been recognized in a small percentage of cases of AFLP.

It is well known that the second half of a healthy pregnancy is an insulin resistant state and circulating levels of cholesterol and triglycerides rise by up to 50% and 300% respectively (11, 12). The first two thirds of pregnancy is an anabolic state with maternal fat deposition. The catabolic state of the final trimester of pregnancy is associated with increased lipid metabolism. Beyond this general observation, fat metabolism is a complex process and little is known about how fatty acid oxidation pathways alter during healthy pregnancy (13-15).
Using new genetic sequencing techniques, I aim to identify novel gene variants associated with AFLP. If my research is successful novel gene variants associated with AFLP, may identify metabolic deficiencies in the mother, which would not only identify women at risk of AFLP, but could prevent offspring morbidity outside of pregnancy. Identification of one or more metabolic disorders associated with AFLP may lead to a targeted prophylaxis or treatment for an affected mother and her offspring resulting in a better quality of life. Understanding the pathophysiology of AFLP would also determine maternal vulnerability to recurrent AFLP in a subsequent pregnancy and long-term vulnerability to a fast-induced energy failure.

As well as a genetic vulnerability, I propose that some women affected by AFLP have reduced fat reserves that prevent adequate generation of energy. This hypothesis arose from the UK Obstetric Surveillance System (UKOSS) data which found that 20% of their cohort of women had a BMI of less than 20. Consequent multi-organ failure causes significant maternal and fetal morbidity and mortality. I will investigate whether there is evidence of sub - clinical impaired fat metabolism in women who have had a pregnancy affected by AFLP.

If our hypothesis is correct, the severity of AFLP may be reduced by simple measures to avoid prolonged fasting, or starvation, and/or treatment of nausea and vomiting in the third trimester.

Determining the exact patho-physiology of AFLP would provide a targeted defect for accurate diagnosis, management and prevention. This study will describe the process through which I have explored the most appropriate methods to investigate fatty acid oxidation in participants affected by AFLP and healthy pregnant women. I have then used the most appropriate method to study this cohort.
1.1 Lipid Metabolism

1.1.1 Energy generation

Metabolism is the term used to describe all chemical reactions that occur within cells of the human body. A proportion of these reactions result in the production of energy from the break down and oxidization of Carbohydrates, Fats and Proteins, which make up key constituents of food (Figure 1-1) (16, 17).

Figure 1-1 Overview of the pathways involved in the production of energy.

Carbohydrates are broken down to sugars, proteins to amino acids and fats to fatty acids and glycerol. Glucose is converted to pyruvate through the pathway of Glycolysis, which is then metabolized to Acetyl CoA and enters the TCA cycle. Oxidative phosphorylation uses energy released from this cycle to generate ATP.

Acetyl Coenzyme A (Acetyl COA), Tricarboxylic Acid Cycle (TCA Cycle), Electron transport chain (ETC Chain) and Adenosine triphosphate (ATP)
The catabolism of carbohydrates, proteins and fats provide the body with the ultimate unit of energy in the form of adenosine triphosphate (ATP). Mitochondria within cellular cytoplasm are referred to as the ‘Power House of the Cell’ as it is here where all aerobic metabolism occurs and where the majority of ATP is formed. ATP is synthesized from adenosine diphosphate (ADP) and inorganic phosphate (Pi) during the process of oxidative phosphorylation. It is ATP that provides energy for all cellular functions in the body (18, 19). See Figure 1-2.

Foods are broken down to their basic components. Carbohydrates are broken down to sugars (glucose, fructose), proteins to amino acids and fats to fatty acids and glycerol. If glucose is readily available, it undergoes a ten-step process called glycolysis in the cytosol of the cell. Pyruvate is formed, which is metabolized to Acetyl CoA and enters the citric acid cycle or tricarboxylic acid cycle (TCA cycle). In this cycle, acetate in the form of Acetyl CoA is oxidized by eight different enzymes the first of which is oxaloacetate (19, 20). The reactions occurring in the TCA cycle are also responsible for the oxidation – reduction reactions that convert nicotinamide adenine dinucleotide (NAD+) to reduced NAD+ (NADH), Flavin adenine dinucleotide (FAD) into FADH2 and guanosine diphosphate (GDP) and Pi into guanosine triphosphate (GTP). FADH2 and NADH undergo oxidative phosphorylation which requires electrons to be transported through protein complexes known as the electron transport chain. This process generates ATP from ADP and inorganic phosphate (Pi) (19, 20).
Adenosine Diphosphate (ADP), Inorganic phosphate (P$_i$), Adenosine Triphosphate (ATP), Electron carriers ($e^-$).

In response to a meal insulin is secreted, which stimulates uptake of glucose into cells and excess glucose to be stored as glycogen in the liver (Glycogenesis). Insulin also promotes the synthesis and storage of fatty acids in the liver and inhibits lipolysis. If there is reduced glucose availability secondary to starvation, liver glycogen stores are initially rapidly depleted. Once all glycogen stores are utilized, pyruvate and Acetyl CoA production for the TCA cycle is diminished resulting in reduced ATP. To overcome this energy deficiency, fatty acid oxidation is a vital source of energy providing approximately 80% of the total energy requirements for an individual during starvation or prolonged exercise (21).

Long chain fatty acids (C16 – C20) are stored as triglycerides in adipose tissue. Triglycerides are transported by plasma lipoproteins from adipose tissue to the liver. Triglycerides are hydrolyzed to fatty acids and glycerol, which is facilitated by lipases such as hormone-sensitive lipase (22). These fatty acids are transported to the mitochondrial matrix where they enter the mitochondrial β-oxidation cycle, producing Acetyl CoA as the end product. The Acetyl CoA generated does not enter the TCA cycle due to diminished levels of oxaloacetate, which has been removed from the cycle to allow production of pyruvate during gluconeogenesis.
This results in a rapid buildup of excess Acetyl CoA, which react to form the main ketone bodies acetone, acetoacetate (AcAc) and 3-\(\beta\)-hydroxybutyrate (3HB). Ketone bodies are an important alternative source of energy for extra-hepatic tissues especially the brain (19-21). This will be discussed further in section 1.1.5.

1.1.2 Lipid Metabolism outside of pregnancy

Lipid or fats are metabolized to fatty acids and glycerol (monoacylglycerol). There are 3 sources of fatty acids;

- Dietary Lipids.
- Fatty acids stored as Triacylglycerols (TAG) in adipose tissue. TAGs are also referred to as triglycerides.
- Liver synthesis of fatty acids that are transported as TAG (23).

Dietary Lipids or fats are metabolized in the intestine to fatty acids and monoacylglycerol by the enzyme pancreatic lipase. Inside the intestinal epithelial cells majority of the fatty acids are in fact re-esterified to glycerol forming triglycerides (24). These triglycerides are transported in plasma as lipoproteins known as chylomicrons. Exported chylomicrons enter the lymphatic system and are transported to various sites including the liver and adipose tissue. The enzyme lipoprotein lipase found on endothelial cells is activated by binding to Apolipoprotein CII, which is present on the surface of chylomicrons. This process results in fatty acids and glycerol being released from chylomicrons. The resultant fatty acids are stored in adipose tissue or used for energy when required (23, 24).

Fatty acids as previously discussed can be stored as triglycerides in adipose tissue and used for energy in the fasted state. However, they can also be re-esterified in the liver to triglycerides and subsequently removed as a separate lipoprotein called very low-density lipoprotein (VLDL).
When insulin levels are high the release of non-esterified fatty acids (NEFA) from the breakdown of triglycerides is inhibited, usually in the postprandial state. Insulin achieves this by inhibiting hormone sensitive lipase (HSL). The other effects of insulin include stimulating production of triglyceride in the liver (which are exported as VLDL) and stimulating lipoprotein lipase where fatty acids are removed from VLDL and stored in adipose tissue (25, 26).

When insulin levels are low with high levels of glucagon (fasting) or epinephrine (exercise) lipolysis is stimulated. Activation of the sympathetic beta-adrenergic receptors on the cell wall of adipocytes results is the production of cyclic adenosine monophosphate (cAMP), which activates the hormone protein kinase. Phosphorylation of protein kinase activates HSL within the cell and inhibits lipoprotein lipase. HSL promotes cleavage of fatty acids from glycerol and therefore, NEFA and glycerol enter the bloodstream. Glycerol can be used to produce glucose in the liver, however this is not an adequate source of glucose for energy. NEFA can enter most cells to produce energy through the process of beta-oxidation (24, 25).
1.1.3 Mitochondrial beta-oxidation

Mitochondrial beta-oxidation of long chain fatty acids is a complex process involving several biochemical steps. See Figure 1-3 a and b.

Figure 1-3 a. The carnitine cycle and fatty acid oxidation

FAT/CD36 (Fatty acid translocase), FACS (Fatty acyl CoA Synthase), CPT1 (Carnitine palmitoyltransferase 1), CPT2 (Carnitine palmitoyltransferase 2), CAT (Carnitine/acylcarnitine translocase), TCA (Tricarboxylic cycle).

b. The mitochondrial beta-oxidation cycle.
Long chain fatty acids are transported across the cell membrane into the cytosol via fatty acid membrane transporters. The three main transporters include fatty acid translocase (FAT/CD36), tissue specific fatty acid transport proteins (FATP) and plasma membrane bound fatty acid binding protein (FABPpm) (27).

Next, fatty acids undergo transformation to cross a selectively permeable membrane to enter the mitochondria. Here free fatty acids are activated to specific coenzyme A esters by the enzyme fatty acyl-CoA synthase (FACS) (28-30). This long-chain acyl-CoA is catalyzed by the enzyme Carnitine palmitoyltransferase 1 (CPT1) found on the outer mitochondrial membrane to form long chain acylcarnitine. A gene variant in the CPT1 enzyme has been found with AFLP. Subsequently, acylcarnitine is shuttled across the inner mitochondrial membrane via the transporter carnitine translocase (CAT) and acylcarnitine can enter the inner mitochondrial matrix (31). The inner mitochondrial membrane transport protein Carnitine palmitoyltransferase 2 (CPT2) converts acylcarnitine to fatty acid acyl-CoA.

Once inside the mitochondria, fatty acid acyl-CoA undergoes four enzyme reactions within the beta-oxidation cycle (Figure 1-3) (27, 29). The four main enzymes involved are acyl CoA dehydrogenase, enoyl CoA hydratase, long chain-3-hydroxyacyl CoA dehydrogenase and 3-ketoacyl CoA thiolase (Figure 1-3b). The third step in this process is catalyzed by the α-subunit long chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) enzyme.

A LCHAD gene variant has been associated with the AFLP (10, 32, 33). The new coenzyme produced through one cycle is shorter and enters the four-step beta-oxidation cycle again. This process is repeated until a short coenzyme is produced. The coenzyme Acetyl-CoA is used for ketogenesis (section 1.1.5) (28-30).

The 4 enzymes involved in mitochondrial beta-oxidation of long chain fatty acids are in fact two proteins found in the inner mitochondrial membrane. Very long-chain acyl-CoA dehydrogenase (VLCAD) is one protein and the other 3 enzymes (LHYD; Long Chain 2,3-enoyl-CoA, LCHAD, LKAT; Long Chain 3-ketoacyl-CoA thiolase) make up the second protein referred to as mitochondrial tri-functional protein (MTP) (34). MTP is a hetero-octamer composed of 4 α-units and 4 β-units.
Genetic mutations involving these proteins and enzymes associated with the carnitine cycle and beta-oxidation, result in disorders of FAO (35).

It is important to mention the role of Malonyl CoA (a three-carbon CoA ester) at this stage as it plays an important role in regulating lipid energy metabolism. Malonyl CoA potently inhibits CPT1 and therefore inhibits FAO. The liver, adipose tissue, heart and skeletal muscle are all sources of this CoA ester. Studies have shown raised insulin levels and low glucagon levels result in raised Malonyl CoA levels, hence inhibiting FAO (36, 37). In the fasted state, levels of insulin and Malonyl CoA are low, therefore FAO is stimulated (38).

1.1.4 Peroxisome proliferator activated receptor (PPAR)

Peroxisome proliferator activated receptor (PPAR) is a nuclear receptor subfamily comprising of 3 main ligand activated transcription factors; PPARα, PPARγ and PPARδ (39). Each transcription factor is known to have a particular tissue distribution pattern and function.

PPARα is predominantly expressed in tissues where fatty acid oxidation occurs. This includes the liver, skeletal muscle, heart, adipose tissue and the kidney (40).

PPARα is one of the most important transcription factors involved in energy metabolism. It is responsible for the upregulation of most of the genes involved in removal of VLDL from the liver, fatty acid oxidation and ketogenesis. Some of these genes include fatty acid binding protein (FABP), CPT1, mitochondrial long and medium chain acyl-CoA dehydrogenases (LCAD, MCAD) and the ketogenesis enzyme, mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS2) (41). PPARα deficiency results in decreased expression of the FAO genes as demonstrated in a study where PPARα null mice developed a fatty liver (42).

As described below in section 1.1.5 Ketogenesis is a vital process for energy production in the fasted state. PPARα is also essential for ketogenesis to occur. This transcription factor is induced by ketogenic stresses such as fasting.
Fasted mice deficient in PPARα develop a phenotype revealing a fatty liver, hypoglycaemia, hypoketosis and raised plasma fatty acids, all of which are suggestive of impaired ketogenesis (43).

A peptide called fibroblast growth factor 21 (FGF21), which is regulated by PPARα, is activated during the fed state to promote lipolysis (44). FGF21 deficient mice have a similar phenotype to PPARα deficiency in the liver.

1.1.5 Lipid metabolism during Pregnancy

Lipid transfer across the placenta to the fetus is limited even though the fetus requires essential fatty acids (EFA) and long chain polyunsaturated fatty acids (LCPUFA) for normal fetal growth. However, it is lipid metabolism that is most affected during pregnancy (45).

During the first two thirds of pregnancy maternal fat stores are laid down secondary to hyperphagia and increased lipogenesis (46, 47). This is facilitated by hypertrophy of adipocytes allowing increased fat storage and by increased adipocyte insulin sensitivity. Although there is a marked increase in fetal growth relative to the embryonic stage, it is limited in absolute terms during this stage (48-52).

This accumulation of fat stops later in pregnancy due to reduced lipoprotein lipase activity and an increase in lipolysis (53-56). Lipolysis produces two products, glycerol and non-esterified fatty acids (NEFA), which are both transferred to the liver (Section 1.1.2). In the liver NEFA and glycerol are converted to acyl-CoA and glycerol-3-phosphate respectively, after which they are both re-esterified to form TAG. TAGs enter maternal circulation in the form of VLDL. Increased lipolysis results in increased VLDL production causing maternal hyperlipidaemia. In the liver, NEFA can be metabolized further by entering the beta-oxidation cycle, producing Acetyl CoA for ketogenesis and energy. This pathway is enhanced in late pregnancy during starvation (57, 58). It is in the latter third of pregnancy where maternal lipid metabolism switches to a catabolic state and coincides with rapid fetal growth in absolute terms (59).
Ketone bodies can readily cross the placenta and be used by the fetus for energy (60, 61). In the maternal circulation, ketone bodies can be used as an energy source for tissue such as skeletal muscle and most importantly the brain. Glycerol is also the preferred substrate for gluconeogenesis during fasting in pregnancy and therefore allows other glucogenic amino acids (e.g. alanine) to cross the placenta to the fetus. Pregnancy hormones play a role in these changes, however it is the switch to an insulin resistant state that increases lipolysis, increases hepatic VLDL production and during starvation or fasting increases gluconeogenesis and ketone body production (62, 63).

The mechanism of the development an insulin resistant state in normal pregnancy is complex. It is thought that several hormonal changes are responsible for the impaired insulin action. Human placental lactogen (HPL) increases significantly in pregnancy and stimulates insulin release from the pancreas (64). HPL has been shown to cause peripheral insulin resistance and also promote lipolysis when fasting, resulting in greater levels of fatty acids, which could affect the action of insulin (65). Human placental growth hormone (hPGH) levels increase during pregnancy and has also been implicated in the development of insulin resistance. One study showed the development of insulin resistance in mice with over-expression of hPGH with similar levels as third trimester pregnancy (66).

Adipokines including leptin, adiponectin, interleukin-6 and Tumour Necrosis Factor-α (TNF-α) have also been implicated in inducing insulin resistance in pregnancy (67, 68). Despite the association with hormonal changes in pregnancy and insulin resistance, the exact mechanism is not known. There are most likely other pregnancy related features that are key in understanding why insulin resistance develops in the third trimester.

The mechanism of the hyperlipidaemia of pregnancy is not completely understood. A key explanation could be the result of increased levels of oestrogen in pregnancy promoting VLDL production in the liver and reducing hepatic lipase activity, therefore further enhancing hyperlipidaemia in pregnancy (69).
The noted changes exhibited include:

- A significant increase in cholesterol and phospholipids (54).
- Increased VLDLs released into maternal circulation, which are not removed from the circulation as a result of reduced lipoprotein lipase activity. Oestrogen has been shown to negatively regulate lipoprotein lipase activity (70).
- Lipoproteins LDL (low density) and HDL (high density) rich in TAG, which firstly may be due to increased cholesterol ester transfer protein activity (CETP) and secondly a consequence of reduced hepatic lipase activity. CETP facilitates the transfer of TAGs to LDL and HDL, whereas reduced hepatic lipase activity allow levels of HDL enriched with TAG to increase by inhibiting its breakdown (54, 71).

If AFLP is a disorder of energy metabolism, the defect can occur at several different points in the pathways associated with energy metabolism. There may be a defect or problem with glycogenolysis and gluconeogenesis or lipolysis (e.g. lipoprotein lipase deficiency), the carnitine shuttle (e.g. CPT1 and CPT2 deficiency), the oxidation of fatty acids and ketogenesis (e.g. Fatty acid oxidation disorders and Inborn errors of ketogenesis).

Glucose metabolism changes throughout pregnancy to ensure appropriate fetal and placental growth and development. It is glucose that predominantly crosses the placenta providing most of the energy for the fetal-placental unit. Placental transfer of lipids is very limited; however, lipids are important in fetal development (72, 73).

Early in pregnancy basal plasma glucose and insulin levels remain normal and it is in the third trimester where both of these levels increase (11). However, pregnant women suffer from a phenomenon known as “accelerated starvation”, characterized by an exaggerated metabolic response to an overnight fast, i.e. a more rapid fall in plasma glucose, raised free fatty acids and increased ketone bodies compared with non-pregnant women (74).
The increase in insulin sensitivity during early pregnancy may explain the decrease in fasting glucose levels during early gestation.

Despite enhanced gluconeogenesis and maternal insulin resistance in the third trimester, hypoglycaemia occurs due to the rapid rate of glucose transfer and utilization by the feto-placental unit (14, 32, 75, 76). There are a number of glucose transporters (e.g. GLUT1) that facilitate this placental transfer of glucose (77). Candidates for mediating these adaptations are placental hormones, such as human placental lactogen, progesterone, cortisol or cytokines in particular tumor necrosis factor-α (TNF-α) (78).

Amino acids are actively transferred across the placenta to the fetus and their concentration is greater in fetal plasma than in the mother. The fetus is unable to produce its own amino acids and thus its' only source is the mother, which is why mothers develop hypoaminoacidaemia (72, 79). Hypoaminoacidaemia has been described especially during fasting, early in pregnancy and continues throughout the remainder of pregnancy (80). Carbohydrates or glucose is the primary energy source for the fetus accounting for 80% of fetal energy consumption. Amino acids and fatty acids provide the remaining 20% of fetal energy requirements (81).

### 1.1.6 Ketone body metabolism

The compounds acetoacetate (AcAc), 3-beta-hydroxybutyrate (3HB) and acetone are collectively referred to as ketone bodies. Ketone bodies are produced in the liver and are an alternate source of energy when glucose is not readily available. Plasma levels of ketone bodies depend upon the rates of ketone body production (Ketogenesis) and ketone body utilization (Ketolysis) must be determined. Ketone body concentrations can range from 0.1nM to 6nM in the fed and fasted state respectively (82, 83).
Ketone bodies are produced following several ketogenic stresses that include a prolonged fast, fever, diarrhoea and vomiting. During a prolonged fast insulin levels are suppressed and lipolysis is activated, resulting in the release of NEFA and glycerol from adipose tissue. These fatty acids are transported by plasma lipoproteins to the liver. Fatty acids enter liver mitochondria through a series of reactions before it can enter the beta-oxidation cycle to produce Acetyl-CoA (See section 1.1.3) (84).

Ketogenesis is facilitated primarily by two key enzymes called mitochondrial HMG-CoA synthase (mHS) and HMG-CoA Lyase (HL). In the liver mitochondria Acetyl CoA (Ac-CoA) and Aceto-acetyl CoA (AcAc-CoA) react with the enzyme mHS to produce 3-hydroxy3-methylglutaryl-CoA (HMG-CoA). Next HMG-CoA reacts with the enzyme HL to form AcAc, which is further partially reduced to 3HB. Both ketone bodies enter the bloodstream and are transported to extrahepatic tissues where ketolysis occurs. Initially, 3HB is converted back to AcAc and the enzyme succinyl-CoA:3-oxoacid CoA transferase (SCOT) activates AcAc to AcAc-CoA. Following this step, the enzyme acetoacetyl-CoA thiolase (T2) reacts with AcAc-CoA causing the transfer of an acetyl group to unbound CoA, resulting in the production of acetyl-CoA which can be used to generate energy (See
Figure 1-4) (84, 85).

**Figure 1-4 The process of Ketogenesis and Ketolysis.**

mHS (HMG-CoA synthase), T2 (Beta-ketothiolase), HL (HMG-CoA lyase), SCOT (Succinyl-CoA-3-oxoacid CoA transferase), TCA cycle (Tricarboxylic cycle), ATP (Adenosine triphosphate).
When glucose levels are low, ketones are an essential source for energy in extrahepatic tissues, especially the brain. Ketogenesis is a complex process which can be adversely affected resulting in inborn errors of ketone body production. The two main inherited disorders of ketogenesis are deficiency of mHS, which involves the \textit{HMGCS2} gene and deficiency of HL, involving the \textit{HMGCL} gene (86, 87).

The majority of individuals with mHS deficiency present with signs and symptoms of hypoglycaemia with laboratory findings showing hypoketotic hypoglycaemia, all of which are seen in disorders of FAO. However, creatinine kinase (CK) levels are not elevated in mHS deficiency and are useful in differentiating between fatty acid oxidation disorders (FAOD) (84). In addition, a paediatric fasting test has been developed to assist in the diagnosis of inborn errors of metabolism. A 24 hour fast was performed in 9 hypoketotic, 2 hyperketotic and 48 control children. Fasting glucose, total ketone body and lactate levels were measured.

The ratio of free fatty acids to total ketone bodies (FFA/KB) was found to be useful in differentiating between hypoketotic, hyperketotic and normal subjects. Therefore, a defect in ketogenesis is suspected if the ratio of FFA/KB is above 2.5 and a ratio below 2.5 is suggestive of defects in ketolysis (88).

Patients with HL deficiency present similarly to those with mHS deficiency. However, unlike mHS deficiency, HL deficient patients produce specific urinary leucine metabolites (3-hydroxy- 3-methylglutarate, 3-methylglutaconate, 3-methylglutarate, 3-hydroxyisovalerate and 3-methylcrotonylglycine) (89).

Inborn errors of Ketolysis include Scot deficiency (\textit{OXCT1} gene) and T2 or Beta-ketothiolase deficiency (\textit{ACAT1} gene) resulting in the inability to breakdown ketones in extrahepatic tissue. With SCOT deficiency Ketolysis is not possible, however with T2 deficiency a proportion of ketolysis can occur (90, 91).
1.1.7 Role of hypoxia in lipid metabolism

Hypoxia at high altitudes is a well-recognized cause of physiological stress and change to normal metabolism. To acclimatize to a low oxygen environment and maximize oxygen delivery to cells of the body, the human body acclimatizes by increasing cardiac output, erythropoiesis and increasing ventilation (92).

Energy or ATP is generated from mitochondrial oxidative phosphorylation for which oxygen is required. If oxygen is unavailable, cells in the body must adapt to overcome the lack of oxygen and continue to produce energy. Hypoxia inducible factors (HIFs) are transcription factors that have a significant role in assisting cells adapt to a hypoxic environment. HIFs exist as three isoforms, HIF-1, HIF-2 and HIF-3, all of which have different roles (93, 94). HIF-1 is involved in upregulating most glycolytic enzymes (e.g. hexokinase), HIF-2 activates anti-oxidant genes like 
*superoxide dismutase (SOD)-2* and HIF-3 does not have an active role in transcription. Compared to HIF-1 and HIF-2 there is much less known about HIF-3. It is thought that HIF-3 inhibits the actions of HIF-1 and HIF-2 (95, 96).

In an oxygenated environment HIFs are targeted by von Hippel-Lindau protein and are degraded. In a hypoxic environment HIFs are not proteasomaly degraded and are able to employ their function as transcription factors (97).

There have been a number of studies to show that hypoxia has an effect on lipid metabolism. In particular, hypoxia is known to increase lipid deposits and up regulate genes associated with lipogenesis (98, 99). A study in rats demonstrated that HIF-2 activation resulted in impaired FAO causing an increase in lipid storage and significant hepatic steatosis (100). Conversely, one study investigating HIF-2 deleted mice reported severe steatosis and another study investigating the activation of HIF-2 in hepatocytes only demonstrated a marginal effect on lipid storage capabilities (96, 101).

Lipid metabolism is clearly affected by transcriptional HIFs, however the pathways involved are complex and other genetic and environmental influences may also affect hepatic lipid metabolism.
Hypoxia or high altitude has an effect on gene expression of those genes involved in carbohydrate metabolism and lipid metabolism (102, 103). Mitochondrial beta-oxidation of fatty acids is a complex process as described in section 1.1.2. There are approximately 25 enzymes involved in the process of fatty acid oxidation and of these 18 gene defects have been found resulting in disorders of FAO (104). All of these enzymes involved in FAO are potential targets for disruption. Studies have identified the effect of hypoxia on enzymes involved in FAO. One study reported that following one day of exposure of rats at high altitude showed a significant decrease in the function of CPT 1 (105, 106).

The ligand-activated transcription factor Peroxisome Proliferator-Activated Receptor alpha (PPARα), is known to promote beta-oxidation of fatty acids in the liver (Section 1.1.3). The expression of approximately 100 genes is affected when PPARα is activated and all of these proteins are involved in lipolysis (107). In hypoxic epithelial cells, the activation of HIF-1 reduces PPARα activity and thereby decreases FAO (108).

One of the aims of my thesis is to determine whether women who have previously suffered from AFLP have a detectable abnormality in lipid metabolism when exposed to a low oxygen environment. To assess the effect of hypoxia on lipid metabolism I performed a pilot study described in Chapter 2.

1.1.8 Fatty Acid Oxidation Disorders (FAOD)

FAOD are inborn errors of metabolism with an incidence of approximately 1 in 10,000 live births. They usually present acutely in the neonatal period and often result in sudden death. The beta-oxidation pathway of long chain fatty acids involves at least 25 enzymes and transport proteins. To date 18 deficiencies of these enzymes have been identified resulting in a FAOD (109, 110). Most disorders of FAO are inherited as an autosomal recessive condition in which the fetus is homozygous and both parents will be obligate heterozygotes, or carriers for the gene defect.
I plan to study women affected by AFLP, a condition which I have hypothesized occurs due to a FAOD in the mother. These women are not as severely affected as neonates with a disorder of FAO and they rarely get recurrent AFLP.

In the 1970’s carnitine palmitoyltransferase deficiency type 2 (CPT2) was identified as the first FAOD (111). Despite this discovery it took a further 10 years to detect the second defect of FAO called medium-chain acyl-CoA dehydrogenase deficiency (MCAD) (112-114). Since the discovery of these two defects, many more enzyme defects affecting FAO have been identified (21, 29).

FAOD may also present in adulthood through two well-recognized clinical presentations. Firstly, patients can present with hypoketotic hypoglycaemia following a history of nausea, vomiting and starvation. Other causes of metabolic stress include any illness, infections and surgical procedures. Additionally, patients may have signs of liver and cardiac disease, as a result of the accumulation of long chain acylcarnitines (115). Secondly, patients can present with a myopathy and a cardiomyopathy as a result of chronic muscle impairment.

These patients may complain of reduced exercise tolerance, muscle weakness and decreased tone. Renal failure may also occur as a result of rhabdomyolysis. Other phenotypes include liver failure and neurological symptoms (116). Liver failure in these patients occur due to an accumulation of long chain fatty acid metabolites that are toxic to the liver and therefore cause hepatocellular injury.

I have already described that long chain fatty acids are essential for the generation of ketones bodies in the liver and act as an alternative source of energy during starvation. However, it is important to note that skeletal and cardiac muscle, obtain significant amounts of energy from fatty acid oxidation, the TCA cycle and eventual production of ATP during the fed state (117). Therefore, defects in FAO will reduce hepatic ketogenesis and impair skeletal and cardiac function.

The accumulation of intermediary metabolites due to FAO defects causes toxicity. For example, 3-hydroxyacyl-CoAs accumulate in patients with LCHAD deficiency, cis-4- decenoate accumulates in patients with MCAD and MADD deficiency and butyl-
CoA/ butyric acid in SCAD deficiency (118). LCHAD associated toxic metabolites are associated with peripheral neuropathy and retinopathy (21, 119-121).

1.1.9 Carnitine

Carnitine (b-hydroxy-g-trimethylaminobutyric acid) has 2 main forms, free carnitine and acylated carnitine. In humans, approximately 85% of carnitine is found in its free form in plasma. L-carnitine an isomer of carnitine is predominantly found in the free form in plasma. Abnormalities associated with carnitine result due to either reduced free carnitine levels or a greater ratio of acylated carnitine to free carnitine (122). Carnitine is synthesized in vivo from essential amino acids predominantly in the liver and kidneys (123).

Primary defects of fatty acid oxidation (except CPT1 deficiency) result in a total reduction of free L-carnitine and/or an increase in acylcarnitine to free L-carnitine ratio. They also result in an accumulation of acylcarnitines.

Acylcarnitines are normally converted back to their respective Acyl-CoA by CPT2 and then enter the beta oxidation cycle. If there is a defect in the carnitine shuttle or beta oxidation cycle this process does not occur and acylcarnitines accumulate (21).

Investigations such as analysis of organic and fatty acids in urine and blood, profile of acylcarnitines and culturing fibroblasts may be useful in trying to diagnose a FAOD (104, 124).

1.1.10 Treatment of FAOD

The mainstay of treatment is to reduce or limit lipolysis and increase gluconeogenesis. Simple measures include avoiding periods of starvation, stress and infection. Primarily all patients are treated with changes to their diet. The diet should be low in fats (long chain), consist mainly of carbohydrates and if necessary
to achieve adequate calorie intake supplemented with medium chain triglycerides (MCT). These dietary measures are necessary to avoid fatty acid oxidation whilst at the same time providing sources of energy. To prevent hypoglycaemia, continuous tube feeds of uncooked cornstarch (e.g. 2g/ kg in toddlers) may be required overnight. MCT oil has been shown to have a number of benefits. A study has shown that MCT is better than carbohydrates at reducing the amount of toxic metabolites produced from fibroblasts that are deficient in LCHAD (125). This same study has postulated that Acetyl-CoA formed from the oxidation of MCT is converted to malonyl-CoA. Malonyl CoA suppresses CPT 1, thereby reducing the transfer of long chain fatty acids into the mitochondria (125). However, MCT oil should not be given to patients with MCAD deficiency as they are unable to break down the medium chain fats in MCT oil and should therefore be avoided completely (126).

1.2 Metabolism in Pregnancy

1.2.1 Maternal whole-body adaptations

Successful pregnancy requires maternal metabolic adaptations to meet the demands of fetal growth and development. The first two thirds of pregnancy is an anabolic state in which the mother accumulates fat stores, but fetal growth is low in absolute terms. It is estimated that a woman will store 3.3kg of fat by fifteen weeks’ gestation (48, 49, 52).

The last third of pregnancy is a catabolic state with rapid absolute fetal growth associated with maternal insulin resistance and elevated blood glucose levels. Insulin resistance also promotes lipolysis, FAO and ketone body production for maternal energy requirements (14, 59).

Physiological adaptations in pregnancy occur soon after conception. The cardiovascular system adapts significantly throughout pregnancy. Blood volume increases as early as 6 weeks and continues to increase into the third trimester (127). Other changes include increased cardiac output, 20% increase in red blood
cell mass, reduced systemic vascular resistance and an increase in ventricular wall muscle mass (128). Throughout pregnancy blood flow to the uterus increases and at term it is as much as 500 – 800 ml/min. Arterial blood pressure decreases early in the first trimester and starts to rise after the second trimester. Hormone induced changes in circulating and vascular factors leads to low peripheral vascular resistance, as well as increased utero-placental blood flow (129).

During pregnancy, the kidneys increase in size, volume and weight. Blood flow through the renal system increases by as much as 80% and glomerular filtration rate increases by 50% in the first trimester (130, 131). The hormone relaxin is responsible for some of these haemodynamic changes (132).

Hepatic arterial blood flow and the size of the liver remain unchanged throughout pregnancy. Healthy peripheral vascular dilatation of pregnancy leads to palmar erythema and mimics one of the signs of liver disease.

Furthermore, spider naevi along the distribution of the superior vena cava and oedema are normal features of pregnancy also found in liver disease (133-135).

When hepatocytes undergo necrosis, changes are seen in a patient’s liver transaminases (aspartate transaminase, alanine transaminase, \( g \)-glutamyl transferase), serum bilirubin, serum albumin and prothrombin time, all of which assess liver injury rather than loss of hepatic function (136). To assess liver function acid - base status and in particular lactate acidosis is a marker of abnormal hepatic function.

In a pregnant state, there is a slight reduction in the normal range of values (See Table 1-2). This is most likely secondary to haemodilution due to the increased circulating volume. The exception is alkaline phosphatase, which increases throughout pregnancy due to placental production of the isoenzyme and fetal bone development (133, 134).

During pregnancy, there is also increased hepatic synthesis of coagulation factors (Factor VII, VIII, X, Fibrinogen), contributing to hypercoagulability and the
prothrombotic state of healthy pregnancy. Serum albumin concentrations fall secondary to plasma volume expansion (134, 135, 137). These maternal adaptations are vital in achieving a successful pregnancy outcome.

### Table 1-2 Levels of bilirubin, albumin and liver enzymes in pregnant and non-pregnant adults (138, 139).

<table>
<thead>
<tr>
<th>LFTs</th>
<th>Non - Pregnant</th>
<th>Pregnant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilirubin µmol/L</td>
<td>0 – 17</td>
<td>4 – 16</td>
</tr>
<tr>
<td>Albumin g/L</td>
<td>35 – 46</td>
<td>28 – 37</td>
</tr>
<tr>
<td>AST IU/L</td>
<td>7 – 40</td>
<td>10 – 30</td>
</tr>
<tr>
<td>ALT IU/L</td>
<td>0 – 40</td>
<td>6 – 32</td>
</tr>
<tr>
<td>GGT IU/L</td>
<td>11 – 0</td>
<td>5 – 43</td>
</tr>
<tr>
<td>ALP IU/L</td>
<td>30 – 130</td>
<td>32 – 418</td>
</tr>
</tbody>
</table>

AST (aspartate transaminase), ALT (alanine transaminase), GGT (γ-glutamyl transferase) and ALP (alkaline phosphatase).

### 1.3 Pathogenesis of Acute Fatty Liver of Pregnancy

In 1934, acute fatty liver of pregnancy (AFLP) was first described as an “acute yellow atrophy of the liver” (140, 141). AFLP has been classically considered a rare
maternal disease of the liver approximately affecting between 1:10 000 to 1:15 000 pregnancies (3). Despite it being such a rare condition it is a well-established cause of maternal and fetal mortality, especially when there is a delay in its diagnosis. The UK obstetric surveillance system (UKOSS) study found just one maternal death out of 57 cases and 7 fetal deaths (3).

Observational studies have shown AFLP to be more common in underweight women, primigravidae, male fetuses (3:1), and multiple pregnancies. Nearly one fifth of women with AFLP had twin pregnancies representing a 14 times increased risk of AFLP with a twin pregnancy. The UKOSS study suggested underweight women are predisposed to developing AFLP, with 20% of their cohort having a body mass index (BMI) less than 20 (3). There is no obvious ethnic or geographical predisposition to AFLP (142, 143).

AFLP is a syndrome of the third trimester of pregnancy. If a liver biopsy were undertaken the microscopic changes seen would include central microvesicular steatosis, canicular cholestasis, and occasionally necrosis of hepatocytes. These changes are not permanent and disappear in the post-partum period (141, 144, 145).

A normal liver has a fat content of approximately 5%, which can increase to 13 – 19% in a woman with AFLP leading to liver failure (141, 146, 147). Despite its name AFLP is a multi-organ syndrome of pregnancy. There have also been reports of micro-vesicular fatty infiltration of the brain, pancreas, kidney and bone marrow (148).

Being such a rare syndrome of pregnancy, AFLP has been a difficult syndrome to study. Much remains to learn about its aetiology, pathogenesis, diagnosis, management and future implications.
1.3.1 What Causes AFLP?

There is a widely-accepted association between AFLP and a defect in mitochondrial beta-oxidation of long chain fatty acids. I have previously stated that the clinical and biochemical presentation of AFLP suggests strong similarities with inherited disorders of FAO. Disorders of FAO can occur at any point within the pathways associated with lipid metabolism.

This includes lipolysis to form fatty acids and glycerol, transport to the liver, uptake of fatty acids by the liver hepatocyte plasma membrane, transport across the mitochondrial membrane and carnitine cycle, the beta-oxidation cycle and hepatic ketogenesis (124). Defects in FAO result in reduced mitochondrial ATP production and reduced ketogenesis resulting in multi-organ dysfunction, similar to that seen in fatty acid oxidation disorders (149).

AFLP has been associated with deficiencies in four enzymes essential in the beta-oxidation cycle, specifically; long chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD), carnitine palmitoyltransferase (CPT1), medium chain acyl-CoA dehydrogenase (MCAD) and short chain acyl-CoA dehydrogenase (5-8).

Mitochondrial beta-oxidation of long chain fatty acids is a complex process involving several biochemical steps as described in section 1.1.2. The third step in this process is catalysed by the α-subunit long chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) enzyme and the deficiency of LCHAD has predominantly been associated with AFLP compared to the other 3 enzymes mentioned above. However, this enzyme deficiency is not seen in all cases of AFLP.

Genetic mutations affecting proteins and enzymes associated with the carnitine cycle and beta-oxidation may also result in disorders of FAO. It has yet to be proven whether these genetic mutations are also associated with AFLP.
1.3.2 LCHAD and AFLP

In 1989, long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) deficiency was first discovered in a 3-day old infant who suffered from sudden infant death syndrome (150). The younger sibling of this infant presented at 5 months old with a history of problems with feeding and reduced consciousness. LCHAD deficiency was diagnosed from cultured skin fibroblasts. A change in diet to include medium chain fatty acids was successful in managing this child (151).

An association between LCHAD deficiency and AFLP was first recognized, when 6 babies were diagnosed with LCHAD deficiency and all mothers had either AFLP, Pre-eclampsia or Haemolysis elevated liver enzymes and low platelets (HELLP) syndrome (9). Further proof of this association was noted in a pregnant woman who delivered at term with AFLP and 4 months later her baby developed a hypoglycaemic coma and was diagnosed with LCHAD deficiency (152).

Twenty-four out of twenty-six Dutch patients with LCHAD deficiency were later found to be homozygous for the 1528G-C transversion in exon 15 of the Hydroxyacyl-CoA Dehydrogenase/ 3-Ketoacyl-CoA Thiolase/ Enoyl-CoA Hydratase, Alpha Subunit (HADHA) gene; the gene encoding LCHAD. The two remaining patients were compound heterozygotes (153). Subsequently, this lead to the development of a new technique to identify the 1528G-C mutation in genomic DNA in 34 LCHAD deficient patients. This technique was successful in identifying an 87% allele frequency of the G1528C mutation in these 34 LCHAD deficient patients.(32). Three further pregnant women who suffered from AFLP had two children who were homozygous for 1528G-C mutation and another who was a compound heterozygote (10).

In another series, eight out of nineteen children presenting with hypoketotic hypoglycaemia were homozygous for the 1528G-C mutation and the remaining eleven children were compound heterozygotes. Fifteen of the nineteen mothers suffered from either AFLP or HELLP syndrome (4). Consequently, a hypothesis that defects in fetal fatty acid oxidation cause maternal AFLP was proposed.
If there is a homozygous 1528G-C mutation in the fetus, long-chain 3-hydroxyacyl metabolites produced by the fetus or placenta accumulate in the mother. These metabolites are highly toxic to the liver and result in reduced hepatic metabolic capacity (4). However, there is evidence that the toxic metabolites are unlikely to come from the fetus as its main energy source is glucose, therefore fetal fatty oxidation is low, thus contradicting the proposed hypothesis (45). However, the placenta expresses enzymes of fatty acid oxidation and in the presence of an unhealthy placenta may result in AFLP in the mother (154).

However, these theories have yet to be proven as there is no conclusive evidence identifying the exact mechanism of how a fatty acid oxidation defect in the fetus causes AFLP. Furthermore, despite this association mothers with a fetus that is LCHAD deficient do not all develop AFLP (155). Other unknown factors are yet to be determined, which are key to understanding AFLP.

1.3.3 Clinical presentation and diagnosis

The onset of AFLP usually occurs in the third trimester, commonly after the 30th week of gestation (156). Women usually present with gradual onset of vague and non-specific symptoms of nausea and vomiting (severe 60%), abdominal pain (60%), fatigue, flu-like illness and peripheral oedema. Jaundice may manifest following 1 – 2 weeks from the onset of symptoms. In extreme cases the first presentation may be with encephalopathy. Occasionally, women with AFLP present with symptoms of diabetes insipidus (3, 157, 158). The diagnosis of AFLP is challenging. It’s presenting signs and symptoms can be mistaken for other obstetric disorders such as preeclampsia and HELLP syndrome.

The UKOSS study of fifty-seven cases showed that over half of all women with AFLP may present with mild high blood pressure (3). Typically, initial investigations of women with AFLP show elevated liver transaminases. The UKOSS study showed all women had raised transaminases with a median elevation of AST 300IU and ALT 300IU (3).
When compared to women with HELLP syndrome transaminase levels are usually greater (ALT > 600IU) (159). Bilirubin (median 101 umol/l), serum creatinine (median 169 umol/l) and white cell count (median 20.7 x 10⁹) are higher in AFLP (3). Furthermore, a coagulopathy is more common in AFLP (87% in UKOSS study) (3, 158) compared with <10% in women with HELLP syndrome. The pathognomonic features include a profound hypoglycaemia and hyperuricaemia, which is out of proportion to pre-eclampsia. Additionally, a leucocytosis, renal impairment and a metabolic acidosis are common (160).

With most cases presenting with non-specific symptoms the clinician or obstetrician should always have a high index of suspicion of a possible diagnosis of AFLP. Table 1-3 illustrates the diagnostic criteria proposed by Ch’ng et al in Swansea (2, 147).

Table 1-3 Swansea Diagnostic Criteria for AFLP (2).

<table>
<thead>
<tr>
<th>Vomiting</th>
<th>Abdominal pain</th>
<th>Polydipsia/ polyuria</th>
<th>Encephalopathy</th>
<th>Elevated bilirubin (&gt;14 mmol/l)</th>
<th>Hypoglycaemia (&lt; 4 mmol/l)</th>
<th>Elevated urate (&gt; 340 mmol/l)</th>
<th>Leucocytosis (&gt; 11X10⁹/l)</th>
<th>Ascites or bright liver on ultrasound scan</th>
<th>Elevated transaminases (aspartate aminotransferase or alanine aminotransferase &gt; 42 IU/l)</th>
<th>Elevated ammonia (&gt; 47 mmol/l)</th>
<th>Renal impairment (creatinine &gt; 150 umol/l)</th>
<th>Coagulopathy (INR &gt; 1.3)</th>
<th>Microvesicular steatosis on liver biopsy</th>
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* Six or more features are required in the absence of another explanation

The gold standard investigation for diagnosing AFLP is a liver biopsy. It was previously accepted that a liver biopsy should not be performed due to the risk of complications secondary to a coagulopathy, however hepatologists can manage the risk of bleeding if necessary. A liver biopsy actually delays the diagnosis and
subsequent management of AFLP. Furthermore, with advances in investigative techniques a liver biopsy is rarely necessary.

Radiological investigations such as magnetic resonance imaging (MRI), computerized tomography (CT) and ultrasound of the liver are not diagnostic investigations. They may illustrate hepatic steatosis, but most often the liver appears normal as the fatty infiltration is microvesicular. However, these investigations are important to rule out other liver abnormalities such as liver infarction or rupture, and biliary disease (143, 160).

1.3.4 Management and Recurrence of AFLP

Early diagnosis of AFLP improves pregnancy outcome. Delivery is the only cure but correcting any coagulopathy may be necessary before delivery. Following a diagnosis of AFLP the mainstay of treatment is stabilization of the patient and prompt delivery of the fetus.

The patient may need airway support, anti-hypertensives to control the blood pressure, treatment of hypoglycaemia, correction of any renal or electrolyte abnormalities and reversal of any coagulopathies. Careful fluid balance is vital to the patient’s management to prevent cerebral and pulmonary oedema secondary to low osmotic pressure. Furthermore, there should be ongoing assessment for any signs of maternal encephalopathy (134, 149, 160). It is vital that this all occurs with a multidisciplinary team in a high dependency setting.

The fetus should also be assessed for fetal well-being which will aid the decision for mode of delivery (135, 147, 161). A safe and quick delivery is recommended. The recent UKOSS study data has shown that current practice in the UK is to perform a caesarean section (75%). Some may consider this to be a high percentage of the population, especially since a caesarean section poses the risk of bleeding secondary to a coagulopathy. Alternatively, this value may represent the severity of the condition and thereby an urgency to deliver early (3). Clinical judgment is required where consideration is given to surgery versus coagulopathy and fetal
versus maternal well-being. Reported cases have shown that despite a reduction in fetal mortality rates there remains a common finding of fetal distress despite the mother being clinically stable. There is no clear explanation as to why this is the case, however if fetal compromise occurs delivery is almost always expedited (147).

Patients with severe AFLP should be transferred to a specialist liver center for further management due to the increased risk of hepatic rupture and bleeding (137). N-acetyl-cysteine (NAC) has anti-oxidant properties that accelerate liver recovery. Traditionally, NAC has been used in the treatment of acetaminophen induced liver failure. However, NAC is increasingly being used in the treatment of acute liver failure.

Studies have demonstrated increased oxygenation and vasodilatation, improving oxygen delivery and blood flow. Other useful effects include it’s anti-inflammatory and antioxidant properties (162, 163).

Complete resolution of normal liver function can take as long as 6 weeks in the post-partum period (134). As recovery is usual, liver transplant secondary to a pregnancy-associated condition is rare. Following AFLP, supportive treatment is desirable until liver impairment improves. However, there have been cases of successful transplantation in patients with multi-organ failure. It has been recommended that liver transplantation only be considered in exceptional cases for example those with worsening coagulopathy and metabolic acidosis, hepatic encephalopathy and liver rupture (137, 164). There have been a few cases reports in the literature where plasma exchange has been used in the management of AFLP, however there have been no randomized control trials for its use in pregnancy associated liver conditions.

The neonate should be assessed by the neonatologists, looking for signs of hypoglycaemia, hepatic dysfunction, neuro-myopathy and cardiomyopathy. The neonate should be screened for disorders of β-oxidation of fatty acids (115, 134, 135, 165).
Isolated cases of recurrent AFLP have been published, but rates of AFLP recurrence are rare (9, 10, 166-168). This could be related to women being advised not to conceive and women avoiding another pregnancy due to the complication of previous AFLP. Although there are case series showing normal pregnancies following a pregnancy affected by AFLP (169, 170).

AFLP is a serious disorder of pregnancy, which if not recognized can result in maternal and fetal mortality. The decreasing mortality rates reflect the improving recognition of this condition and early delivery of the fetus (3, 158).

This however is not an easy feat, as the presenting clinical features can be mistaken for pre-eclampsia or HELLP syndrome.

Once a diagnosis has been made the treatment is supportive with delivery of the baby as soon as possible. It is important to enlist help early from hepatologists and intensivists, as often the biochemical status does not represent the disease severity. All patients should be offered genetic counseling for any future pregnancies.

We have much to learn about the pathogenesis of this condition especially with its association with FAO disorders.

My PhD thesis uses several techniques to investigate whether women who have had AFLP have subclinical signs or symptoms of a disorder of fatty acid oxidation. I used several novel techniques to;

1. Review the clinical parameters of women with AFLP admitted to hospital and/or a liver ITU.
2. Identify women with this rare disorder, AFLP. The social media networking site Facebook has a AFLP support group from which women were recruited.
3. Assess in vivo mitochondrial function and energy turnover within the liver using phosphorus MRS (Pilot Study).
4. Stress the fatty acid oxidation pathway using Hypoxia (Pilot Study).
5. Stress the fatty acid oxidation pathway using a fast and fat burning exercise to search for subclinical defects in FAO.
6. **Search for novel proteins associated with a past history of AFLP.**

7. **Search for novel gene variants associated with families affected by AFLP using whole exome and whole genome sequencing.**

### 1.4 Hypothesis

i. I hypothesise that women with AFLP have a defect in fatty acid oxidation that prevents ketogenesis.

ii. I hypothesise that women who have had AFLP have a genetically inherited, subclinical defect in fatty acid oxidation that leads to impaired ketogenesis during prolonged fasting.

### 1.5 Research Aims and Objectives

i. To determine whether women with AFLP have detectable ketonuria at the time of clinical presentation.

ii. To determine whether women who have had AFLP can generate ketone bodies following a prolonged fast and fat-burning exercise.

iii. Using a proteomics analysis, to discover novel serum proteins in women with a past history of AFLP following a 24-hour fast and fat burning exercise.

iv. To determine whether women and their families affected by AFLP have a gene variant associated with fatty acid oxidation (FAO).
CHAPTER 2

PILOT EXPERIMENTAL STUDIES
Chapter 2  

2.1 Introduction

AFLP is a rare condition of pregnancy, which makes it difficult to study. There are several clinical and biochemical features of AFLP that suggest strong similarities with inherited disorders of FAO. There is also a well-recognized association between AFLP and a defect in mitochondrial beta-oxidation of long chain fatty acids.

It is in the mitochondrion where ATP is synthesized from ADP and inorganic phosphate (Pi) during the process of oxidative phosphorylation. It is ATP that provides energy for all cellular functions in the body (18, 19). Defects in FAO result in multi-organ dysfunction secondary to reduction in ketogenesis and ATP generation (149).

The focus of this research study was to identify changes in energy metabolism in women previously affected by AFLP compared to non-AFLP women as well as healthy pregnant women. I needed to develop a suitable experimental protocol to test my hypothesis that women who have had AFLP have a genetically inherited, subclinical defect in fatty acid oxidation that leads to impaired ketogenesis during prolonged fasting.

To determine the most appropriate study protocol to investigate energy metabolism in women who had AFLP, 3 pilot studies were carried out.
2.2 Pilot Studies

2.2.1 Pilot Study 1: Assessment of Hepatic intracellular ATP production with Phosphorus MRS

Magnetic Resonance Spectroscopy (MRS) is a method that recognizes the chemical content and structure of magnetic resonance visible nuclei of which there are 3; 1H, 13C and 31P. We are now able to assess in vivo mitochondrial function and energy turnover within human tissue using phosphorus MRS.

MRS was first used on rat skeletal muscle and allocated individual resonances to inorganic phosphate (Pi), phosphocreatinine (PCr) and adenosine triphosphate (ATP) (171). The first 31P MRS performed in an adult human brain illustrated seven normal peaks, Pi, PCr, Phosphomonoesters (PME), Phosphodiester (PDE) and the 3 phosphate groups of ATP (α, β, and γ) (172).

The liver is the largest and most highly metabolic organ of the human body. 31P MRS has become a useful non-invasive method to accurately measure hepatic function and aid diagnosis of pathological conditions (e.g. cirrhosis, malignancy and steatosis).

A phosphorus spectrum signals seven different peaks as described above. In liver disease, we are likely to see a raised PME (cell membrane products for synthesis) and a low PDE (cell membrane break-down products), as a result of damage to hepatic cells and a rapid turnover of phospholipids in hepatic cell membrane. A study of 49 patients with liver disease secondary to different pathologies like cirrhosis showed an increased PME/PDE ratio and reducing plasma albumin concentrations (173).

Other MRS study findings have included raised PME/ATP compared to healthy controls in patients with cirrhosis and primary biliary cholangitis (174, 175). The majority of the studies that followed, investigated metabolic changes in liver disease, in particular cirrhosis and fibrosis. The results have shown an increase in PME and decrease in PDE and ATP (176).
Liver disease specific to pregnancy is a result of the development of AFLP, Obstetric cholestasis or HELLP syndrome. 31P MRS was used to investigate changes in hepatic metabolism in a study of 7 women with HELLP syndrome. The results for 6 women (one woman was excluded as the pregnancy was terminated at 21 weeks), showed 5 normal phosphorus spectra and one abnormal spectrum. This spectrum showed an increase in PME and a decrease in ATP compared to healthy controls. However, the patient responsible for the abnormal spectrum was diagnosed with severe HELLP in comparison to the other women with HELLP syndrome (177).

31P MRS could play a useful role in the non-invasive assessment of the metabolic state of the liver in conditions specific to pregnancy. MRS assessment is being used in neonates and there have been a number of projects at UCL using MRS. Due to the availability of this test, I contacted the medical physics and bioengineering department at UCL to discuss my study. It was my aim to use 31P MRS to investigate whether women previously affected by AFLP had a defect in their ability to generate hepatic ATP.

2.2.1.1 MRS Protocol

To establish a working protocol, I performed a pilot study in 6 healthy, non-pregnant volunteers, in collaboration with Dr. David Price of the Department of Medical Physics and Bioengineering at UCLH.

All volunteers were invited to the radiology and imaging department at UCLH after a 12-hour fast (water could be consumed freely) to have a 31P MRS scan of the liver using a 3-Tesla MRI scanner. A radiographer performed the scan after a thorough checklist had been carried out to ensure there were no contraindications to having a MRI scan.

Volunteers were asked to lie on their backs with a coil placed over the liver. The scan lasted approximately 30-45 minutes. A panic button was provided if the volunteer wanted the scan to end at any point.
A pillow, headphones, earplugs and leg rests were provided to ensure the participant was comfortable. Once the scan was complete, the volunteer was free to leave.

As a result of this study and other studies being carried out by Dr. David Price, he developed a 14cm surface coil that sits over the liver and is associated with the 3-Tesla MRI scanner and multi-nuclear (phosphorus; 31P) channel. Dr. Price had experience with coils of different size and the 14cm coil was found to be the most appropriate. This coil and MRS allows measures of ATP peaks to be identified within the liver. From 6 volunteers, we demonstrated clear phosphorus peaks reflecting normal ATP production in the liver. The phosphorus spectrum, including ATP peaks from one healthy volunteer is shown below (See Figure 2-1). Further results are illustrated in Appendix 1.

**Figure 2-1 Normal Phosphorus spectrum of a healthy volunteer.**

I had planned to recruit women who have had a past history of AFLP compared to healthy controls to participate in this study. However, on further investigation I deduced that I would most likely only see a significant change in hepatic ATP production if these women were unwell or if their fatty acid oxidation pathways were significantly stressed. I was planning on using starvation as a stress factor however, following discussion with Dr Price we felt that a bigger stress would be required to show a difference. Due to practical difficulties stressing the FAO pathway and then moving women in a timely fashion (from the sports institute) to the MRI scanner, it was thought unfeasible to continue with this study.
2.2.2 Pilot Study 2: Assessment of Lipid response to sub-acute hypoxia

I next designed a study to stress the maternal fatty acid oxidation pathway using hypoxia with the aim to identify a FAO defect that might predispose a mother to AFLP. There have been a number of studies to show that hypoxia has an effect on lipid metabolism. Hypoxia is known to increase lipid deposits and up regulate genes associated with lipogenesis (98, 99).

2.2.2.1 Hypoxia Protocol

Nine healthy female volunteers were invited to the Institute of Sport, Exercise and Health (ISEH) following an 18-hour fast. Twenty milliliters of blood and a mid-stream urine were collected. The participants remained fasted whilst resting in a hypoxia chamber (12% Oxygen, equivalent to 4500m) for a further 6 hours, whilst being monitored with an oxygen finger probe and with heart rate and blood pressure monitors (Figure 2-2).

At 20, 22 and 24 hours of fasting, a further 20mL blood samples were collected. All samples were centrifuged (3000rpm for 10 minutes) and plasma and serum were collected to measure ketone bodies (β-hydroxybutyrate), non-esterified fatty acids (NEFA), lipid profile, insulin and glucose. A further aliquot of blood was centrifuged at 3000rpm for 10 minutes, in order to store plasma and serum in an -80°C freezer. The mid-stream urine specimen was stored in an -80°C freezer.

Figure 2-2 The Institute’s, Exercise and Health and the Hypoxia Chamber.
Descriptive characteristics of the 9 female volunteers are shown in Table 2-1. The age range was 19 – 39 years (Mean age 29 years) and BMI ranged from 18.7 – 30.5 kg/m² (Mean BMI 24 kg/m²).

Table 2-1 Baseline characteristics of the 9 healthy participants.

<table>
<thead>
<tr>
<th>Participant</th>
<th>Age</th>
<th>Height (cm)</th>
<th>Weight (kg)</th>
<th>BMI (kg/m²)</th>
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<td>21.6</td>
</tr>
<tr>
<td>9</td>
<td>34</td>
<td>167</td>
<td>53</td>
<td>19</td>
</tr>
<tr>
<td>Mean</td>
<td>29</td>
<td>168</td>
<td>67.3</td>
<td>24</td>
</tr>
</tbody>
</table>

The results show that between 18 – 24 hours of fasting and hypoxic conditions, serum levels of β-hydroxybutyrate (5HB) rise and the ratio of free fatty acid NEFA/BHB falls. Insulin levels fall whilst glucose levels remain stable (Figure 2-3). Oxygen saturations returned to baseline at the end of the study. The lowest average oxygen saturation was 85% at 2 hours of hypoxia (Figure 2-4).
Figure 2-3 Changes in serum metabolites throughout 6 hours of hypoxia in 9 healthy non-pregnant women (Median +/- IQR).

- **a.** Median Value of Insulin (mIU/L)
  - Time in Hypoxia Chamber (Hours) (18-24 hours of fasting)

- **b.** Median Value of Glucose (mmol/L)
  - Time in Hypoxia Chamber (Hours) (18-24 hours of fasting)

- **c.** Median Value of B-hydroxybutyrate (mmol/L)
  - Time in Hypoxia Chamber (Hours) (18-24 hours of fasting)

- **d.** Median Value of NEFA (mmol/L)
  - Time in Hypoxia Chamber (Hours) (18-24 hours of fasting)

- **e.** *
Chapter 2 Pilot Experimental Studies

Fasting & Hypoxia Study

![Graph showing median NEFA/BHB values over time in hypoxia chamber (18-24 hours of fasting).]

- a. Measure of Insulin, b. Measure of Glucose, c. Measure of β-hydroxybutyrate, d. Measure of NEFA and e. Ratio of NEFA to β-hydroxybutyrate. * P < 0.05 significant difference.

Figure 2-4 Oxygen saturations throughout the hypoxia study and fast only study.

The Non-Parametric Wilcoxon Signed Rank Test was used to statistically analyze the study data. This pilot study showed a significant difference in ketone production by subjecting participants to hypoxia. In particular, median β-hydroxybutyrate levels rose significantly during the 6 hours of hypoxia from 0.08 to 0.67mmol/L (p=0.001, 95% CI (0.27, 0.8)) and median NEFA levels rose from 0.61 to 0.9mmol/L (p=0.006, 95% CI (0.69, -0.11)). Glucose and insulin levels fell from a median of 5.6 to 4.6mmol/L (p=0.1162, 95% CI (0.1, 1.0)) and 9.2 to 5.3mIU/L (p= 0.1615, 95% CI (-7.0, 12.4) respectively. There were no significant differences in both glucose and insulin levels at the end of 6 hours of hypoxia. I hypothesized that women affected by AFLP will be less able to generate ketone bodies.
Despite my results demonstrating that hypoxia does stress the FAO pathway, I felt that potentially fat burning exercise would be a better physiological stress than hypoxia. This was suggested by Dr Phil Hennis who was the scientist helping with the study design. Furthermore, with the hypoxia study I was unable to quantify fat oxidation but with the exercise study quantifying fat oxidation would be possible and important to my study.

I developed an exercise protocol to ensure maximum fat oxidization. In order to discover the most effective way to stress the oxidation of NEFA to ketone bodies, I tested their response to ‘fat burning’ exercise.

2.2.3 Pilot Study 3: Fasting and Fat burning Exercise study

2.2.3.1 Exercise and Fat oxidation
It is well recognized that exercising for a longer duration increase fat oxidation rates, however there is conflicting evidence as to the most appropriate exercise intensity to work at to achieve greater fat oxidation (178, 179).

One study determined fat oxidation rates to be lower at a low exercise intensity compared to at a moderate intensity and then returning to a lower fat oxidation rate at higher intensity of exercise (180). This demonstrated that a particular exercise intensity for an individual to determine maximal fat oxidation does exist. In 2002, Achten et al developed an exercise test or “Fatmax” test, to determine the maximal fat oxidation rate (MFO) (181). This study involved 18 healthy men exercising initially at a work rate of 95W on a cycle ergometer. The work rate increased by 35W every five minutes until the respiratory exchange ratio (RER) was greater than 1.0. Once this point had been reached the workload increased every two minutes until exhaustion. To validate the “Fatmax” test the same 18 healthy men participated in another test which involved exercising for a continuous period at a constant work rate. This work rate was paralleled to the initial test with incremental increases in work rate. The “Fatmax” test was validated as no difference was identified in the fat oxidation rate between the test with the constant load and the incremental step test (181). The “Fatmax” test involves exercising with incremental increases in work rate either on a cycle ergometer or
treadmill. During the test, breath by breath samples are measured for each different stage. Indirect calorimetry can then be used to calculate total fat and carbohydrate oxidation rates in the body (181). This test produced results to demonstrate MFO rates, the exercise intensity at which MFO was determined (Fatmax) and the exercise intensity at which fat oxidation is minimal or cannot be measured (RER > 1.0).

With existing evidence, available on the accuracy of the “Fatmax” test and determination of MFO and the intensity at which this occurs, it seemed reasonable to develop an exercise protocol in which I aimed to perform repeated episodes of exercise where mostly fat is being oxidized. By validating this method to stress the lipolytic pathway I could assess lipid metabolism in women who have suffered from AFLP.

The exercise study proposed in my research project was aimed to test FAO. The use of cycle ergometry allowed calculation of fat and carbohydrate metabolism. The exercise was alternated with rest periods and of low intensity posing no significant risk to the mother or fetus.

2.2.3.2 Study protocol
All exercise studies were carried out at the Institute of Sport, Exercise and Health, 170 Tottenham Court Road, London. The laboratory contained all equipment necessary to perform the exercise study including a wet laboratory to process and store all blood samples. Before commencing the study, each participant had already been provided with a patient information leaflet. They were asked to fill out a health questionnaire and sign a consent form (Appendix 2). All volunteers were aware that the study duration was 6 hours.
Chapter 2 Pilot Experimental Studies

2.2.3.3 Calibration
A two-part calibration was performed prior to commencing the study and half way through the study. The first part calibrated flow volume using a 3 litre syringe. The mouth piece was carefully secured to the end of the syringe, which connected the computer system to the syringe. Next four smooth but slow pumps of the syringe were performed, followed by four smooth fast pumps. The computer system recorded the volume each pump performed by the syringe. The computer software automated the volume calibration and either passed or failed the calibration.

The second part involved calibration of the gas analyzer. A gas cylinder containing a mixture of 15% Oxygen and 5% Carbon dioxide was automatically passed through the medisoft ergocard system. The analyzer adjusted itself automatically according to the calibration gases.

2.2.3.4 Medisoft Ergocard professional testing system
The medisoft ergocard professional testing system (Vitalograph, Medisoft, Belgium) is specifically designed to perform cardio-pulmonary exercise testing. It has the resources to measure several variables by analyzing breath by breath measurements at rest and during exercise. Our study was mostly interested in measurements of volume of oxygen ($\dot{V}O_2$), volume of carbon dioxide ($\dot{V}CO_2$) and the respiratory exchange ratio (RER).

Breath-by-breath analysis occurred for 2-minutes during each 5-minute stage of the exercise test. The remaining $\dot{V}O_2$ and $\dot{V}CO_2$ values for those 2-minutes were averaged and used to calculate fat oxidation. The initial part of the exercise test determined the exercise intensity at which maximum fat oxidation rates occurred. This test was performed on a recumbent cycle ergometer (Figure 2-5).

Figure 2-5 The recumbent cycle ergometer at the Institute of Sport, Exercise and Health.
During the test, I measured:

- Maximal fat oxidation rates (MFO)
- The exercise intensity at which MFO occurred
- The exercise intensity at which fat oxidation rates are negligible (FATMIN; RER > 1.0). See Figure 2-6.

**Figure 2-6 A typical fat oxidation curve with increasing exercise intensity.**

MFO (Maximal fat oxidation rates), FATMAX (Exercise intensity at which maximum fat oxidation rates occur), FATMIN (Exercise intensity where fat oxidation rates are zero).
2.2.3.5 Indirect Calorimetry

Indirect calorimetry is a well-recognized method to ascertain whole body fat oxidation rates through measuring oxygen consumption and carbon dioxide production. It was in the 1920’s when innovative research first identified using pulmonary gas exchange measurements to calculate the amount of energy utilized whilst both resting and exercising (182). Following on from this research, we can now ascertain the oxidation of fat and carbohydrate molecules and their contribution to total energy expenditure.

The chemical structure of carbohydrate and fat molecules are different. Therefore, the amount of oxygen required and carbon dioxide produced for oxidization will be different between the two. Fat oxidation rates (g·min-1) were calculated using Microsoft excel and the work rate producing the maximum fat oxidation rate (MFO) were used for the remainder of the study. The calculation used was proposed by Jeukendrup and Wallis (183).

\[
\text{Fat Oxidation (g·min-1) = 1.65·VO}_2 - 1.701·VCO_2
\]

\[
\text{CHO oxidation (g·min-1) = 4.21·VCO}_2 - 2.962·VO_2
\]

2.2.3.6 Respiratory Exchange Ratio (RER)

The respiratory exchange ratio is a ratio between Carbon Dioxide (VCO\textsubscript{2}) released and Oxygen consumed (VO\textsubscript{2}) at a tissue level:

\[
\text{RER} = \frac{\text{VCO}_2}{\text{VO}_2}
\]

It is an indirect measure of fat or carbohydrate utilization for fuel at different intensities during a period of steady state exercise. The oxidation of one molecule of carbohydrate and one molecule of fat is shown below.

Oxidation of a molecule of Carbohydrate:
6 O2 + C6H12O6 → 6 CO2 + 6 H2O + 38 ATP

$$\text{RER} = \frac{\dot{V}_{CO_2}}{\dot{V}_{O_2}} = \frac{6 \text{ CO}_2}{6 \text{ O}_2} = 1.0$$

Oxidation of a molecule of Fatty Acid:

23 O2 + C16H32O2 → 16 CO2 + 16 H2O + 129 ATP

$$\text{RER} = \frac{\dot{V}_{CO_2}}{\dot{V}_{O_2}} = \frac{16 \text{ CO}_2}{23 \text{ O}_2} = 0.7$$

2.2.3.7 Exercise Trial

I recruited 10 healthy, non-pregnant female volunteers for this pilot study. Following an 18-hour fast all participants arrived at 8.00am at the Institute of Sport, Exercise and Health. Water could be consumed freely throughout the study. Initially all volunteers rested for 30 minutes during which a consent form was signed and a baseline blood pressure (BP), heart rate and oxygen saturation were measured. BMI was next determined by checking the weight and height of each volunteer. An initial blood test and urine sample were also collected.

The volunteers were then ready to commence the exercise bike test. The sport’s laboratory was set to maintain a temperature of 21°C. To start, the work rate had been programmed to an intensity of 10 watts. The workload increased in incremental steps of 10 watts every 5 minutes until an RER of 1.0. This RER represents oxidation of carbohydrate and not fat. Once an RER of 1.0 had been reached the exercise was stopped. During the last 2 minutes of each stage, respiratory gas measurements were collected using the medisoft ergocard gas analyzer. Once maximum fat oxidation had been determined it was that specific work rate that was programmed for the remainder of the study.

The participants cycled for 30 minutes and rested for 30 minutes over the next 5.5 hours. A further BP, heart rate and oxygen saturation were measured before commencing each exercise cycle. Another 3 blood tests were taken every 2 hours.
At the end of the study a final urine sample was collected. All samples were processed and stored in an \(-80^\circ\text{C}\) freezer for future screening.

All 10 volunteers completed the same study on a separate day where they were only required to fast and no exercise was performed. During the day, they rested in a quiet room where 4 blood tests were taken every 2 hours.

2.2.3.8 Indications for termination of the study included
- Chest Pain
- Severe desaturation (SPO\(_2\) < 80%)
- Pallor
- Dizziness
- Respiratory difficulties
- Loss of coordination
- Confusion

2.2.3.9 Results
All 10 participants were female, healthy and non-pregnant. They all took part in the fasting and exercise study and repeated the study on a separate day with just fasting alone. The aim was to recruit parous, healthy non-pregnant women, however I faced significant difficulty in recruiting volunteers for this all-day study. Therefore, I decided to recruit non-parous women as well as parous women if possible. The patient characteristics are shown in Table 2-2. The sample group had a mean age of 26 years and a mean BMI of 21.
Table 2-2 Participant baseline characteristics for the fasting and exercise study.

<table>
<thead>
<tr>
<th>Participant</th>
<th>Age (Years)</th>
<th>Height (cm)</th>
<th>Weight (kg)</th>
<th>BMI (kg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>171</td>
<td>68</td>
<td>23</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>160</td>
<td>60</td>
<td>23</td>
</tr>
<tr>
<td>3</td>
<td>31</td>
<td>158</td>
<td>58</td>
<td>23</td>
</tr>
<tr>
<td>4</td>
<td>22</td>
<td>165</td>
<td>51.5</td>
<td>19</td>
</tr>
<tr>
<td>5</td>
<td>32</td>
<td>172</td>
<td>55</td>
<td>19</td>
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<td>51</td>
<td>18</td>
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<td>7</td>
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<td>53</td>
<td>20</td>
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<tr>
<td>8</td>
<td>20</td>
<td>170</td>
<td>69</td>
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<tr>
<td>9</td>
<td>37</td>
<td>169</td>
<td>59</td>
<td>21</td>
</tr>
<tr>
<td>10</td>
<td>25</td>
<td>166</td>
<td>67</td>
<td>24</td>
</tr>
<tr>
<td>Mean</td>
<td>26</td>
<td>166</td>
<td>59</td>
<td>21</td>
</tr>
</tbody>
</table>
Figure 2-7 Metabolite differences between fasting only group and exercise and fasting group during the last 6 hours of the study.

a. Exercise Vs Fast Only Study

- B-Hydroxybutyrate (mmol/L)
- Median Value
- Time of Fast & Exercise (Hours)

b. Exercise Vs Fast Only Study

- NEFA (mmol/L)
- Median Value
- Time of Fast & Exercise (Hours)

c. Exercise Vs Fast Only Study

- Glucose (3.9-5.1 mmol/L)
- Median Value
- Time of Fast & Exercise (Hours)

d. Exercise Vs Fast Only Study

- Insulin (2.6-24.9 mIU/L)
- Median Value
- Time of Fast & Exercise (Hours)

e.
2.2.4 Hypoxia Study Vs Fasting and Exercise Study

Nine healthy women participated in the hypoxia study and 10 healthy women participated in the exercise study. The mean baseline characteristics of both groups of women are shown in Table 2-3.
Table 2-3 Mean participant characteristics for the hypoxia study and exercise study.

<table>
<thead>
<tr>
<th></th>
<th>Age Range</th>
<th>Mean Age (Years)</th>
<th>BMI Range</th>
<th>Mean BMI (kg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypoxia Study</td>
<td>19 - 39</td>
<td>29</td>
<td>18.7 – 30.5</td>
<td>24</td>
</tr>
<tr>
<td>Exercise Study</td>
<td>19 - 36</td>
<td>26</td>
<td>18.3 – 24.1</td>
<td>21</td>
</tr>
</tbody>
</table>

Direct comparisons between both groups cannot accurately be determined as different participants took part in the two studies and as stated above the groups were not matched for parity. However, some differences have been identified in both groups (Figure 2-8).

Figure 2-8 Metabolite differences between the hypoxia study and exercise study during the last 6 hours of the study.
a. Measure of β-hydroxybutyrate, b. Measure of NEFA, c. Measure of Glucose and d. Measure of Insulin. * P < 0.05 significant difference.

Table 2-4 Difference in metabolic parameters at 24 hours between the exercise study and hypoxia study (Non-Parametric Wilcoxon Signed Rank Test)

<table>
<thead>
<tr>
<th></th>
<th>Glucose</th>
<th>Insulin</th>
<th>NEFA</th>
<th>β-Hydroxybutyrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median difference</td>
<td>-0.55</td>
<td>-2.95</td>
<td>0.65</td>
<td>0.32</td>
</tr>
<tr>
<td>95% CI for</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pop. median</td>
<td>(0.27, 1.42)</td>
<td>(1.25, 7.64)</td>
<td>(-1.33, -0.19)</td>
<td>(-0.53, 0.33)</td>
</tr>
<tr>
<td>P-value</td>
<td>0.013</td>
<td>0.003</td>
<td>0.008</td>
<td>0.842</td>
</tr>
</tbody>
</table>

The results show that fasting and exercise caused a bigger change in Glucose, Insulin and NEFA than fasting with hypoxia, however, the standard error bars are significantly large. Normalisation of data was confirmed using Graphpad Prism.
statistical software and the D’Agostina-Pearson and Kolmogorov-Smirnov normality tests.

I decided to adopt the fast and exercise study to stress the FAO pathway of women with AFLP because the results show a larger difference in FAO when compared to hypoxia and fasting alone. The exercise study was also a more suitable study as I could quantify fat metabolism through indirect calorimetry, which was not possible with the hypoxia study.

2.3 Summary

Following the 3 different pilot studies, the results suggest that fasting and exercise stress the fatty acid oxidation pathway effectively. As a result, I proceeded to recruit women to take part in a fast and fat burning exercise study. The next chapter describes the methodology of this study and other methods involved in testing my hypothesis.
CHAPTER 3
METHODOLOGY
AND MATERIALS
Chapter 3 Methodology and Materials

One of the main aims of my thesis is to identify whether women previously affected by AFLP have a defect in energy metabolism, in particular through fatty acid oxidation, which is unmasked in the third trimester of pregnancy. I performed several pilot studies (Chapter 2), before I determined a final optimal study protocol. In brief, I identified women with a past history of AFLP, I extracted DNA from them and their family (The family included the affected woman who had AFLP, the father of the children and first-degree relatives including the affected child and unaffected siblings) and used a regime of fasting with fat-burning exercise to stress their ability to generate energy. I searched for novel gene variants associated with energy metabolism in women with AFLP and following the study protocol, I compared differences in the products of FAO in women who had AFLP with those who did not have AFLP and women in the third trimester of pregnancy.

3.1 Overview of Final Methodology to fulfil objectives

i. Investigation of Families previously affected by AFLP

In all family members (Father of children, affected child and unaffected sibling) where a mother had been affected by AFLP I studied the whole exome and/or whole genome to identify novel gene variants that may be causal of this condition.

ii. Investigation of non-pregnant women with and without a past history of AFLP

In women who have had AFLP I used fasting with fat burning exercise to stress the FAO pathway to identify novel functional defects in lipid metabolism compared with women who did not have AFLP.

iii. Investigation of pregnant women without AFLP

In a separate cohort of healthy pregnant women (BMI 18 – 30kg/m²) I used fasting with fat burning exercise stress to characterize alterations in FAO during the third trimester.

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3.2 Study Design

3.2.1 Fasting with Fat-burning Exercise

This cohort study was performed on 3 groups of women (i) non-pregnant women previously affected by AFLP, (ii) non-pregnant women unaffected by AFLP and (iii) pregnant women in the third trimester. This study was carried out at The Institute of Sport, Exercise and Health (ISEH). Ethical approval for my study entitled ‘Investigation into the Aetiology of Acute Fatty Liver of Pregnancy’ was granted by the NRES Committee London – Bromley (REC reference: 14/LO/1489) and the Joint UCLH/UCL Research and Development department.

3.2.2 Sample Size Calculations

Sample size calculations were performed using standard sample size formulae from the center for applied statistics courses 2015, Institute of Child Health (Director Professor Angie Wade). I performed a pilot study of 10 healthy volunteers comparing FAO in ‘fasting with exercise’ to ‘fasting’ alone. An independent t-test provided a mean difference of the main end-product of fatty acid oxidation (β-hydroxybutyrate) as being 0.40mmol/L. The exercise and fasting study showed a mean of 0.98 mmol/L and standard deviation 0.45. Using this information along with a 95% confidence and study power of 80 I needed to recruit 20 healthy non-pregnant volunteers.

Individuals who suffer with a disorder of fatty acid oxidation are unable to produce ketone bodies or produce a reduced amount. I hypothesize that women who have had AFLP have a disorder of FAO and will be unable to produce ketones in the same way as a healthy individual. I would expect to see greater than 50% reduction in the ketones produced in women who have had AFLP compared with healthy controls. Individuals with a FAO disorder are unable to produce ketone bodies, therefore it seemed reasonable that if women who have had AFLP have a subclinical disorder of FAO, if stressed they would have a greater than 50% reduction in ketones.
If I assume the standard deviation remains the same, a significance of 95% and study power of 80, I will require a sample size of 13 women who have suffered from AFLP. If I used a power of 90 I would need to recruit almost double the number of women. This would have been difficult due to the rarity of AFLP and difficulty in recruiting for my all-day study. Therefore, I felt it was acceptable to use a power of 80 when deciding my sample size.

3.2.3 Statistical Analysis

Simple descriptive statistics have been used to compare the study participants. Baseline characteristics of non-pregnant women who have previously suffered from AFLP, non-AFLP women, pregnant women and non-pregnant women have been summarized using means and standard deviations or medians and interquartile range where appropriate. Statistical analysis was performed using IBM SPSS for Mac, version 22 and GraphPad Prism version 7. Normality of data obtained was determined through visual assessment of histograms and confirmed using Graphpad Prism statistical software and the D'Agostina-Pearson and Kolmogorov-Smirnov normality tests. When comparing the difference between two means, an unpaired t-test or the Non-Parametric Wilcoxon Signed Rank Test was the statistical test employed for continuous variables. A statistically significant result was determined with a 2-tailed P-value < 0.05.
3.3 Inclusion Criteria for Participants

I identified separate criteria for the 3 individual groups of participants that have been recruited to the study. All participants provided informed consent prior to taking part in the study. The non-AFLP participants were divided into 2 groups; 1 group fasted for 24 hours (n=23) and the second group fasted for 18 hours (n=5).

i. AFLP group (n=13):

- Fulfilled the Swansea criteria for diagnosis of AFLP (Table 1-3)
- Currently not pregnant, aged 18 – 49 years’ old and with no medical problems
- Family members include; Woman who had AFLP, Father of the children, and first-degree relatives including the child born in the pregnancy where the mother had AFLP and the sibling born in another pregnancy where the mother did not have AFLP.

ii. Non-AFLP women (n=28):

- Non-pregnant
- Aged 18 – 49 years’ old
- Currently healthy with no significant medical problems
- BMI 18 – 30kg/m²

iii. Pregnant Group (n=12):

- Healthy pregnant women with no significant medical problems
- Gestational age 32 – 39 weeks
- Single Intrauterine Pregnancy
- BMI at booking (10-12 weeks’ gestation) 18 – 30kg/m²
3.4 Exclusion Criteria for participants

i. Exclusion Criteria for whole exome and genome sequencing study

- Known gene variant G1526C for LCHAD deficiency in the participant
- Known disorder of fatty acid oxidation in the participant
- If fetal tissue was unavailable from AFLP-related intrauterine death or stillbirth.

ii. Exclusion Criteria for Fasting and Exercise Study

a. Pregnant women

- Heart disease/ Heart Failure
- Persistent vaginal bleeding
- Placenta Praevia
- Risk of preterm labour
- Small for gestational age/ Growth restricted babies
- Pregnancy induced hypertension
- Incompetent cervix/ cervical cerclage in situ
- Diabetes/ Gestational Diabetes
- Severe anaemia
- All exclusion criteria as for non-pregnant women

b. Non-Pregnant women

- Known disorder of fatty acid oxidation in the participant
- Known liver disease in the participant e.g. fatty liver, hepatitis, cirrhosis
- Heart disease/ Heart Failure
- Symptomatic arrhythmia’s/ syncope
- Uncontrolled Asthma
- Thrombosis
- Resting room oxygen saturations < 85%
3.5 Experimental Design and Study Protocol

This is a cohort study to investigate the pathogenesis of AFLP. Participants consented to all or some of the study elements.

3.5.1 Participants

I initially attempted to identify participants on several AFLP support groups, however I soon realized that these had been abandoned for a single patient group for women with AFLP, established on the social networking group Facebook. I went on to contact the group administrator to get consent to post on the group forum. Following an initial expression of interest in our research by families affected by AFLP posted on Facebook, I had an overwhelming response to a preliminary patient engagement day held at UCL.

Families affected by AFLP attended from all over the UK and expressions of interest came from all over the world. All families that attended the patient awareness day offered their view on our research proposal and were very keen to become involved in all elements of our research. I recruited 13 families previously affected by AFLP from the ‘AFLP support group page’ on Facebook and London based hospitals including UCLH.

Prior to the study, I asked all women previously affected by AFLP to obtain a copy of their medical notes. If this was not possible I was given permission to approach their hospital for a copy of their relevant medical records. Once I had read the medical records and the diagnosis of AFLP had been confirmed according to the Swansea criteria for AFLP, I provided information packs regarding the study prior to seeking consent. All women who contacted me from the Facebook group did have AFLP and this was confirmed through an interview with them and reading their medical records.
Healthy pregnant women were recruited from midwifery booking clinics, antenatal clinics as well as antenatal classes, all of which took place at the Elizabeth Garret Anderson wing, University College London Hospital. All pregnant women provided verbal consent to review their medical records to ensure they met our research criteria for recruitment.

3.6 Exercise Study

I recruited 3 groups of volunteers for this study:

i. Non-pregnant women previously affected by AFLP (n=13).

ii. Non-pregnant women without a past history of AFLP (n=28). Twenty-three women acted as controls for AFLP cases and 5 women acted as controls for pregnant women (shorter fast).

iii. Healthy Pregnant women (n = 12).

3.6.1 The Metabolic Study of women who had a past pregnancy affected by AFLP

All participants fasted for 18 hours, unsupervised at home. The fast commenced at 14.30 the day before the study. Water could be consumed freely. Eighteen hours later, at 08.30 on the following day they attended the ISEH. Over the next 6 hours the participants underwent intermittent fat-burning exercise as described in Chapter 2, Section 2.2.3.2. Whilst on the exercise bike the participant wore a light facemask, which collected breath-by-breath gas measurements.

Peripheral blood (20mL) was taken at 18 hours, 20 hours, 22 hours and 24 hours of fasting. Plasma and serum was stored in an -80°C freezer for future study.
3.6.2 The metabolic study of healthy non-pregnant women unaffected by AFLP

Twenty-three participants performed the same protocol as the AFLP group.

3.6.3 The Metabolic study of women in third trimester of pregnancy

All pregnant women underwent an overnight fast, similar to that for a routine glucose tolerance test (GTT). The fast commenced at 20.30 and water could be consumed freely. Twelve hours later, at 08.30 the participants attended the ISEH.

Over the next 6 hours the participants underwent intermittent fat-burning exercise as described in Chapter 2. During the study, blood pressure and heart rate were monitored every hour. Peripheral maternal blood (20mL) was taken every 2 hours at 12 hours, 14 hours, 16 hours and 18 hours of fasting. Plasma, serum and whole blood was stored in an -80°C freezer for future study. A full antenatal check was also performed with fetal heart rate monitoring at 12 hours, 14 hours, 16 hours and 18 hours of the study.

3.6.4 The Metabolic study of non-pregnant women

Five non-pregnant participants performed the same protocol as the pregnant group.

3.7 Proteomics

Proteomics is the large-scale analysis of proteins. Proteins are made up of amino acids. This technology enables a measure of the concentration of multiple proteins in a sample. The proteome changes following stresses occurring within a cell.
In this study, I sent plasma samples for proteomic analysis before and after the exercise study. Proteomics were carried out by SomaLogic at UCL genomics, ICH. SomaLogic use SOMAmer reagents and a SOMAscan TM assay. It is this reagent (DNA based molecules) that binds to high affinity and high specificity proteins. The SOMAscan platform can concurrently measure over 1000 proteins in any given sample. The SOMAscan assay is performed using the SomaLogic Quality system. All equipment within the unit is maintained and operated in accordance with standard operating procedures.

3.7.1 Proteomic Protocol

3.7.1.1 Samples for Proteomic Analysis
Fifty-six plasma samples were transported to Somalogic, ICH. Two samples each from 13 women who had previously suffered from AFLP and 13 women who did not have AFLP, from before and after metabolic exercise.

3.7.1.2 Multiplexed SOMAmer affinity assay
Initially SOMAmer reagents (labeled with a 5′ fluorophore, photocleavable linker and biotin) are immobilized on beads coated with streptavidin. This reagent is next incubated with the plasma sample containing the proteins, where protein complexes form on the beads. The unbound proteins are then washed away leaving the protein complexes to be tagged with biotin. The photocleaver linker uses ultraviolet light to release the SOMAmer protein complexes from the beads. These protein complexes are further incubated in a separate buffer which contained a polyanionic competitor. This buffer allows the breakdown of non-specific protein interactions. The desired SOMAmer protein complexes are recaptured with another set of biotin tagged proteins. Next, further unbound SOMAmer reagents are washed away.
Chapter 3 Methodology and Materials

The final few steps involve the bound SOMAmer reagents being released from the beads in a separate denaturing buffer. The SOMAmer reagents are now ready to be quantified using a microarray chip containing complementary sequences is where the final SOMAmer reagents are hybridized to and where the proteins are quantified.

3.7.1.3 Data analysis
SomaLogic have developed normalization procedures to ensure the data produced is reliable. This involves placement of hybridization control sequences into the assay eluate before hybridization takes place.

This is then measured for each sample array correcting for systematic effects. The raw data has been analyzed by Dr. Jorge Garcia-Hernandez at the Farr Institute, UCL.

3.8 DNA Extraction
I recruited 7 families with a confirmed diagnosis of AFLP. The families included mother, father, affected child and in 3 families a sibling unaffected by AFLP.

During a pre-arranged day, each family attended the Clinical Research Facility in the EGA Maternity Wing, UCLH or the ISEH. The procedure was explained and consent was confirmed. Local anaesthetic cream (Emla) or spray was used for the children to reduce any discomfort. Five mL of venous blood was taken under sterile conditions from each member of the family. A plaster/ dressing was placed over the puncture site.

DNA was extracted immediately from 200µl of whole blood using the QIAamp DNA Blood Mini Kit (QIAGEN Ltd, Manchester, UK). I produced 2 aliquots of extracted DNA for each family member.
Chapter 3 Methodology and Materials

The DNA was stored at -20°C until the samples were transported to GOSgene, ICH, for either whole exome sequencing (WES) or whole genome sequencing (WGS). DNA concentration was assessed by Dr. Hywel Williams using a Qubit 3.0 Fluorometer (Life technologies) and following instructions provided by the manufacturer.

The DNA from 3 families were sent for WES and the DNA from 4 families were sent for WGS. When I commenced my study, the plan was to send all samples for WES, however, Gosgene changed their protocols and were no longer sending samples for WES and all samples were being sent for WGS. The sequencing protocols differ slightly, however the analysis remained the same for all families, where only the protein coding region of the genome was analyzed.

Before WES or WGS could be performed a sample of the blood collected from the mother was tested for the common gene variant G1528C of the LCHAD enzyme, as we know this is rarely associated with AFLP. In all families in which I studied WGS and WES, this gene variant was absent.

3.8.1 Whole exome sequencing

Whole exome sequencing was performed in collaboration with Dr. Hywel Williams (GOSgene, Institute for Child Health). Whole exome sequencing selectively sequences the protein coding regions of the genome and is a cost-effective way of identifying novel gene variants in rare disorders such as AFLP. The protein coding region of the genome (exomes) constitute < 2% of the entire genome. However, it is widely known that greater than 85% of Mendelian disorders disrupt this protein coding region of the genome and are more likely to have a functional or damaging effect (184, 185). It therefore seems reasonable to first search for gene variants in this subset of the genome.
For this study, we used the illumina platform HiSeq2500 in Hong Kong (Beijing Genomics Institute, BGI) where 2x100bp read length DNA sequences were produced. The exome sequencing pipeline involves several different steps (Figure 3-1). An exome DNA library is created from genomic DNA. Initially, genomic DNA is fragmented and the ends of these fragments are bound by oligonucleotide adaptors. Next, the DNA is enriched for sequences corresponding to exons by aqueous phase hybridization capture. It is here where the fragments are hybridized to biotinylated DNA or RNA baits. Streptavidin coated beads are added and the resultant complexes are washed to provide targeted DNA sequences for amplification (Illumina TruSeq).

Following the Illumina TruSeq exome enrichment DNA clusters are generated. First, DNA molecules produced in the library preparation stage are attached to the surface of an 8-channeled flow cell, by an automated Cluster station. Following this bridge amplification of the DNA molecules occur, creating clusters.

The next step involves sequencing by synthesis, which involves the addition of DNA polymerase to all 4 nucleotides, which are each conveying a unique fluorescent label and have a corresponding fluorophore attached to it. The nucleotides are recognized by their fluorescent emissions. As the strand elongates the clusters are excited by light and the emitted colour is recorded by an optical detector.

Once the nucleotides have been incorporated the fluorophore is cleaved, which allows the next nucleotide to be incorporated in the next cycle.

We used the Agilent SureSelect v4 (Agilent, Santa Clara, California, USA) chemistry to target the exome and run the samples using the Illumina next generation platform with the resultant data analyzed by us and the UCL bioinformatics group within GOSgene.
Figure 3-1 Exome sequencing process.

I identified 3 families previously affected by AFLP. I extracted DNA from each family member (Table 3-1).

Table 3-1 Three families identified for WES.

<table>
<thead>
<tr>
<th>Family</th>
<th>Mother</th>
<th>Father</th>
<th>Affected Child</th>
<th>Unaffected Child</th>
<th>Total samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Family 1</td>
<td>✔</td>
<td>✔</td>
<td>Boy</td>
<td>Girl</td>
<td>4</td>
</tr>
<tr>
<td>Family 2</td>
<td>✔</td>
<td>✔</td>
<td>Twin Boys</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>Family 3</td>
<td>✔</td>
<td>✔</td>
<td>Boy</td>
<td>Girl</td>
<td>4</td>
</tr>
</tbody>
</table>
3.8.2 Whole exome sequencing protocol

3.8.2.1 Sample
DNA was extracted (n=12), transferred to Gosgene and assessed as described in above. Beijing Genomics required at least 1.0ug of DNA.

3.8.2.2 Exome Capture and Sequencing
Prepared samples of high quality DNA were transferred to Beijing Genomics and were processed using the Agilent SureSelect v4 sample preparation and capture protocol. Processed samples were then run on an Illumina HiSeq2500 sequencer producing sequenced reads at an average of 100 times exome coverage (100X), which were transferred back to Gosgene, ICH.

3.8.2.3 Exome Analysis
The bioinformatics team at Gosgene aligned the sequenced reads to the human reference genome (hg19), using the Burrows-Wheeler Alignment Tool (BWA) software. The software package GATK further processed the alignments, including base quality score recalibration, indel realignment, duplicate removal and to perform SNP and INDEL discovery and genotyping using standard hard filtering parameters and variant quality score recalibration. The variant data could now be analyzed using the Ingenuity Variant Analysis Software from Ingenuity Systems (www.ingenuity.com).

A BAM file containing a binary format of the aligned sequencing data was also produced (using GATK) for each individual sample, where variants could be visualized using the Integrative Genomics Viewer:

(www.broadinstitute.org/igv/UserGuide)
3.8.2.4 Filter Pipeline

The Ingenuity variant analysis software uses a data filter pipeline composed of various stages. A data filter pipeline was developed, to reduce the final number of variants assessed.

Firstly, samples not passing a call quality log score of 20 (1 in 100 chance that the call is a false positive) or read depth (number of reads mapped to a given site in one sample of multiple sets of samples) of at least 10 were removed (Figure 3-2). Therefore, 99% of the calls were accurate.

**Figure 3-2 Ingenuity Variant Analysis Filter 1.**

![Filter 1](image)

Secondly, variants not present in the coding sequence or canonical splice regions were removed. Next a filter to focus on analysis of frameshift, splice site, stop-gain, stop-loss and undefined mutations (Figure 3-3).

**Figure 3-3 Ingenuity Variant Analysis Filter 3.**
The next filter removed common variants with an allele frequency of >0.5% in the 1000 genomes project, >0.5% of all in the ExAC (The Exome Aggregation Consortium) and >0.5% of all NHLBI ESP (exome sequencing project) exomes.

Figure 3-4 Ingenuity Variant Analysis Filter 4.

The remaining variants were filtered against known genetic variants associated with energy metabolism and fatty acid oxidation disorders identified from OMIM database and published literature (Table 3-2 and Figure 3-5). Rare variants identified were subject to further assessment and validation.
Table 3-2 Genes involved in energy metabolism.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene</th>
<th>Gene</th>
<th>Gene</th>
<th>Gene</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>AADAC</td>
<td>GLS</td>
<td>LDHB</td>
<td>MGLL</td>
<td>SLC1A3</td>
<td>SLC38A3</td>
</tr>
<tr>
<td>ACAD9</td>
<td>GLS2</td>
<td>LDHc</td>
<td>PCDHGA5</td>
<td>SLC22A5</td>
<td>SLC38A5</td>
</tr>
<tr>
<td>ACADM</td>
<td>GLUD1</td>
<td>LIPA</td>
<td>PDHA1</td>
<td>SLC25A20</td>
<td>TALDO1</td>
</tr>
<tr>
<td>ACADS</td>
<td>HADH</td>
<td>LIPC</td>
<td>PDHA2</td>
<td>SLC2A1</td>
<td>TAZ</td>
</tr>
<tr>
<td>ACADVL</td>
<td>HADHA</td>
<td>LIPE</td>
<td>PDHB</td>
<td>SLC2A10</td>
<td>TCF12</td>
</tr>
<tr>
<td>CEL</td>
<td>HADHB</td>
<td>LIPF</td>
<td>PKF</td>
<td>SLC2A11</td>
<td>TKT</td>
</tr>
<tr>
<td>CPT1A</td>
<td>HK1</td>
<td>LIPG</td>
<td>PKM</td>
<td>SLC2A12</td>
<td>FGF21</td>
</tr>
<tr>
<td>CPT2</td>
<td>HK2</td>
<td>LIPH</td>
<td>PNLIP</td>
<td>SLC2A2</td>
<td></td>
</tr>
<tr>
<td>DAGLA</td>
<td>HK3</td>
<td>LIPI</td>
<td>PNLIPPRP3</td>
<td>SLC2A3</td>
<td></td>
</tr>
<tr>
<td>DAGLB</td>
<td>HMGCL</td>
<td>LIPK</td>
<td>PNPLA2</td>
<td>SLC2A4</td>
<td></td>
</tr>
<tr>
<td>DDR2</td>
<td>HNRNPU</td>
<td>LIPM</td>
<td>PNPLA3</td>
<td>SLC2A5</td>
<td></td>
</tr>
<tr>
<td>ETFA</td>
<td>HSD17B10</td>
<td>LIPN</td>
<td>PPARG</td>
<td>SLC2A6</td>
<td></td>
</tr>
<tr>
<td>ETFB</td>
<td>IDH1</td>
<td>ME1</td>
<td>PPARA</td>
<td>SLC2A7</td>
<td></td>
</tr>
<tr>
<td>ETFDH</td>
<td>KCNA4</td>
<td>ME2</td>
<td>NR1C1</td>
<td>SLC2A8</td>
<td></td>
</tr>
<tr>
<td>G6PD</td>
<td>LDHA</td>
<td>ME3</td>
<td>SDHA</td>
<td>SLC2A9</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3-5 Ingenuity Variant Analysis Filter 5.
3.9 Whole Genome Sequencing

Whole genome sequencing (WGS) determines the entire DNA sequence of the human genome. WGS enables assessment of single nucleotide variants (SNV), Copy number variants (CNV), structural variations and insertions and deletions of bases in both protein coding and non-coding regions of the genome. Compared to WES sequencing of the whole genome provides a more uniform coverage therefore producing reliable DNA sequencing.

I identified 4 well-phenotyped families that have previously been affected by AFLP. I extracted DNA from each family member (Table 3-3).

Table 3-3 Four families identified for WGS.

<table>
<thead>
<tr>
<th>Family</th>
<th>Mother</th>
<th>Father</th>
<th>Affected Child</th>
<th>Unaffected Child</th>
<th>Total samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Family 4</td>
<td>✓</td>
<td>✓</td>
<td>Girl</td>
<td>N/A</td>
<td>3</td>
</tr>
</tbody>
</table>
3.9.1 Whole genome sequencing protocol

3.9.1.1 Genome Sequencing

DNA was extracted (n=15) and transferred to Gosgene. Prepared samples of high quality DNA were transferred to Beijing Genomics and were processed for sequencing. The initial preparation of DNA for WGS is the same as for WES. DNA is fragmented followed by oligonucleotide adaptors ligated to each fragmented end. With WES, the next step would involve DNA enrichment by the aqueous phase hybridization capture. However, with WGS there are no further steps to perform and the DNA library is complete and ready to be sequenced (Cluster generation, sequencing by synthesis and data analysis).

Processed samples were then run on an Illumina HiSeq X Ten sequencer producing sequenced reads at an average of 30 times genome coverage (30X), which were transferred back to Gosgene.

3.9.1.2 Genome Analysis

The bioinformatics team at Gosgene aligned the sequenced reads to the human reference genome (hg19), using the GENALICE hardware and software solution which performs read mapping and variant calling to produce standard vcf and BAM files. The variant data was analyzed using the Ingenuity Variant Analysis Software and BAM files.
3.9.1.3 Filter Pipeline

The ingenuity data filter pipeline used for whole exome data (Section 3.8.2.4) was also used for analysis of the whole genome data for the 4 different families. Figure 3-6 shows a summary of this study.

3.10 General Methods

3.10.1 Biological samples (handling, processing and storage)

Blood, urine and DNA were collected from participants following the participant’s consent and in accordance with the study protocol. The samples were processed and frozen immediately after collection.

All blood samples for metabolic and biochemical analysis were centrifuged at 3000rpm for 10 minutes and sent to Dr. Gill Rumsby (Consultant Biochemist) at UCLH biochemical laboratory, 60 Whitfield street and to Dr. Helen Aitkenhead (Clinical Scientist) at Great Ormond Street Hospital, CBL laboratories. Measurements of NEFA, β-hydroxybutyrate, Glucose, Insulin, Lipid profile and Lactate were made using routine validated assays.

Extracted DNA was sent to Dr. Hywel Williams at GOSgene, Institute for Child Health, 30 Guilford St, London WC1N 1EH for whole exome and whole genome sequencing and bioinformatics analysis.

At the Clinical Research Facility, UCLH and the Institute of Sport, exercise and health, I processed, stored and disposed of Blood, urine and DNA in accordance with all applicable legal and regulatory requirements, including the Human Tissue Act 2004 and any amendments thereto.
Figure 3-6 Summary of Study.

**Recruitment**
- AFLP group (n=13) & Non-AFLP women (n=23)
- Pregnant group (n=12) & Non-Pregnant women (n=5)

**Cases**
- Previous AFLP
- Pregnant Women (32-39 weeks)

**Controls**
- Non-AFLP women
- Non-Pregnant women
Exercise Study

Proteomics

AFLP families only
Affected mother, father, affected child and/or unaffected child

DNA extraction

Whole Exome Sequencing (WES)
n=3

Whole Genome Sequencing (WGS)
n=4
CHAPTER 4

ACUTE FATTY LIVER
OF PREGNANCY
PHENOTYPE
Chapter 4  Acute Fatty Liver of Pregnancy Phenotype

4.1 Introduction

Establishing an accurate phenotype for women who develop AFLP has been difficult as women often present critically unwell in the third trimester and require urgent childbirth. The Swansea criteria for diagnosing AFLP were proposed by Ch’ng et al in 2002 following a review of 142 patients with abnormal liver function tests from a total of 4377 deliveries (2). Out of 142 patients, 68 women had Pre-eclampsia, 30 developed HELLP syndrome, 23 obstetric cholestasis and 5 women developed AFLP. The Swansea criteria (Table 1-3) were developed from general observations and biochemical features identified in this small group of affected women, however they are currently widely cited.

The UK Obstetric Surveillance System (UKOSS) audited the number of women with AFLP using the Swansea criteria. Throughout the UK, between February 2005 and August 2006, 57 women were diagnosed with AFLP. The aim of the study was to accurately document the incidence, management and outcomes of women with AFLP in the UK, as well as to evaluate the proposed diagnostic criteria. This group identified an incidence of 5 cases per 100,000 maternities which is a lower incidence compared to other reports. Furthermore, maternal and neonatal outcomes were better than previous cited literature. The study findings also supported the criteria proposed by Ch’ng et al and suggested all future studies adopt this criteria to allow accurate comparisons of results between different studies (3).

Between June 2008 and July 2013, a retrospective study of 56 women with AFLP assessed clinical features, laboratory results, management and outcomes (186). Nausea and vomiting without apparent cause was the main symptom experienced by these women. This study also suggested elevated levels of serum transaminase (>200 U/L) and bilirubin (>60 mmol/L) were of greater importance than any other laboratory result when diagnosing AFLP.
Increasing bilirubin levels were associated with fetal and neonatal death. Raised PT and INR levels were also positively correlated with serious maternal complications (186).

I propose that AFLP occurs during the insulin resistant third trimester of pregnancy in women with a defect in fatty acid oxidation, but not just a deficiency in Long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD). It was Ibdah and colleagues who found that some women with AFLP have a mutation in the LCHAD gene. This enzyme catalyses the third step in the beta oxidation of fatty acids, where the end-product is acetyl-CoA (4). Nineteen out of 24 children with hypoketotic hypoglycaemia, were themselves found to be LCHAD deficient. Eight children were LCHAD homozygoses and the remaining 11 were compound heterozygote for the common gene variant (G1528C). Furthermore, 79% (15/19) of the women heterozygous for the LCHAD gene defect and who were pregnant with an LCHAD deficient fetus, developed AFLP (12/19) or HELLP syndrome (3/19). The diagnosis was made by the referring obstetric clinician using standard diagnostic criteria. The remaining 5 women who did not have any gene defects and did not develop AFLP, delivered offspring with complete tri-functional protein deficiency (4) This study proposed that AFLP was the result of a fetal fatty acid oxidation disorder in a heterozygous mother. However, outside of the cohort studied by Ibdah et al, most women with AFLP are not heterozygous for LCHAD deficiency and not all mothers who deliver LCHAD deficient babies develop AFLP.

Nevertheless, this study has greatly helped our understanding of the pathogenesis of AFLP by implicating a defect in fatty acid oxidation.

Women with AFLP also share many of the presenting symptoms and signs of a fatty acid oxidation (FAO) defect (See Error! Reference source not found.). A defect in maternal or fetal LCHAD is a rare explanation of the FAO defect, characteristic of AFLP. In my study cohort, 28 women had been tested and were negative for the common LCHAD variant G1528C. The offspring of 5 women were tested for the same gene variant, which was absent.
Chapter 4 Acute Fatty Liver of Pregnancy Phenotype

In this chapter, my aim is to further characterize the clinical symptoms and signs of women who develop AFLP and to refine the diagnostic criteria.

4.2 Hypothesis

AFLP is a gestational metabolic disorder with the same clinical and biochemical features as a disorder of Fatty Acid Oxidation.

4.3 Specific Aims

i. To develop an accurate phenotype for women affected by AFLP.

ii. To collect clinical, biochemical and radiological information from 13 women previously affected by AFLP identified by myself and 20 women admitted to the King’s College Liver ITU between January 2005 to December 2015.

iii. To develop accurate diagnostic criteria for AFLP.

4.4 Methodology

4.4.1 Study Design

This observational study was carried out at University College London Hospital (UCLH). Women were identified from the AFLP support group on Facebook and King’s College London Hospital between April 2015 and January 2017. We were granted ethical approval for our study by the NRES Committee London – Bromley (REC reference: 14/LO/1489), the Joint UCLH/UCL Research and Development department and the King’s College Hospital Research and Development office (REF KCH16-133).
4.4.2 Women with a previous history of AFLP

Families affected by AFLP were identified from the AFLP support group on the social networking site Facebook (n=8) and maternity units in London (n=13). They were included in the study if they met the Swansea criteria for diagnosis of AFLP. All women or offspring were negative for the common missense mutation G1528C in the LCHAD gene. The recruitment process is described in detail in Chapter 3.

4.4.3 Patients admitted to King’s College London liver ITU

The Liver ITU at King’s College London provided an accurate record of all pregnant women and/or post-partum women that had previously been admitted to the Liver ITU over the past 10 years (January 2005 to December 2015). I obtained the medical records for each patient, assessed their electronic patient records and reviewed their biochemical investigations. The radiological investigations were all reviewed again by a senior radiologist at the hospital. Through this process, I identified 20 women affected by AFLP and had been a patient in the Liver ITU. All available baseline characteristics and retrospective data were collected and reviewed. I was unable to interview these women as they were not current patients in the hospital.

4.4.4 Statistical Analysis

Baseline characteristics of women who have previously suffered from AFLP, have been summarized using means and standard deviations or medians and interquartile range where appropriate. Statistical analysis was performed using IBM SPSS for Mac, version 22 and GraphPad Prism version 7.
4.5 Results

4.5.1 Recruitment into the study

In this study, women previously affected by AFLP were divided into 2 groups. The first group (Group 1) is comprised of 13 women who suffered from AFLP identified by myself. From the 13 women, 8 women were recruited from the support group on Facebook, 4 women were recruited from UCLH and 1 woman volunteered from another London maternity unit. The medical records of all participants were scrutinized to ensure they met the Swansea criteria for diagnosis of AFLP.

The second group (Group 2) consisted of 20 women who had been admitted to the King’s Liver ITU. I reviewed the patient records over a period of 4 months (November 2016 to February 2017). A list of 56 pregnant or postpartum women admitted to the Liver ITU at King’s College Hospital were identified. Twenty women met the Swansea criteria after review of their medical records and were included in the study. The remaining women did not meet the study inclusion criteria and were admitted to the Liver ITU for another reason.

All clinical phenotyping data was obtained from the initial presentation to hospital. When analyzing data from Kings College Liver ITU, baseline data was collected from information provided from the local hospital at admission where the patient was transferred from.

4.5.2 Maternal Phenotype and Baseline Characteristics

The data recorded was dependent on the detail of record keeping, so inevitably, there was missing data: an issue when collecting information retrospectively (See Table 4-1). The pre-pregnancy height and weight were not available in a total of 22 cases and the birthweight for a total of 10 pregnancies were not accessible. Pre-pregnancy BMI data was only available in 17 women.
Table 4-1 Maternal Baseline characteristics at the time of delivery

<table>
<thead>
<tr>
<th></th>
<th>Group 1 (UCLH study n=13)</th>
<th>Group 2 (Kings’ study n=20)</th>
<th>Group 1 &amp; 2 (n=37)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median 25\textsuperscript{th} 75\textsuperscript{th} Range</td>
<td>Median 25\textsuperscript{th} 75\textsuperscript{th} Range</td>
<td></td>
</tr>
<tr>
<td>Age (Group 1 n=13) (Group 2 n=20)</td>
<td>30 28 34.5 27-41</td>
<td>30.5 28 37 21-52</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m\textsuperscript{2}) (Group 1 n=11) (Group 2 n=6)</td>
<td>22 21 23.5 20-28</td>
<td>22.5 18.8 28.3 15-29</td>
<td></td>
</tr>
<tr>
<td>Nulliparous (Group 1 n=13) (Group 2 n=20)</td>
<td>11 (84.6%)</td>
<td>15 (75%)</td>
<td></td>
</tr>
<tr>
<td>Multiparous (Group 1 n=13) (Group 2 n=20)</td>
<td>2 (15.4%)</td>
<td>5 (25%)</td>
<td></td>
</tr>
</tbody>
</table>

The results show that most women with AFLP are nulliparous (> 75%).

4.5.2.1 Offspring Gender

Differences in both offspring gender and parity have been implicated in AFLP. See Table 4-2 and Table 4-3. The tables illustrate the fetal characteristics of 17 offspring in Group 1, (13 women had 17 offspring, 9 singletons and 4 twin pregnancies). Group 2 comprised of 26 offspring from 20 women (15 singleton, 4 twins, 1 triplet) and 6 with gender not available. Available data for 20 offspring is given. The results show a greater percentage of male offspring born to women previously affected by AFLP.

Table 4-2 Offspring characteristics

<table>
<thead>
<tr>
<th>AFLP Offspring</th>
<th>Group 1 (n=17)</th>
<th>Group 2 (n=20)</th>
<th>Group 1 &amp; 2 (n=37)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>12 (70.6%)</td>
<td>11 (55.0%)</td>
<td>23 (62.2%)</td>
</tr>
<tr>
<td>Female</td>
<td>5 (29.4%)</td>
<td>9 (45.0%)</td>
<td>14 (37.8%)</td>
</tr>
</tbody>
</table>
Table 4-3 AFLP Singleton and multiple pregnancies women affected by in AFLP.

<table>
<thead>
<tr>
<th>AFLP pregnancies</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 1 &amp; 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Singleton</td>
<td>9 (69.2%)</td>
<td>15 (75%)</td>
<td>24 (64.9%)</td>
</tr>
<tr>
<td>Preganacies affected by Twins</td>
<td>4 (30.8%)</td>
<td>4 (20%)</td>
<td>8 (24.2%)</td>
</tr>
<tr>
<td>Preganacies affected by Triples</td>
<td>0 (0%)</td>
<td>1 (5%)</td>
<td>1 (3.0 %)</td>
</tr>
</tbody>
</table>

Almost 25% of all pregnancies experienced a twin pregnancy.

All women developed AFLP in the third trimester of pregnancy. The birth weight was known for 27 offspring in total with an average birth centile of 58th centile for singletons and 34th centile for the twin offspring. See Table 4-4.

Table 4-4 Baseline characteristics of the offspring

<table>
<thead>
<tr>
<th>AFLP Women</th>
<th>Group 1 &amp; 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>Gestation (weeks) (n=32)</td>
<td>36+2</td>
</tr>
<tr>
<td>Birthweight (g) Singletons (n=19)</td>
<td>2736.3</td>
</tr>
<tr>
<td>Customized Birth Centile (%) Singletons (n=19)</td>
<td>58</td>
</tr>
<tr>
<td>Birthweight (g) Twins/Triplets (n=8)</td>
<td>2321.1</td>
</tr>
<tr>
<td>Customized Birth Centile (%) Twins/Triplets (n=8)</td>
<td>33.75</td>
</tr>
</tbody>
</table>
4.5.3 Maternal Characteristics, delivery and outcome

There is no known association between ethnicity and AFLP. In this study 21 of 31 cases were White British (68%), 2 Black African (6.5%), with other ethnicities making up the remaining 25% of cases. The ethnicity was unknown in 2 cases.
Table 4-5 Maternal characteristics associated with the delivery and outcome.

FLS (Flu-like symptoms), EMCS (Emergency caesarean section), NVD (Normal vaginal delivery), SVD (Spontaneous vaginal delivery), DIC (Disseminated intravascular coagulation), RPOC (retained products of conception), PPH (Post-partum haemorrhage), CVVP (Continuous venous-venous haemofiltration), ITU (Intensive care unit)

<table>
<thead>
<tr>
<th>AFLP Women</th>
<th>Ethnicity</th>
<th>Presenting Signs &amp; Symptoms</th>
<th>Blood pressure at presentation (mm/Hg)</th>
<th>Mode of Delivery</th>
<th>Complications and Management</th>
<th>Maternal Outcome Mean gestation 36+2 weeks (31 – 41 weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>White British</td>
<td>1 week anorexia 1 week FLS 1 week extreme fatigue</td>
<td>Normotensive</td>
<td>EMCS</td>
<td>Nil Supportive care</td>
<td>Full recovery 0 days in ITU</td>
</tr>
<tr>
<td>2</td>
<td>White British</td>
<td>1 week anorexia 1 week FLS 1 week polydipsia 1 week vomiting 3 day’s diarrhoea intermittent abdominal pain</td>
<td>130/80</td>
<td>SVD</td>
<td>Acute Renal Failure requiring haemofiltration DIC Encephalopathy Sepsis</td>
<td>Full recovery 5 days in ITU</td>
</tr>
<tr>
<td>3</td>
<td>White British</td>
<td>1 week anorexia 1 week polydipsia 1 week extreme fatigue Intermittent Vomiting</td>
<td>160/100</td>
<td>EMCS</td>
<td>Nil Supportive care</td>
<td>Full recovery 0 days in ITU</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>4</td>
<td>White Other</td>
<td>2-3 week's anorexia 2-3 week's polydipsia 1 week abdominal pain 1 week Polyuria</td>
<td>Mild</td>
<td>EMCS</td>
<td>Encephalopathy</td>
<td>Full recovery 2 days in ITU</td>
</tr>
<tr>
<td>5</td>
<td>Pakistani British</td>
<td>2 week’s polydipsia 2 week’s extreme fatigue 1 week FLS 2 week’s nausea &amp; vomiting</td>
<td>125/74</td>
<td>EMCS</td>
<td>Nil Supportive care</td>
<td>Full recovery 0 days in ITU</td>
</tr>
<tr>
<td>6</td>
<td>White British</td>
<td>1 week polydipsia 2 week’s extreme fatigue 1 week FLS 2 day’s nausea &amp; vomiting 2 days Confused Jaundiced</td>
<td>99/63</td>
<td>EMCS</td>
<td>Encephalopathy</td>
<td>Full recovery 5 days in ITU</td>
</tr>
<tr>
<td>7</td>
<td>White British</td>
<td>1 week polydipsia 1 week extreme fatigue 1 week FLS 4 day’s anorexia 4 days vomiting</td>
<td>Mild</td>
<td>EMCS</td>
<td>Nil Supportive care</td>
<td>Full recovery 1 day in ITU</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6 week’s nausea</td>
<td>Intermittent vomiting</td>
<td>4 week’s polydipsia</td>
<td>4 week’s extreme fatigue</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>-----------------</td>
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<td>--------------------------</td>
</tr>
<tr>
<td>8</td>
<td>Indian British</td>
<td>6 week’s nausea</td>
<td>Intermittent vomiting</td>
<td>4 week’s polydipsia</td>
<td>4 week’s extreme fatigue</td>
<td>Unknown</td>
</tr>
<tr>
<td>9</td>
<td>White British</td>
<td>4 week’s anorexia</td>
<td>4 week’s extreme fatigue</td>
<td>4 week’s polydipsia</td>
<td>8 week’s nausea &amp; vomiting</td>
<td>Jaundiced</td>
</tr>
<tr>
<td>10</td>
<td>White British</td>
<td>2 week’s anorexia</td>
<td>2 week’s extreme fatigue</td>
<td>2 week’s nausea &amp; vomiting</td>
<td>Intermittent abdominal pain</td>
<td>Jaundiced</td>
</tr>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>11</strong></td>
<td>White British</td>
<td>4 week’s polydipsia &amp; polyuria 6 week’s extreme fatigue 4 weeks’ nausea &amp; vomiting Intermittent abdominal pain Jaundiced</td>
<td>136/66</td>
<td>EMCS</td>
<td>Haemoperitoneum Encephalopathy Ascites</td>
<td>Full recovery 11 days in ITU</td>
</tr>
<tr>
<td><strong>12</strong></td>
<td>White British</td>
<td>2 week’s anorexia 12 week’s extreme fatigue 4 week’s polydipsia 3-4 days’ nausea &amp; vomiting 1 week FLS Jaundiced</td>
<td>130-150/90-100</td>
<td>EMCS</td>
<td>DIC Sepsis</td>
<td>Full recovery 0 days in ITU</td>
</tr>
<tr>
<td><strong>13</strong></td>
<td>White British</td>
<td>1 week anorexia 2 week’s polydipsia &amp; polyuria 1 week nausea &amp; vomiting 2 week’s extreme fatigue</td>
<td>142/75</td>
<td>EMCS</td>
<td>Supportive care</td>
<td>Full recovery 0 days in ITU</td>
</tr>
<tr>
<td><strong>14</strong></td>
<td>White British</td>
<td>1 week vomiting 1 week reflux Weight Loss Jaundiced</td>
<td>142/78</td>
<td>SVD</td>
<td>Supportive care</td>
<td>Full recovery 12 days in ITU</td>
</tr>
<tr>
<td>No.</td>
<td>Race/Religion</td>
<td>Symptoms/Complications</td>
<td>Blood Pressure</td>
<td>Treatment</td>
<td>Outcome</td>
<td></td>
</tr>
<tr>
<td>-----</td>
<td>---------------------</td>
<td>----------------------------------------------------------------------------------------</td>
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<td></td>
</tr>
<tr>
<td>15</td>
<td>Bengali Muslim</td>
<td>1 week generally unwell/fatigue Jaundiced</td>
<td>127/75</td>
<td>SVD</td>
<td>Full recovery 3 days in ITU</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>White British</td>
<td>4 day’s nausea 4 day’s vomiting Confused</td>
<td>120/75</td>
<td>EMCS</td>
<td>Full recovery 48 days in ITU</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>White British</td>
<td>2 weeks’ flu like symptoms 2 weeks vomiting Jaundiced on admission</td>
<td>140/92</td>
<td>EMCS</td>
<td>Full recovery 5 days in ITU</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>White British</td>
<td>Epigastric pain Nausea &amp; vomiting</td>
<td>160-170/98-105</td>
<td>EMCS</td>
<td>Pancreatitis, Oliguric on CVVP Full recovery 13 days in ITU</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Indian Muslim</td>
<td>1 week of nausea &amp; vomiting Itching</td>
<td>150/90</td>
<td>EMCS</td>
<td>Supportive care          Full recovery 16 days in ITU</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>White British</td>
<td>Abdominal pain Generally unwell Fatigue &amp; Drowsy Jaundiced</td>
<td>125/50</td>
<td>SVD</td>
<td>Oliguric treated with CVVP Full recovery 11 days in ITU</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>Unknown</td>
<td>Abdominal pain 2 weeks generally unwell/fatigue Vomiting Polydipsia &amp; polyuria Confused</td>
<td>143/85</td>
<td>EMCS</td>
<td>PPH 1000mls, Encephalopathy Full recovery 2 days in ITU</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><strong>22</strong></td>
<td>Black African</td>
<td>1 week nausea 1 week vomiting Polydipsia Jaundiced</td>
<td>135/90</td>
<td>EMCS</td>
<td>Oliguric Atrial Fibrillation Full recovery 22 days in ITU</td>
<td></td>
</tr>
<tr>
<td><strong>23</strong></td>
<td>White Other</td>
<td>Epigastric pain Nausea &amp; vomiting Jaundiced</td>
<td>150/89</td>
<td>EMCS</td>
<td>PPH 1.5 litres Hyperlactaeemia Full recovery 10 days in ITU</td>
<td></td>
</tr>
<tr>
<td><strong>24</strong></td>
<td>Black African</td>
<td>2 days unwell Vomiting Reduced fetal movements</td>
<td>140/90</td>
<td>EMCS</td>
<td>PPH 1.6L, B-Lynch uterotronics Unknown 6 days in ITU</td>
<td></td>
</tr>
<tr>
<td><strong>25</strong></td>
<td>White British</td>
<td>2 weeks of FLS Vomiting Polydipsia</td>
<td>130/80</td>
<td>EMCS</td>
<td>Unknown Full recovery 5 days in ITU</td>
<td></td>
</tr>
<tr>
<td><strong>26</strong></td>
<td>Unknown</td>
<td>1 week abdominal pain 1 week nausea &amp; vomiting Jaundiced</td>
<td>156/86</td>
<td>EMCS</td>
<td>Ascites Post EMCS wound infection Full recovery 10 days in ITU</td>
<td></td>
</tr>
<tr>
<td><strong>27</strong></td>
<td>White British</td>
<td>Abdominal pain Vomiting Polydipsia</td>
<td>137/73</td>
<td>EMCS</td>
<td>Abdominal wall haematoma laparotomy Liver biopsy: moderate cholestasis, marked hepatocyte ballooning and macrovesiculation Unknown 25 days in ITU</td>
<td></td>
</tr>
<tr>
<td>No.</td>
<td>Race</td>
<td>Condition</td>
<td>Symptoms</td>
<td>Blood Pressure</td>
<td>Method</td>
<td>Complications</td>
</tr>
<tr>
<td>-----</td>
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<td>--------</td>
<td>---------------</td>
</tr>
<tr>
<td>28</td>
<td>White British</td>
<td>1 week generally unwell/fatigue</td>
<td>Abdominal pain, Diarrhoea, Jaundiced</td>
<td>125/78</td>
<td>Kiwi/ Ventouse</td>
<td>PPH 10 litres, Hysterectomy, laparotomy to evacuate haematoma</td>
</tr>
<tr>
<td>29</td>
<td>White British</td>
<td>3 day’s abdominal pain</td>
<td>3 day’s vomiting</td>
<td>160/80</td>
<td>Induction of Labour</td>
<td>NVD</td>
</tr>
<tr>
<td>30</td>
<td>Other; Mixed/ Romanian</td>
<td>2 week’s abdominal pain</td>
<td>2 week’s vomiting</td>
<td>120/80</td>
<td>EMCS</td>
<td>Post-partum bilateral Bell’s palsy</td>
</tr>
<tr>
<td>31</td>
<td>White British</td>
<td>3 week’s abdominal pain</td>
<td>3 week’s nausea, 3 week’s anorexia, 3 weeks fatigue, 3 week’s polydipsia</td>
<td>150/96</td>
<td>EMCS</td>
<td>Unknown</td>
</tr>
<tr>
<td>32</td>
<td>White British</td>
<td>2 week’s abdominal pain</td>
<td>2-3 weeks’ nausea &amp; vomiting</td>
<td>120/80</td>
<td>EMCS</td>
<td>Placental Abruption</td>
</tr>
<tr>
<td>33</td>
<td>Unknown</td>
<td>Nausea &amp; vomiting</td>
<td>Abdominal pain after caesarean section for an IUD of one twin</td>
<td>117/82</td>
<td>EMCS</td>
<td>Unknown</td>
</tr>
</tbody>
</table>
The data collected clearly illustrates most babies were delivered by emergency caesarean section (n=26/33) compared to vaginal delivery (n=7/33) (Figure 4-1). One case needed a ventouse to assist with a vaginal delivery.

**Figure 4-1 Mode of Delivery in all 33 cases of AFLP**

4.5.4 Clinical signs and symptoms

The most common symptoms were nausea and vomiting, polydipsia and abdominal pain. See Table 4-6. Clinical jaundice is recognized when serum bilirubin levels are greater than 30 µmol/L, which was the case in 30/33 women from this cohort. Interestingly, despite a mean bilirubin level of 99.7 µmol/L only 39.4% of cases were recorded as having jaundice on admission to hospital.
Table 4-6 Presenting Signs & Symptoms of AFLP in Group 1 & 2.

<table>
<thead>
<tr>
<th>Signs &amp; Symptoms</th>
<th>Number (N)</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nausea &amp; Vomiting</td>
<td>28</td>
<td>84.8</td>
</tr>
<tr>
<td>Polydipsia</td>
<td>17</td>
<td>52</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>16</td>
<td>48.5</td>
</tr>
<tr>
<td>Fatigue</td>
<td>15</td>
<td>45.5</td>
</tr>
<tr>
<td>Jaundiced</td>
<td>13</td>
<td>39.4</td>
</tr>
<tr>
<td>Anorexia</td>
<td>9</td>
<td>27.3</td>
</tr>
<tr>
<td>Flu Like Symptoms</td>
<td>8</td>
<td>24.3</td>
</tr>
<tr>
<td>Polyuria</td>
<td>4</td>
<td>12.1</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>2</td>
<td>0.6</td>
</tr>
</tbody>
</table>

The diagnosis of AFLP can be challenging. It’s presenting signs and symptoms can be mistaken for other obstetric disorders such as pre-eclampsia and HELLP syndrome. Accurate measurement of blood pressure can aid in differentiating the three conditions. The National Institute for Health and Care Excellence (NICE) guidelines characterize hypertension in pregnancy as mild (140/90–149/99 mmHg), moderate (150/100–159/109 mmHg) and severe hypertension (>160/110 mmHg). Mean arterial blood pressure (MAP) can be calculated to describe an individual’s average blood pressure in a single cardiac cycle which represents tissue perfusion. A MAP of < 117mmHg signifies normal or mild hypertension and a MAP > 117mmHg characterizes moderate to severe hypertension. In 32 cases of AFLP, 29 women had a normal blood pressure or mild hypertension and 3 women had moderate/ severe hypertension.
Figure 4-2). This would not be the case in women presenting with Pre-eclampsia or HELLP syndrome, where blood pressure readings would be predominantly moderate to severe.

Figure 4-2: Mean arterial blood pressure measurement in the 32 cases of AFLP

4.5.5 Biochemistry

Despite the vague signs and symptoms described by women presenting with AFLP, the diagnosis can be reached with the assistance of simple investigations. See Table 4-7.
Table 4-7 Biochemistry results at time of presentation in all cases of AFLP

<table>
<thead>
<tr>
<th>Test</th>
<th>Mean</th>
<th>SD</th>
<th>SEM</th>
<th>Range</th>
<th>Normal range for pregnancy (187, 188)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Liver Function</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose mmol/L (n=30)</td>
<td>3.1</td>
<td>1.02</td>
<td>0.19</td>
<td>1-4.9</td>
<td>4 – 4.5</td>
</tr>
<tr>
<td>ALT IU/L (n=29)</td>
<td>412.5</td>
<td>265.2</td>
<td>49.2</td>
<td>33-1100</td>
<td>6 – 32</td>
</tr>
<tr>
<td>Alb g/L (n=29)</td>
<td>24.2</td>
<td>4.2</td>
<td>0.8</td>
<td>17-34</td>
<td>28 – 37</td>
</tr>
<tr>
<td>Bil µmol/L (n=31)</td>
<td>99.7</td>
<td>45.1</td>
<td>8.1</td>
<td>23-204</td>
<td>3 – 14</td>
</tr>
<tr>
<td>INR (n=28)</td>
<td>2.04</td>
<td>0.89</td>
<td>0.17</td>
<td>1.2-4.5</td>
<td>0.8 – 0.94</td>
</tr>
<tr>
<td>Lactate Mmol/L (n=17)</td>
<td>3.36</td>
<td>1.50</td>
<td>0.36</td>
<td>1.3-6.5</td>
<td>0.5 – 2</td>
</tr>
<tr>
<td><strong>Renal Function</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urea mmol/L (n=28)</td>
<td>9.75</td>
<td>3.6</td>
<td>0.68</td>
<td>3.8-18.6</td>
<td>2.4 – 3.8</td>
</tr>
<tr>
<td>Cr µmol/L (n=33)</td>
<td>201.03</td>
<td>76.7</td>
<td>13.35</td>
<td>100-419</td>
<td>55 – 73</td>
</tr>
</tbody>
</table>

These results show that women with AFLP present with impaired synthetic liver function (hypoglycaemia, raised lactate and prolonged international normalised ratio (INR)). Liver injury was noted biochemically with moderate transaminitis (elevated ALT and AST), hypoalbuminaemia and elevated bilirubin. They also have impaired kidney function (elevated urea and creatinine). Unfortunately, I was unable to collect acid-base status in the form of pH.
4.5.6 Haematology

Table 4-8 Haematology results at time of presentation in all cases of AFLP.

<table>
<thead>
<tr>
<th>Test</th>
<th>Mean</th>
<th>SD</th>
<th>SEM</th>
<th>Range</th>
<th>Normal range for pregnancy (187)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Full blood Count</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hb g/dL (n=28)</td>
<td>12.4</td>
<td>1.72</td>
<td>0.33</td>
<td>8.2-15.4</td>
<td>11.0 – 14.0</td>
</tr>
<tr>
<td>Platelets x10^9/L (n=28)</td>
<td>164</td>
<td>66.6</td>
<td>12.6</td>
<td>64-320</td>
<td>150 – 400</td>
</tr>
</tbody>
</table>

Unlike women with HELLP syndrome, my study women had normal mean haemoglobin and platelet levels are normal. See Table 4-8.

4.5.7 Additional tests at King’s college hospital.

The Liver ITU at King’s hospital additionally measured a separate liver transaminase and ferritin levels in some women. A lipid profile on 14 of the 20 women admitted to the unit were also measured (Table 4-9 and Table 4-10).

Table 4-9 Additional characteristics measured in the women admitted to Kings Liver Unit

<table>
<thead>
<tr>
<th>Test</th>
<th>Mean</th>
<th>SD</th>
<th>SEM</th>
<th>Range</th>
<th>Normal range for pregnancy (187, 188)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST IU/L (n=19)</td>
<td>247.7</td>
<td>179.4</td>
<td>41.17</td>
<td>33-715</td>
<td>11 – 30</td>
</tr>
<tr>
<td>Ferritin µg/L (n=16)</td>
<td>132.4</td>
<td>114.5</td>
<td>28.62</td>
<td>12-370</td>
<td>12 – 150</td>
</tr>
</tbody>
</table>
Table 4-10 Lipid profile levels

<table>
<thead>
<tr>
<th>Test</th>
<th>Mean</th>
<th>SD</th>
<th>SEM</th>
<th>Range</th>
<th>Normal range for pregnancy (188)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol mmol/L</td>
<td>1.98</td>
<td>0.75</td>
<td>0.2</td>
<td>1-3.6</td>
<td>5 – 9.0</td>
</tr>
<tr>
<td>(n=14)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglyceride mmol/L</td>
<td>1.4</td>
<td>0.75</td>
<td>0.2</td>
<td>0.6-3.2</td>
<td>1.5 – 5.0</td>
</tr>
<tr>
<td>(n=14)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Pregnant levels of total cholesterol increase during each trimester and by the third trimester range from 5.0 – 9.0 mmol/L (188). Serum triglycerides levels also increase during pregnancy.

I studied 12 pregnant women in the third trimester following a 12-hour fast (Chapter 5) and found a mean total cholesterol of 6.8 mmol/L (range 5.3-10.1 mmol/L) and mean triglyceride level 2.4 mmol/L (range 0.9-4.1 mmol/L). In the women at King’s hospital with AFLP the mean cholesterol and triglyceride levels were 1.98 mmol/L and 1.4 mmol/L respectively. Both results are lower than the levels for healthy pregnancy.

4.5.8 Urinalysis

Twelve women who presented with AFLP had urinalysis that specifically recorded the absence of ketonuria. A further 7 women had urinalysis that recorded NAD. Other findings included 2+ proteinuria (n=1/19) and trace or + proteinuria (n=4/19). Bilirubinuria was not documented in any of the results (Table 4-11).
Table 4-11 Urine Dipstick Results on admission to hospital

<table>
<thead>
<tr>
<th>Urine Result</th>
<th>Number of cases (n=19)</th>
<th>Average (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO Ketones</td>
<td>12</td>
<td>63.2</td>
</tr>
<tr>
<td>NAD</td>
<td>7</td>
<td>36.8</td>
</tr>
<tr>
<td>Protein 2+ or more</td>
<td>1</td>
<td>5.3%</td>
</tr>
<tr>
<td>Protein Trace or 1+</td>
<td>4</td>
<td>21.1%</td>
</tr>
<tr>
<td>Other (e.g. blood, leucocytes)</td>
<td>3</td>
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</tr>
<tr>
<td>Unknown</td>
<td>14</td>
<td>42.4</td>
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</table>

4.5.9 Radiological findings

Eleven of the 20 AFLP patients admitted to the Liver ITU at King’s College Hospital had their Computed Tomography (CT) scans reviewed retrospectively by a senior radiologist. All CT scans were performed post-partum and 4 patients had multiple CTs during their admission.

Historically, single measurements of hepatic attenuation were used to quantify liver fat content or hepatic steatosis. Using this method, a normal liver attenuation ranged from 50 – 65 HU (Hounsfield unit). Fatty infiltration was diagnosed with a hepatic attenuation < 48 HU (189). One study found a hepatic attenuation of 40 HU to represent fatty infiltration of 30%. (190, 191) More recent, methods have shown a result of <104HU (calculated by \([L - 0.3 \times (0.75 \times P + 0.25 \times A)] / 0.7\]), where L, P and A represents liver, main portal vein and abdominal aorta attenuation) to represent steatosis of at least 5%. A value of > 104 HU signifies no hepatic
steatosis. This method is considered the standard for measuring hepatic steatosis (192).

4.5.9.1 Radiological Results:

i There were no signs of chronic liver disease or portal hypertension in any of the cases.

ii Hepatic steatosis: All 11 patients demonstrated evidence of hepatic steatosis on portal venous phase imaging, with an average attenuation of 53 HU (Range 10.6 – 85.1 HU).

iii Liver volume: Two women were noted to have a liver volume of less than 1 litre. A repeat CT scan was performed greater than 10 days after the initial CT in 3 women, which showed an increase in liver volume of 37%, 62% and 174% respectively. Only one of these 3 women had an initial small liver volume.

iv Ascites was present in 91% of cases (10/11) and 18% of cases (n= 2/11) were complicated by an abdominal wall haematoma during the scan.

v There was no CT evidence of acute pancreatitis in any of the cases (Table 4-12).
Table 4-12 Retrospective CT scan findings in patients with AFLP.

<table>
<thead>
<tr>
<th>AFLP Case</th>
<th>Days post-partum</th>
<th>Liver Volume (cc)</th>
<th>Liver volume increase (%)</th>
<th>Chronic Liver disease</th>
<th>Biliary Dilatation</th>
<th>Portal Vein</th>
<th>Hepatic Vein</th>
<th>Hepatic Artery</th>
<th>Steatosis 1 (If &lt;104HU is fatty liver)</th>
<th>Steatosis 2</th>
<th>Kidneys</th>
<th>Pancreas</th>
<th>Signs of increase portal pressure</th>
<th>Ascites</th>
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<td>0</td>
<td>1133.9</td>
<td>No</td>
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<td>Patent</td>
<td>Patent</td>
<td>68.0</td>
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<td>Normal</td>
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<td>No</td>
<td>Perihepatic haematoma</td>
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<tr>
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<td>1</td>
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<td>No</td>
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<td>89.4</td>
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<td>No</td>
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<td>Peripheral Oedema</td>
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<td>Patent</td>
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<td>Patent</td>
<td>75.7</td>
<td>Right hydronephrosis</td>
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<td>No</td>
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<td>Massive ascites</td>
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<td>17</td>
<td>2</td>
<td>2186.1 37.4</td>
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<td>Patent</td>
<td>58.9</td>
<td>Right hydronephrosis</td>
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<td>No (LAI &lt;5)</td>
<td>Small right kidney</td>
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</table>

Small Peripheral oedema

Large Peripheral oedema

Small ascites

Moderate Large

Large

Small
Chapter 4 Acute Fatty Liver of Pregnancy Phenotype

4.6 Discussion

This study has identified key findings that better defines the phenotype of AFLP.

- The most common symptoms experienced by these women are nausea and vomiting, polydipsia and abdominal pain.
- Offspring have a normal birth weight for gestational age.
- AFLP women suffer predominantly from synthetic liver failure and renal failure.
- Women with AFLP have hypocholesterolaemia and hypotriglyceridaemia.
- The absence of ketonuria was seen in women with AFLP despite prolonged fasting in the third trimester of pregnancy.

From these results, I have been able to provide further insight into the aetiology of AFLP. With prospective studies' it may be possible to upgrade the current diagnostic criteria for AFLP. My findings also recommend additional tests that could be performed on all women presenting to hospital with the symptoms described. This is discussed further below.

4.6.1 Pregnancy characteristics of women with AFLP

The women in this study present with a median age of approximately 30 years (range 21-52) and have a median BMI of 22 (range 15-29). From 17 women 5 had a BMI of less than 20. It has been suggested that there may be an association between low BMI and developing AFLP. In UKOSS, 20% (9/57) of women were underweight with a BMI of less than 20 (3). In the women I studied, 29.4% of women had a low BMI. However, in my cohort the number of women with a BMI documented was very small and to confirm the association between low BMI and AFLP further studies would be required.
From scrutinizing the records of 33 pregnancies affected by AFLP, I have shown that AFLP most commonly occurs during the third trimester of primigravid pregnancies (26/33; 78.8%).

Furthermore, women with multiple pregnancies (Twins/Triplets 9/33; 27.3%) and carrying a male fetus (23/37; 62%, 2:1 male to female ratio) are disproportionately represented amongst women with AFLP. The mean birthweight of singleton offspring was 2736.3g (58th centile, n=19) and 2321.1g (34th centile, n=8) for twin offspring. Birthweight in both groups are normal for gestational age, which is a feature that has never been characterized before in previous reports.

These observations support some of what is currently known about AFLP. Previous studies have shown that greater than 50% of women with AFLP were nulliparous and nearly one fifth of these women had twin pregnancies, representing a 14 times increased risk of AFLP with a twin pregnancy (3, 186, 193). The onset of AFLP always occurs in the third trimester, commonly after the 30th week of gestation (156). The women in my cohort presented after 31 week’s gestation with a mean gestational age of 36 weeks.

These findings tell us that women experience a normal pregnancy until the third trimester of pregnancy. Importantly, the placenta is functioning normally, which is represented by well grown offspring. Conversely, babies born to women with pre-eclampsia and HELLP are more likely to deliver growth restricted babies secondary to poor placentation, oxidative stress and maternal endothelial dysfunction (194). This is a key distinguishing feature of AFLP compared to pre-eclampsia.

The risk of developing AFLP increases with twin pregnancies and male fetuses, for which there is no definitive answer as to why this is the case. One study reported that pregnant women have 10% greater energy intake with male fetuses compared to female fetuses, therefore concluding that male fetuses have higher energy requirements (195). However, these findings have not been reproduced in the literature.
With twin or triplet pregnancies the energy requirements are considered greater than singleton pregnancies because there are more fetuses. However, again this has not been proven.

4.6.2 Maternal symptoms and signs associated with AFLP

In my study, the most common symptom was nausea and vomiting (n=28/33, 85%), followed by polydipsia (n=17/33, 52%) and abdominal pain (n=16/33, 49%). Women usually present with gradual onset of vague and non-specific symptoms.

Most published cohort studies identify vomiting and abdominal pain as the most common symptoms. Interestingly, the second most frequent complaint in my study was polydipsia, which is thought to be a rare feature of AFLP (157, 160). Jaundice was noticed in only 39% (n=13/33) of women I studied, despite a mean bilirubin of 99.7 mmol/L. Patients with a bilirubin greater than 30 mmol/L should have evidence of jaundice. The lack of clinically recorded jaundice suggests that timing of clinical examination is important.

Women with AFLP have raised levels of bilirubin, which is almost certainly a result of hepatocellular injury. The breakdown of red blood cells results in the formation of bilirubin (a waste product) which is transported in blood bound to albumin. Bilirubin is removed from the circulation by hepatocytes, where it is conjugated with glucuronic acid making it water soluble (Conjugated bilirubin). It can now be excreted in bile and removed through the gut. In cases of hepatic parenchymal damage the metabolism of bilirubin (especially excretion of conjugated bilirubin) is impaired resulting in hyperbilirubinaemia. (196)

Hepatic encephalopathy was identified in 18.2% (n=6/33) of women with AFLP. This is a known serious complication and indicates severe liver failure secondary to cerebral oedema. In normal circumstances ammonia that is released from the gut enters the portal circulation of the liver where it is converted to urea.
However, with hepatic failure ammonia accumulates and enters systemic circulation causing neuronal dysfunction and encephalopathy. Brain oedema can occur in combination with a rise in ammonia (197).

### 4.6.3 Liver Function tests

In my cohort study, women with AFLP suffered from synthetic liver failure (hypoglycaemia, prolonged INR and raised lactate). At presentation hypoglycaemia (glucose < 4.0 mmol/L) was present in 23/30 cases (76.7%). It would have been useful to have seen the acid-base (pH) results as this is an important feature in determining severity of liver failure.

Seven cases had a glucose level greater than 4.0 mmol/L (23.3%), however two of these women were given intramuscular steroid injections for fetal lung maturity, which could have increased the blood glucose level. The glucose levels for 3 women with AFLP were not recorded.

A prolonged INR was seen in all women where the results were available (31/31). INR is a test performed to assess how well the blood coagulates in an individual. Coagulation factors (proteins) are synthesized by hepatic parenchymal cells. In liver injury, the production of these proteins is impaired resulting in a prolonged INR (198).

Both INR and prothrombin time (PT) are assays that evaluate the extrinsic pathway of the coagulation cascade, otherwise known as the tissue factor pathway. This pathway includes several reactions to produce thrombin which activates release of the main clotting factor VIII. The assays INR and PT are evaluated along with the activated partial thromboplastin time (aPTT) which measures the intrinsic path of the coagulation cascade also known as the contact activation pathway. Both of these pathways come together to form fibrin the most important haemostatic plug (199).
Twenty-eight women had a prolonged INR and 3 women showed increased APTT levels in the absence of an INR result. Blood clotting parameters were unavailable for 2 women. Raised liver transaminases and conjugated hyperbilirubinaemia were identified in all cases where results were available. The liver also synthesizes albumin which is reduced in liver disease. Hypoalbuminaemia was noted in the study women (158, 200).

4.6.4 Renal Function

All women in this study had renal failure (raised urea and creatinine). The pathogenesis of acute kidney injury (AKI) in AFLP is not well understood. In acute liver failure (ALF) approximately 55% of all patients develop renal failure (201). Another study showed that of 308 patients with acute liver failure 133 (43%) also developed AKI (202). These patients met the RIFLE (Risk, Injury, Failure, Loss and end stage renal disease) criteria for AKI (203). It is clear from the literature that the frequency of AKI in ALF patients is significant, however pathogenic studies to investigate this association are lacking. There have been several studies investigating risk factors associated with the development of AKI in critically ill patients, which include sepsis, contrast agents, diabetes, increasing age, hypovolaemia and shock. Collectively, these studies have implicated a number of complex pathological processes that may be responsible for the AKI seen in ALF including the following:

- Hypotension, vasodilatation and cardiac injury may contribute to reduced renal blood flow (204).
- ALF activates the sympathetic nervous system, this further contributes to the effect of variation in perfusion pressures to the already vulnerable kidney (201).
- Renal vasoconstriction can be amplified by activation of the renin-aldosterone-angiotensin (204).
ALF is associated with increased circulating level of cytokines like IL-1 and TNF-a this could contribute to cell death in the kidneys, however the evidence for this needs further investigation (205).

There have been a few reported cases of AFLP where renal pathology was histologically assessed. The main features demonstrated renal tubular epithelium swelling and necrosis with crystal formation within the lumen of nephrons. Several cases were shown to have fine fatty vacuolization of the renal tubules (206, 207). There have also been isolated case reports of glomerular lesions in AFLP however, this is a rare finding and has not been demonstrated in any women with AFLP in my study (208).

The acute kidney injury seen in women with AFLP could be a result of several complex mechanisms as described above, however the exact mechanism is not yet known.

4.6.5 Fat Metabolism

The first two thirds of pregnancy is an insulin sensitive, anabolic state associated with maternal fat deposition. The third trimester is an insulin resistant, catabolic state associated with increased lipolysis and fatty acid oxidation (14, 15, 59). Circulating levels of cholesterol and triglycerides rise by up to 50% and 300% respectively (11, 12). I have shown that women with AFLP in my study have decreased levels of both cholesterol and triglyceride which is a result of acute liver failure.

I have proposed that women with AFLP have a defect in in fatty acid oxidation. If this is true, lipid levels should not fall as lipolysis can still occur. The liver is crucial in the production, storage and transport of fatty acids and lipoproteins. It is likely that reduced cholesterol and triglyceride levels in AFLP occur secondary to synthetic parenchymal hepatic failure (209-211).
Reported studies have shown the reduction of cholesterol and triglyceride levels in liver disease such as liver cirrhosis (209, 212).

Conversely, women in the third trimester and in particular, women with pre-eclampsia/HELLP have increased lipid levels (84, 213). Therefore, an early measure of maternal serum lipids could be another way to differentiate between AFLP and pre-eclampsia. One study identified women with elevated lipid levels before pregnancy were at a greater risk of developing pre-eclampsia.

These women subsequently had higher levels of cholesterol and triglyceride during pregnancy (214). Elevated serum triglyceride levels can be caused by increased insulin resistance, decreased β-oxidation and decreased clearance of triglycerides from circulation. Insulin normally suppresses fatty acid release from adipose tissue by activating hormone sensitive lipase (HSL) and promoting re-esterification of fats. This leads to a reduction in secretion of hepatic VLDL. Simultaneously, insulin activates lipoprotein lipase, which accelerates removal of lipoproteins rich in triglyceride from the circulation. This process is impaired with insulin resistance, leading to raised cholesterol and triglyceride levels (25, 215).

Differences in lipid profiles could be an important distinguishing feature between AFLP and pre-eclampsia. A measure of serum lipid levels, is not currently a part of the criteria to diagnose AFLP and has only been observed by some centers (186, 193).

### 4.6.6 Urinary Analysis

During the third trimester of pregnancy, the insulin-resistant mother who fasts induces an accelerated activation of lipolysis, fatty acid oxidation and the production of ketone bodies for energy. As women with AFLP have severe prolonged nausea and vomiting ranging from days to weeks, the production of urinary ketone bodies should be very evident.
All 19 women who presented with AFLP had an absence of ketonuria. Twelve out of 19 women with AFLP had a specific result stating no ketonuria. Seven out of 19 women with AFLP had a urinalysis stating “NAD”.

Ketone bodies (acetoacetate (AcAc) and 3-beta-hydroxybutyrate (3HB)), are important sources of alternate energy for extra-hepatic tissue when glucose supplies are low.

Fatty acids enter mitochondria and undergo fatty acid oxidation to produce acetyl-CoA. See Chapter 1 for a detailed explanation. Ketogenesis involves a series of enzyme reactions, initially using acetyl-CoA to produce ATP.

If there is a defect in this pathway this results in energy failure and the absence of ketonuria despite starvation (84). The absence of ketonuria supports the notion that AFLP is associated with a defect in fatty acid oxidation or ketogenesis.

4.6.7 Liver Imaging

Specific features in the CT scans reviewed, included the presence of pre-existing chronic liver disease, portal hypertension, vascular shunting, liver volume and any additional complications.

Radiologically AFLP is not associated with pre-existing liver disease or portal hypertension. In my study, CT scans were available for 11 women. All initial scans were performed between day 0 to day 4 post-delivery. All cases reviewed in this study showed the presence of steatosis. CT scans are a useful tool to identify steatosis and other complications such as a haematoma. Although, there have been reports of inaccurate detection of mild steatosis with CT compared to moderate and severe steatosis. Furthermore, several studies have shown MRI to be more accurate in detecting steatosis (216, 217).
It would have been useful to quantify the degree of steatosis further in these 11 women; however, this can only be performed on pre-contrast CT scans, which were unavailable.

In the 3 cases where a repeat CT was performed 10 days after the initial CT, increased liver volume was noted. Despite the small number of cases, this shows that significant liver regeneration can be expected in patients after 10 days or even longer during the post-partum period. CT scans have been widely evaluated as an accurate tool in assessing total liver volume. Formula using body surface area and 3D reconstruction of the liver have been used to measure liver volume. This has been validated by several studies including data measured in western populations (218). A small liver has a total liver volume of less than 1 litre.

CT scans are not routinely performed in healthy pregnancy, therefore data for liver volume changes in pregnancy is unavailable. However, 2 women in this study had a liver volume of less than a litre and where multiple scans were performed liver regeneration was identified. Accurately measuring liver volume is important as studies have shown that liver volume correlates with disease severity and therefore can help predict prognosis in chronic liver failure (219).

The CT scans for 2 women in this study identified abdominal wall haematoma’s. CT scans will remain the most suitable investigation in the management of haemorrhagic complications. In acute liver failure CT scans have a role to investigate liver capsule haematomas, infarction, and rupture.
4.6.8 Maternal and Fetal Mortality

In the UK, maternal and fetal morbidity and mortality rates have improved year on year since 1952. In 2005, the UK obstetric surveillance system (UKOSS) study of AFLP observed just 1 maternal death out of 57 AFLP cases. There were 7 perinatal deaths associated with AFLP giving a perinatal mortality rate of 11% (3). There were no maternal deaths amongst the 33 cases of AFLP that I have studied (1998 – 2015).

4.7 Conclusions

From the findings of this study, I propose a new set of criteria for the diagnosis of AFLP (See

Lactate

Table 4-13). In addition to the Swansea criteria, 4 additional features should be included.

i  Normal grown fetus for gestational age

ii  Absence of ketonuria, despite prolonged fasting (nausea and vomiting)
iii Hypocholesterolaemia and hypotriglyceridaemia

iv Lactate

Table 4-13 Proposed new criteria for the diagnosis of AFLP

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<tr>
<th>Criteria</th>
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<tbody>
<tr>
<td>Normal Birth weight for gestation</td>
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<tr>
<td>Absence of ketonuria</td>
</tr>
<tr>
<td>Raised Lactate</td>
</tr>
<tr>
<td>Total cholesterol (&lt; 3.0mmol/L)</td>
</tr>
<tr>
<td>Total triglyceride (&lt; 2.0mmol/L)</td>
</tr>
<tr>
<td>Vomiting</td>
</tr>
<tr>
<td>Abdominal pain</td>
</tr>
<tr>
<td>Polydipsia/ polyuria</td>
</tr>
<tr>
<td>Encephalopathy</td>
</tr>
<tr>
<td>Elevated bilirubin (&gt;14 mmol/l)</td>
</tr>
<tr>
<td>Hypoglycaemia (&lt; 4 mmol/l)</td>
</tr>
<tr>
<td>Elevated urate (&gt; 340 mmol/l)</td>
</tr>
<tr>
<td>Leucocytosis (&gt; 11X10^9/l)</td>
</tr>
<tr>
<td>Ascites or bright liver on ultrasound scan</td>
</tr>
<tr>
<td>Elevated transaminases (aspartate aminotransferase or alanine aminotransferase &gt; 42 IU/l)</td>
</tr>
<tr>
<td>Elevated ammonia (&gt; 47 mmol/l)</td>
</tr>
<tr>
<td>Renal impairment (creatinine &gt; 150 umol/l)</td>
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<tr>
<td>Coagulopathy (INR &gt; 1.3)</td>
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<td>Microvesicular steatosis on liver biopsy</td>
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<td>* Six or more features are required in the absence of another explanation</td>
</tr>
</tbody>
</table>


Secondly, I propose clinical features that will assist clinicians in differentiating between AFLP, pre-eclampsia and HELLP syndrome (Table 4-14).

**Table 4-14 Features differentiating between AFLP, Pre-eclampsia and HELLP syndrome.**

<table>
<thead>
<tr>
<th></th>
<th>AFLP</th>
<th>Pre-eclampsia &amp; HELLP Syndrome</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fetal features</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal Fetal Growth</td>
<td>Fetal Growth Restriction</td>
<td></td>
</tr>
<tr>
<td>Stillbirth</td>
<td>Stillbirth</td>
<td></td>
</tr>
<tr>
<td>Pre-term</td>
<td>Pre-term</td>
<td></td>
</tr>
<tr>
<td><strong>Maternal symptoms</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nausea &amp; vomiting</td>
<td>Headaches</td>
<td></td>
</tr>
<tr>
<td>Polydipsia</td>
<td>Changes in vision</td>
<td></td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>Upper abdominal pain</td>
<td></td>
</tr>
<tr>
<td><strong>Maternal signs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal/ Mild Hypertension</td>
<td>Moderate/Severe Hypertension</td>
<td></td>
</tr>
<tr>
<td>Jaundice</td>
<td>Brisk reflexes</td>
<td></td>
</tr>
<tr>
<td>Hepatic encephalopathy</td>
<td>Pulmonary Oedema</td>
<td></td>
</tr>
</tbody>
</table>
### Biochemical Results

<table>
<thead>
<tr>
<th>Urinalysis</th>
<th>Absence of Ketonuria</th>
<th>Proteinuria</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Synthetic Liver failure</strong></td>
<td>Liver failure</td>
<td></td>
</tr>
<tr>
<td>- Hypoglycaemia</td>
<td>- Raised transaminases</td>
<td></td>
</tr>
<tr>
<td>- Hypoalbuminaemia</td>
<td>- DIC</td>
<td></td>
</tr>
<tr>
<td>- Raised transaminases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Raised INR</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Moderate/ Severe renal failure</strong></th>
<th><strong>Mild renal failure</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hyperbilirubinaemia</strong></td>
<td><strong>Thrombocytopenia</strong></td>
</tr>
<tr>
<td><strong>Hypcholesterolaemia</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Hypotriglyceridaemia</strong></td>
<td></td>
</tr>
</tbody>
</table>

### 4.7.1 Study Limitations

One of the limitations of my study was that it was a retrospective observational study. I was unable to contact the women identified from the King’s Liver ITU database, therefore I relied upon data that had been recorded in the hand held and electronic medical records. One main difficulty was dealing with missing data. I attempted to collect as much information as possible, however missing data prevented completion of some data sets. Despite these limitations, I obtained sufficient information to draw clear conclusions on the phenotype of women who previously suffered from AFLP.
4.8 Summary

This study has identified novel features that refine our current phenotype of women who present with AFLP and which fit with a proposed pathophysiology of a maternal defect in fatty acid oxidation. The most common documented defect in women with AFLP has been a defect in FAO through the common missense mutation of the LCHAD gene (LCHAD deficiency) although this accounts for less than 10% of all cases of AFLP (4). Although none of the women I studied had LCHAD deficiency, a defect anywhere along the pathway of fat metabolism would fit with the refined clinical phenotype. In particular, the absence of ketonuria in all women who had urinalysis, despite prolonged nausea, vomiting and fasting in the third trimester.

Low cholesterol and triglyceride levels are probably secondary to acute synthetic liver failure. This observation has not previously been associated with AFLP in the past.

From this study, I have therefore identified two simple investigations that can aid in the diagnosis of AFLP. I recommend that women who present with AFLP should have their lipid profile checked (to identify acute liver failure) and urine analysis to test for the absence of ketones (to identify a defect in FAO). Other important tests include liver transaminases, lactate, bilirubin and glucose. I have further demonstrated that babies born to these women have a normal birth weight for gestational age.

I propose that AFLP occurs during the insulin resistant state of the third trimester in a previously well mother who has an unidentified subclinical defect in fat metabolism. When she develops prolonged nausea and vomiting for a multitude of potential reasons, the fast unmasks the previously subclinical defect in energy
metabolism that leads to multi-organ failure. An antidote could be early admission to hospital for parenteral glucose.

I conclude that AFLP is therefore a metabolic disorder namely a disorder of fat metabolism (Figure 4-3) and is quite unlike pre-eclampsia or HELLP syndrome which have a vascular pathophysiology.

Figure 4-3 Proposed metabolic pathway in AFLP.

The defect in lipid metabolism could occur at any level in the pathway from lipolysis to energy generation.
Following the study of these women with AFLP there are still the following unanswered questions:

i. Why do these women vomit in the first place?

ii. The onset of AFLP is acute as the baby is well grown, but what is the stimulus?

iii. Where in the pathway of maternal lipid metabolism does the defect occur?

iv. What is the role of the male fetus?

v. Twin pregnancies suggest a placental dose origin, is the placenta key to the pathogenesis?
CHAPTER 5

FAT-BURNING

EXERCISE STUDY
Chapter 5 Exercise Study

5.1 Introduction

5.1.1 Abstract

Aims:

My aims were

i To establish whether women in the third trimester produce ketone bodies more rapidly compared with healthy non-pregnant women.

ii To establish whether women who have had Acute Fatty Liver of pregnancy (AFLP) have a reduced ability to generate ketone bodies from fatty acid oxidation following a 24-hour fast and fat-burning exercise.

Methods:

Pregnant subjects and their non-pregnant controls were fasted for a total of 18 hours, whilst women who had AFLP in a previous pregnancy and their non-pregnant controls were fasted for 24 hours. For the last 6 hours of fasting, all subjects underwent 6 hours of intermittent fat-burning exercise. Four blood samples were taken at 2 hourly intervals for the last 6 hours of the study and stored for measurement of metabolites of fat and glucose metabolism.

Results:

i Following an 18-hour fast and fat burning exercise, women in the third trimester of pregnancy produce a higher concentration of β-hydroxybutyrate (5-HB) compared with healthy non-pregnant women.

ii Following a 24-hour fast and fat burning exercise, non-pregnant women who have previously had AFLP produced a similar concentration of ketones (5HB) as women who had not had AFLP.
Conclusions:

In this study, I confirmed that fasting in the third trimester of pregnancy leads to a significantly greater production of ketones compared with the non-pregnant state: a phenomenon known as accelerated starvation. I have also demonstrated that women who have previously suffered from AFLP do not appear to have a defect in ketone body production outside of pregnancy.

5.1.2 Background

The clinical and biochemical phenotype of women with AFLP suggests it is a metabolic disorder of fatty acid oxidation. This is supported by my own findings (See Chapter 4). Investigations into the pathogenesis of AFLP whilst the mother is still pregnant are very difficult, due to the urgent need to deliver the baby and support the mother’s health. I therefore developed a novel study to assess fatty acid oxidation in women who had recovered from AFLP.

The study involved subjecting participants to a prolonged fast, which would lead to fat metabolism, followed by cardio-pulmonary exercise at a level that predominantly utilized fat as fuel. Cardiopulmonary exercise testing (CPET) is a test that involves measuring respiratory oxygen uptake (\(\dot{V}O_2\)), the production of carbon dioxide (\(\dot{V}CO_2\)) and ventilation. CPET is increasingly employed as a clinical tool to assess exercise capability.

Using CPET one can predict functional capacity, especially in patients with cardiac or respiratory disease. However, CPET testing can also be modified to evaluate other metabolic responses (220).

Energy expenditure such as fat oxidation can be measured using an adaptation of indirect calorimetry. Indirect Calorimetry is a method that has been developed to measure substrate oxidation by measuring oxygen consumption and carbon dioxide excretion both at rest and during exercise (183). During this process, expired gases are collected in large bags called Douglas bags.
A dry gas meter measures the volume of air in these bags following which a small sample of the expired air is drawn out from the Douglas bag to analyse the gases oxygen and carbon dioxide. Over the years techniques have developed whereby computerized systems can now be used to continuously measure the volume of oxygen and carbon dioxide (221). Douglas bags remain the gold standard for measurement of indirect calorimetry, however computerised systems are more favourable due to the speed and ease of performing the measurements (222). It is important to ensure equipment is regularly validated and careful calibration is performed as small changes in volumes of oxygen and carbon dioxide can have significant effects on fat and carbohydrate oxidation (223).

Achten and colleagues established a method to determine the level of exercise that leads to the maximal rate of fat oxidation. The “Fatmax” test involves exercising with incremental increases in work rate where indirect calorimetry is simultaneously used to calculate total fat and carbohydrate oxidation rates in the body (224).

In this study, I used a modified protocol of CPET and indirect calorimetry to maximally stress the fatty acid oxidation pathway. I measured ketone production in women who had previously suffered from AFLP and compared their response with healthy non-pregnant women and I also studied healthy pregnant and in the third trimester.

Under steady exercise on a stationary recumbent bike, I measured the respiratory exchange ratio (RER), which is a ratio of carbon dioxide production to oxygen uptake ($\frac{\text{VCO}_2}{\text{VO}_2}$) (181). Under these steady state conditions RER equals the Respiratory quotient (RQ), which represents the fuel used for metabolism at a tissue level. A RQ or RER of greater than 1.0 indicates the use of carbohydrates as the main fuel for metabolic processes. A value of less than 1.0 and closer to 0.7 represents fat as the predominant fuel. Measuring metabolic events at a tissue level can be difficult, which is why RER is used as a representative of RQ. RER can be measured during steady state exercise using gas exchange (225).
5.2 Hypothesis

i. I hypothesise that during the third trimester of pregnancy, women will have an increased capacity to generate energy from fatty acid oxidation and ketone production compared with non-pregnant controls.

ii. I hypothesise that following a period of fasting and fat-burning exercise, women who have had AFLP will have a reduced ability to generate energy from fatty acid oxidation and ketone body production.

5.3 Specific aims

i. To investigate whether women in the third trimester produce ketones following a shorter fast than healthy non-pregnant women.

ii. To establish whether women who have had AFLP have a limited ability to generate ketone bodies from FAO following a 24-hour fast and fat-burning exercise.

iii. To investigate differences in other elements of energy metabolism (including glucose, insulin and NEFA) between women who have had AFLP and healthy controls.

iv. To collect and store plasma before, during and at the end of the study for proteomic analysis.
5.4 **Methodology (Details in Chapter 3)**

5.4.1 **Study Design**

This cohort study was performed on women previously affected by AFLP, healthy non-pregnant women (non-AFLP) and pregnant women in the third trimester (pregnant) and non-pregnant women (non-pregnant). This study took place at The Institute of Sport, Exercise and Health (ISEH), UCL between April 2015 and June 2017.

5.4.2 **Exercise Study Participants**

I conducted 2 exercise studies.

- **Study 1: The metabolic response of pregnant women to fasting and fat-burning exercise.**

  This study comprised of healthy pregnant women in the third trimester of pregnancy (32-39 week’s gestation; ‘pregnant’ (n=12) and ‘non-pregnant’ women (n=5)). The pregnant women had a BMI of 18 – 30kg/m$^2$ at 10-12 weeks’ gestation and a single intrauterine pregnancy. The non-pregnant controls also had a BMI of 18 – 30kg/m$^2$ (See Table 5-1). These women were recruited from the UCLH low risk antenatal clinic at the Elizabeth Garrett Anderson wing.

- **Study 2: The metabolic response of women who have had AFLP to fasting and fat-burning exercise.**

  This study included women previously affected by AFLP (AFLP n=13) and healthy, non-pregnant women (Non-AFLP n= 23). The cases were recruited from the AFLP support group on the social networking site Facebook and from other London maternity units. Healthy controls were recruited by advertising on the University College London (UCL) forum, the Institute for Women’s Health (IFWH) and at University College London Hospital (UCLH).
5.4.3 Study Protocol

All participants attended the ISEH to take part in the study. Study 1 involved a 12-hour fast and Study 2 an 18-hour fast prior to both groups starting 6 hours of fat-burning exercise. At the ISEH all participants completed a health screening questionnaire and consent form. Baseline measurements for height, weight, blood pressure and heart rate were documented. An initial blood test was taken prior to commencing six hours of fat-burning exercise. Throughout the fat-burning exercise further blood tests were taken at 2h, 4h and 6h. Samples were centrifuged, processed and stored for further analysis. Certain samples were sent to the biochemistry laboratory at Whitfield street, London where routine validated assays were used to measure serum glucose, insulin and lipid profile. Beta-hydroxybutyrate and NEFA levels were measured using routine validated techniques at the Camelia Botnar Laboratories at GOSH. Plasma was stored for proteomic analysis.

The respiratory exchange ratio (RER) was employed to ensure participants remained in the fat burning zone throughout the study. RER is the ratio between Carbon Dioxide (\(\text{CO}_2\)) released and Oxygen consumed (\(\text{VO}_2\)) at a tissue level. It indirectly measures fat or carbohydrate utilization for fuel at various intensities during steady state exercise. The oxidation of one molecule of carbohydrate and fat leads to a RER of 1.0 and 0.7, respectively. During the exercise study, I confirmed each participant remained in the fat burning zone as continuous real-time measures of RER were recorded. All participants exercised with an RER of less 1.0 and aimed to achieve an RER close to 0.7 (Figure 5-1). If I noted the RER increasing I reduced the exercise intensity by 5 watts. For a detailed study protocol see section 3.6.
Figure 5-1 RER of less than 1.0 representing fat oxidation

The red line represents the volume of carbon dioxide produced and the blue line represents the volume of inhaled oxygen. The ratio of the 2 gases produces the respiratory exchange ratio at the tissue level (Orange line). An RER of less than 1.0 and closer to 0.7 signifies fat oxidation. The RER remains less than 1.0 throughout this study.

5.4.4 Statistical Analysis

Sample size calculations were performed using the results from the pilot study described in section 3.2.2. In this pilot study 10 healthy volunteers participated in (i) a fasting and exercise study and then on a separate day (ii) fasting study alone. An independent t-test provided a mean difference of the main end-product of fatty acid oxidation ($\beta$-hydroxybutyrate) as being 0.40mmol/L. The exercise and fasting study showed a mean of 0.98 mmol/L and standard deviation 0.45. Furthermore, I assumed that after fasting and fat-burning exercise, women who have had AFLP would generate 50% less SHB compared with women who did not have AFLP. If the standard deviation remained the same, a significance of 95% and study power of 80%, would require a sample size of 13 women who have suffered from AFLP and a control sample size of n=20 for study 2. The aim for study 1 was to recruit and study as many pregnant women and controls as I could in the time period available.
Baseline characteristics of all women have been summarized using means and standard deviations or medians and interquartile range as appropriate.

A mixed model analysis with random intercept and random slope has been performed using Stata software (StataCorp. 2015. *Stata Statistical Software: Release 14*. College Station, TX: StataCorp LP.) to analyze differences between the groups. Restricted maximum likelihood (REML) estimation was used to build up the models to prevent biased estimates. Also, likelihood ratio tests (LRT) were used to compare the random effects of the models when the fixed effects are the same. A statistically significant result was determined when the P-value < 0.05. This type of analysis takes into account the correlation between individuals at different time intervals which increases the power of the analysis. This analysis was performed by Jorge Garcia – Hernandez, Research associate in statistics (Farr Institute, UCL).

### 5.4.4.1 Mixed statistical models

Multilevel mixed models or hierarchical models are used when observations from individuals are grouped. In this study, I made repeated measurements of the same metabolite (e.g. Glucose levels at four different time points) in the same individual. Fixed effects are those attributable to a finite set of levels/categories of a factor (e.g., time, fasting and group (pregnant/not pregnant)) that are of specific interest, whereas random effects are those attributable to an essentially infinite set of levels of a factor (e.g. patient/participant) observed in a random sample from the population of interest. This mixed model analysis uses both fixed and random effects in the same analysis.

Variability in the data can be explained by the extent to which the intercept and slope vary in the mixed model. The slope is the rate of change and shows how a unit increase in the explanatory variable (e.g. time) affects the response (a particular blood test result). The y-intercept shows you what's predicted for the response when the explanatory value (time) is zero (Figure 5-2). It can be shown that the optimal mixed model for these data is one with random intercepts and random slopes.
This implies that the intercepts of the regression lines of the relevant response versus time for all patients vary around a mean intercept and the slopes of these lines also vary around a mean regression line. I have created mixed models for each response which looked at the effect of group (i.e. pregnant/ not pregnant) after adjusting for the time of fast. I also included an interaction term of group versus time. A significant interaction implies that any differences between the groups is not consistent for all times. I determined the estimated coefficients for the factors of interest for these models, together with their associated 95% confidence intervals and a p-values.

**Figure 5-2 y-intercept and slope**

The y-intercept is the point where the line crosses the vertical y-axis which represents a time of zero. The slope of a given line measures how much the value of units (y) changes for every unit of change in time (x), e.g. For every unit of time that the x-variable increases, the y-variable increases.
5.5 Results

5.5.1 Recruitment into the study

Study 1 included 12 pregnant women in the third trimester and 5 non-pregnant women. Study 2 included 13 women who previously suffered AFLP and 23 healthy women. Both studies took place between April 2015 and June 2017 at UCLH.

The medical records of the participants previously affected by AFLP and the healthy pregnant women were reviewed prior to entry into the study. All participants were asked to fill out a health screening questionnaire to ensure they had no contraindications to participating in the fat-burning exercise study.

Study 2, included 7 parous women and 16 women who had never been pregnant (nulliparous).

5.5.2 Study 1: The metabolic response of pregnant women to fasting and fat-burning exercise.

5.5.2.1 Baseline characteristics of participants

Study 1 investigated metabolic differences in response to fasting and fat-burning exercise between healthy women in the third trimester of pregnancy (n=12) and healthy, non-pregnant women (n=5). Both pregnant and non-pregnant participants were interviewed and had measures of height (cm), weight (kg) and BMI (kg/m²) recorded prior to starting the exercise protocol (See Table 5-1). There was a difference in age, weight and BMI between the pregnant and non-pregnant women.
Table 5-1 Study 1 Baseline Phenotype of healthy pregnant and non-pregnant women.

<table>
<thead>
<tr>
<th>Participants</th>
<th>Pregnant women (third trimester) n=12</th>
<th>Non-pregnant women n=5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
<td>25&lt;sup&gt;th&lt;/sup&gt;</td>
</tr>
<tr>
<td>Age (Years)</td>
<td></td>
<td>33</td>
</tr>
<tr>
<td>Height (cm)</td>
<td></td>
<td>166.5</td>
</tr>
<tr>
<td>Booking Weight (kg)</td>
<td></td>
<td>65.2</td>
</tr>
<tr>
<td>Booking BMI (kg/m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td></td>
<td>23.4</td>
</tr>
<tr>
<td>Nulliparous</td>
<td></td>
<td>9 (75%)</td>
</tr>
<tr>
<td>Parous</td>
<td></td>
<td>3 (25%)</td>
</tr>
</tbody>
</table>
5.5.3 Study 1: Measurement of study metabolite in pregnant (n=12) v non-pregnant women (n=5) following 18 hours of fasting and including 6 hours of fat-burning exercise.

i \( \beta \)-hydroxybutyrate (5HB)

Healthy pregnant women produced approximately 51% more \( \beta \)-hydroxybutyrate (5-HB) (mean 0.86 +/- SEM 0.09 mmol/L) following 18 hours of fasting and 6 hours of fat burning exercise compared with non-pregnant women (mean 0.42 +/- SEM 0.09 mmol/L), \( p=0.01 \). See Table 5-8 and Figure 5-3.

Figure 5-3 Study 1: Changes in serum \( \beta \)-hydroxybutyrate after 12 hours of fasting and throughout 6 further hours of fat-burning exercise in pregnant women (n= 12) and healthy non-pregnant women (n=5); (95% CI ([0.11, 0.76]) \( p=0.01 \)).

Both pregnant and non-pregnant women produced increased levels of 5HB over the last 6 hours of fat-burning exercise. However, when applying the mixed model analysis, pregnant women produced significantly higher levels of 5HB compared with non-pregnant women (95% CI (0.008, 0.15), \( p=0.03 \)) with time. See Table 5-2 and Figure 5-3.
Table 5-2 Study 1: Parsimonious final model with random intercept and slope for plasma β-hydroxybutyrate (mmol/L).

A mixed model analysis of plasma β-hydroxybutyrate (5HB) levels in pregnant (n=12) vs non-pregnant women (n=28 adjusted for the length of fast) following 6 hours of fat-burning exercise was performed. This showed that during fat-burning exercise there is no difference in 5HB levels between women in the third trimester of pregnancy and non-pregnant women (95%CI (-0.24, 0.03), p=0.13), however there is a significant interaction with time (95%CI (0.008, 0.15), p=0.03). In other words, when fasted, pregnant women do not produce more 5HB than non-pregnant women, they simply produce ketones earlier in a fast.

<table>
<thead>
<tr>
<th></th>
<th>Coefficients</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
<td>0.18</td>
<td>(0.14, 0.21)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Fast</td>
<td>-0.01</td>
<td>(-0.13, 0.11)</td>
<td>0.85</td>
</tr>
<tr>
<td>Pregnant vs Non-pregnant (mmol/L)</td>
<td>-0.11</td>
<td>(-0.24, 0.03)</td>
<td>0.13</td>
</tr>
<tr>
<td>Interaction with time Pregnant vs Non-pregnant</td>
<td>0.08</td>
<td>(0.008, 0.15)</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Random effects

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>SD slope</td>
<td>0.10</td>
<td>(0.07, 0.12)</td>
</tr>
<tr>
<td>SD intercept</td>
<td>0.11</td>
<td>(0.08, 0.15)</td>
</tr>
<tr>
<td>Correlation</td>
<td>0.99</td>
<td>(-1,1)</td>
</tr>
</tbody>
</table>

ii. Non-esterified fatty acids (NEFA)

Healthy pregnant women produced lower levels of NEFA (mean 1.02 +/- SEM 0.05 mmol/L) following 18 hours of fasting and 6 hours of fat burning exercise compared with non-pregnant women (mean 1.35 +/- SEM 0.26 mmol/L), p=0.09. See Table 5-8 and Figure 5-4.
When applying the mixed model analysis NEFA levels increased over time in all participants. However, over the last 6 hours of fasting and fat-burning exercise, pregnant women produced significantly less NEFA when compared with non-pregnant women (95% CI (-0.25, -0.001), p=0.05) with time. See
Table 5-3 Study 1: Parsimonious final model with random intercept and slope for NEFA mmol/L.

A mixed model analysis of plasma NEFA (mmol/L) levels in pregnant (n=12) v non-pregnant women (n=28 where the length of fast has been adjusted for in the analysis) following 6 hours of fat-burning exercise was performed. This showed that during fat-burning exercise women in the third trimester of pregnancy produce 0.15 mmol/L less NEFA (95%CI (-0.34, 0.04)) compared with non-pregnant women, but this result is not statistically significant (p = 0.12). However, there is a significant interaction between pregnant and non-pregnant women with time (95%CI (-0.25, -0.001), p=0.05). In other words, pregnant women produce similar plasma NEFA levels in response to fasting as non-pregnant women, but earlier in a fast.
### Chapter 5 Exercise Study

<table>
<thead>
<tr>
<th></th>
<th>Coefficients</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Time</strong></td>
<td>0.31</td>
<td>(0.24, 0.38)</td>
<td>0.0001</td>
</tr>
<tr>
<td><strong>Fast</strong></td>
<td>-0.02</td>
<td>(-0.19, 0.15)</td>
<td>0.81</td>
</tr>
<tr>
<td>Pregnant vs Non-</td>
<td>-0.15</td>
<td>(-0.34, 0.04)</td>
<td>0.12</td>
</tr>
<tr>
<td>pregnant (mmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interaction with time</td>
<td>-0.12</td>
<td>(-0.25, -0.001)</td>
<td>0.05</td>
</tr>
<tr>
<td>Pregnant vs Non-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pregnant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Random effects</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD slope</td>
<td>0.16</td>
<td>(0.13, 0.21)</td>
<td></td>
</tr>
<tr>
<td>SD intercept</td>
<td>0.10</td>
<td>(0.05, 0.2)</td>
<td></td>
</tr>
<tr>
<td>Correlation</td>
<td>0.69</td>
<td>(-0.50, 0.98)</td>
<td></td>
</tr>
</tbody>
</table>

#### iii. Glucose

At the end of 18 hours fast including 6 hours of fat-burning exercise, pregnant women had lower glucose levels (mean 3.61 +/- SEM 0.10 mmol/L) compared with non-pregnant controls (mean 4.26 +/- SEM 0.14 mmol/L), p=0.001. See Table 5-8 and Figure 5-5.

**Figure 5-5 Study 1:** Changes in plasma glucose levels after 12 hours of fasting and throughout 6 further hours of fat-burning exercise in pregnant women (n= 12) and healthy non-pregnant women (n=5); (95% CI ((-1.03, -0.28)) p= 0.002).
When applying the mixed model analysis glucose levels fell in both pregnant and non-pregnant women. Throughout the last 6 hours of the study, glucose levels were lower in pregnant compared with non-pregnant women (95% CI (-1.12, -0.31), p=0.001). However, over time both pregnant and non-pregnant women had a similar fall in plasma glucose levels (95% CI ((-0.07, 0.12)) p=0.56). See Table 5-4.
Table 5-4 Study 1: Parsimonious final model with random intercept and slope for Glucose mmol/L.

A mixed model analysis of plasma Glucose (mmol/L) levels in pregnant (n=12) vs non-pregnant women (n=28 where the length of fast has been adjusted for in the analysis) following 6 hours of fat-burning exercise was performed. This showed during fat-burning exercise women in the third trimester of pregnancy produce significantly less glucose 0.71mmol/L (95% CI (-1.12, -0.31); p<0.001) compared with non-pregnant women. In other words, pregnant women produce less plasma glucose levels in response to fasting than non-pregnant women and this is not affected by time.

<table>
<thead>
<tr>
<th></th>
<th>Coefficients</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Time</strong></td>
<td>-0.28</td>
<td>(-0.34, -0.23)</td>
<td><strong>0.0001</strong></td>
</tr>
<tr>
<td><strong>Fast</strong></td>
<td>-0.14</td>
<td>(-0.50, 0.22)</td>
<td>0.43</td>
</tr>
<tr>
<td><strong>Pregnant vs Non-pregnant (mmol/L)</strong></td>
<td>-0.71</td>
<td>(-1.12, -0.31)</td>
<td><strong>0.001</strong></td>
</tr>
<tr>
<td><strong>Interaction with time</strong></td>
<td><strong>Pregnant vs Non-pregnant</strong></td>
<td>0.03</td>
<td>(-0.07, 0.12)</td>
</tr>
</tbody>
</table>

**Random effects**

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SD slope</strong></td>
<td>0.12</td>
<td>(0.09, 0.16)</td>
</tr>
<tr>
<td><strong>SD intercept</strong></td>
<td>0.39</td>
<td>(0.31, 0.49)</td>
</tr>
<tr>
<td><strong>Correlation</strong></td>
<td>-0.37</td>
<td>(-0.62, -0.05)</td>
</tr>
</tbody>
</table>

iv. **Insulin**

Healthy pregnant women had lower plasma levels of insulin (mean 3.49 +/- SEM 0.63 mIU/L) following 18 hours of fasting and 6 hours of fat burning exercise compared with non-pregnant women (mean 4.74 +/- SEM 1.10 mIU/L), p=0.32. See Table 5-8 and

161
Figure 5-6 Study 1: Changes in plasma Insulin levels after 12 hours of fasting and throughout 6 further hours of fat-burning exercise in pregnant women (n= 12) and healthy non-pregnant women (n=5); (95% CI ((-3.82, 1.33)) p= 0.32).

When the mixed model analysis was applied insulin levels decreased similarly in both pregnant and non-pregnant women over time (95% CI (-1.54, 0.05); p = 0.07).
Table 5-5 Study 1: Parsimonious final model with random intercept and slope for Insulin mIU/L.

A mixed model analysis of serum insulin (mIU/L) levels in pregnant (n=12) v non-pregnant women (n=28 where the length of fast has been adjusted for in the analysis) following 6 hours of fat-burning exercise was performed. This showed during fat-burning exercise women in the third trimester of pregnancy produced 0.28 mIU/L less insulin compared with non-pregnant women, however this is not statistically significant (95% CI (-3.1, 3.7)); p = 0.87). In other words, pregnant women produce similar plasma insulin levels in response to fasting as non-pregnant women.
v. **Cholesterol**

At the end of 18 hours fast including 6 hours of fat-burning exercise, pregnant women had higher plasma levels of cholesterol (mean 7.05 +/- SEM 0.36 mmol/L) compared with non-pregnant women (mean 4.58 +/- SEM 0.52 mmol/L), p=0.002. See Table 5-8 and

Figure 5-7.

**Figure 5-7 Study 1: Changes in serum cholesterol levels after 12 hours of fasting and throughout 6 further hours of fat-burning exercise in pregnant women (n= 12) and healthy non-pregnant women (n=5); (95% CI ((1.09, 3.85)) p= 0.002).**
When applying the mixed model analysis cholesterol levels were significantly higher in pregnant women compared with non-pregnant women (95% CI (1.1, 3.4), p<0.001). However, throughout the duration of the study, serum cholesterol levels did not change significantly in any participants (95% CI (-0.01, 0.16); p=0.09). See Table 5-6.
Table 5-6 Study 1: Parsimonious final model with random intercept and slope for Cholesterol mmol/L.

A mixed model analysis of serum Cholesterol (mmol/L) levels in pregnant (n=12) v non-pregnant women (n=28 where the length of fast has been adjusted for in the analysis) following 6 hours of fat-burning exercise was performed. This showed during fat-burning exercise women in the third trimester of pregnancy significantly produced 2.25 mmol/L (95%CI (1.1, 3.4); p = 0.001) more cholesterol compared with non-pregnant women. In other words, pregnant women produce greater cholesterol levels in response to fasting as non-pregnant women with no effect of time.

<table>
<thead>
<tr>
<th>Coefficients</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
<td>-0.005</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>(-0.05, 0.04)</td>
<td></td>
</tr>
<tr>
<td>Fast</td>
<td>-0.08</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>(-1.13, 0.98)</td>
<td></td>
</tr>
<tr>
<td>Pregnant vs Non-pregnant (mmol/L)</td>
<td>2.25</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>(1.1, 3.4)</td>
<td></td>
</tr>
<tr>
<td>Interaction with time</td>
<td>0.08</td>
<td>0.09</td>
</tr>
<tr>
<td>Pregnant vs Non-pregnant</td>
<td>(-0.01, 0.16)</td>
<td></td>
</tr>
<tr>
<td>Random effects</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD slope</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.02, 0.21)</td>
<td></td>
</tr>
<tr>
<td>SD intercept</td>
<td>1.09</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.9, 1.34)</td>
<td></td>
</tr>
<tr>
<td>Correlation</td>
<td>-0.17</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(-0.65, 0.41)</td>
<td></td>
</tr>
</tbody>
</table>
vi. **Triglyceride**

Healthy pregnant women had higher plasma levels of triglyceride (mean 2.33 +/- SEM 0.23 mmol/L) following 18 hours of fasting and 6 hours of fat burning exercise compared with non-pregnant women (mean 0.9 +/- 0.11 mmol/L), p=0.001. See Table 5-8 and Figure 5-8.

**Figure 5-8 Study 1:** Changes in serum triglyceride levels after 12 hours of fasting and throughout 6 further hours of fat-burning exercise in pregnant women (n= 12) and healthy non-pregnant women (n=5); (95% CI ((0.66, 2.21)) p= 0.001).

When the mixed model analysis was applied triglyceride levels decreased similarly in both pregnant and non-pregnant women (95%CI (-0.05, 0.06); p=0.93). See
Table 5-7 Study 1: Parsimonious final model with random intercept and slope for Triglyceride mmol/L.

A mixed model analysis of serum triglyceride (mmol/L) levels in pregnant (n=12) v non-pregnant women (n=28 where the length of fast has been adjusted for in the analysis) following 6 hours of fat-burning exercise was performed. This showed during fat-burning exercise women in the third trimester of pregnancy significantly produced 1.41 mmol/L
more triglyceride compared with non-pregnant women (95%CI (0.84, 1.98); p = 0.001). In other words, pregnant women produce greater triglyceride levels in response to fasting as non-pregnant women with no effect of time.

<table>
<thead>
<tr>
<th></th>
<th>Coefficients</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
<td>-0.04</td>
<td>(-0.07, -0.004)</td>
<td>0.03</td>
</tr>
<tr>
<td>Fast</td>
<td>-0.14</td>
<td>(-0.64, 0.36)</td>
<td>0.58</td>
</tr>
<tr>
<td>Pregnant vs Non-pregnant (mmol/L)</td>
<td>1.41</td>
<td>(0.84, 1.98)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Interaction with time Pregnant vs Non-pregnant</td>
<td>0.003</td>
<td>(-0.05, 0.06)</td>
<td>0.93</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Random effects</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>SD slope</td>
<td>0.08</td>
<td>(0.06, 0.1)</td>
<td></td>
</tr>
<tr>
<td>SD intercept</td>
<td>0.58</td>
<td>(0.47, 0.7)</td>
<td></td>
</tr>
<tr>
<td>Correlation</td>
<td>-0.47</td>
<td>(-0.67, -0.2)</td>
<td></td>
</tr>
</tbody>
</table>

vii. HDL

Healthy pregnant women had higher plasma levels of HDL (mean 2.33 +/- SEM 0.26 mmol/L) following 18 hours of fasting and 6 hours of fat burning exercise compared with non-pregnant women (mean 1.88 +/- 0.27 mmol/L), p=0.33. However, the difference is not statistically significant. See Table 5-8 and Figure 5-9.

Figure 5-9 Study 1: Changes in serum HDL levels after 12 hours of fasting and throughout 6 further hours of fat-burning exercise in pregnant women (n= 12) and healthy non-pregnant women (n=5); (95% CI ((-0.49, 1.38)) p= 0.33).
viii. **LDL**

Healthy pregnant women had higher plasma levels of LDL (mean 3.88 +/- SEM 0.34 mmol/L) following 18 hours of fasting and 6 hours of fat burning exercise compared with non-pregnant women (mean 2.32 +/- 0.62 mmol/L), p=0.03. See Table 5-8 and Figure 5-10.

**Figure 5-10 Study 1:** Changes in serum LDL levels after 12 hours of fasting and throughout 6 further hours of fat-burning exercise in pregnant women (n= 12) and healthy non-pregnant women (n=5); (95% CI ((0.12, 2.96)) p = 0.03).
Table 5-8 Study 1: Final plasma concentration of each metabolite at the end of an 18 hour fast and 6 hours of fat burning exercise

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Pregnant (third trimester) n= 12</th>
<th>Non-pregnant n=5</th>
<th>95% CI</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
<td>25&lt;sup&gt;th&lt;/sup&gt;</td>
<td>75&lt;sup&gt;th&lt;/sup&gt;</td>
<td>Range</td>
</tr>
<tr>
<td>β-hydroxybutyrate (mmol/L)</td>
<td>0.78</td>
<td>0.68</td>
<td>1.01</td>
<td>0.45-1.62</td>
</tr>
<tr>
<td>NEFA (mmol/L)</td>
<td>0.995</td>
<td>0.89</td>
<td>1.08</td>
<td>0.8-1.43</td>
</tr>
<tr>
<td>NEFA/SHB</td>
<td>1.19</td>
<td>1.03</td>
<td>1.53</td>
<td>0.81-2.02</td>
</tr>
<tr>
<td>Glucose mg/dL</td>
<td>3.65</td>
<td>3.33</td>
<td>3.8</td>
<td>3.1-4.2</td>
</tr>
<tr>
<td>Insulin mIU/L</td>
<td>3.6</td>
<td>1.35</td>
<td>5.25</td>
<td>0.5-7.2</td>
</tr>
<tr>
<td>Cholesterol mmol/L</td>
<td>6.65</td>
<td>6.25</td>
<td>8.05</td>
<td>5.3-9.4</td>
</tr>
<tr>
<td>Triglyceride mmol/L</td>
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<td>2.65</td>
<td>1.4-3.3</td>
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<td>HDL mmol/L</td>
<td>2.05</td>
<td>1.73</td>
<td>2.38</td>
<td>1.6-4.3</td>
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<tr>
<td>LDL mmol/L</td>
<td>3.55</td>
<td>2.95</td>
<td>4.65</td>
<td>2.5-6.3</td>
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</table>
5.5.4  Study 2: The metabolic response of women who have had AFLP to fasting and fat-burning exercise.

5.5.4.1  Baseline characteristics of participants
There were no differences in height, weight and BMI between past AFLP cases and non-AFLP controls. However, women previously affected by AFLP were on average 9 years older than non-AFLP controls. See Table 5-9. Only 30% (7/23) of women who had not had AFLP were parous. My results may have been influenced by the disparity in parity and age. To address this issue I would need to recruit more controls.
Table 5-9 Study 2: Baseline Phenotype of previous AFLP and non-AFLP women.

<table>
<thead>
<tr>
<th>Participants</th>
<th>AFLP Case (n=13)</th>
<th>Median</th>
<th>25th</th>
<th>75th</th>
<th>Range</th>
<th>Mean</th>
<th>SD</th>
<th>SEM</th>
<th>Control (n=23)</th>
<th>Median</th>
<th>25th</th>
<th>75th</th>
<th>Range</th>
<th>Mean</th>
<th>SD</th>
<th>SEM</th>
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<tbody>
<tr>
<td>Age (Years)</td>
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<td>33</td>
<td>43.5</td>
<td>31-47</td>
<td>37.5</td>
<td>5.5</td>
<td>1.53</td>
<td></td>
<td>28</td>
<td>21</td>
<td>36</td>
<td>18-49</td>
<td>29</td>
<td>8.72</td>
<td>1.82</td>
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<td>Height (cm)</td>
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<td>162.5</td>
<td>157.8</td>
<td>167.5</td>
<td>152-176</td>
<td>163.5</td>
<td>7.13</td>
<td>1.91</td>
<td></td>
<td>167</td>
<td>160</td>
<td>171</td>
<td>157-181</td>
<td>166.7</td>
<td>6.63</td>
<td>1.38</td>
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<td>Weight (kg)</td>
<td></td>
<td>59</td>
<td>54.2</td>
<td>65.5</td>
<td>46-109</td>
<td>62.6</td>
<td>15.6</td>
<td>4.32</td>
<td></td>
<td>60.9</td>
<td>57</td>
<td>68</td>
<td>51-91</td>
<td>63.3</td>
<td>9.96</td>
<td>2.08</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td></td>
<td>23</td>
<td>19.9</td>
<td>25.5</td>
<td>19-35</td>
<td>23.6</td>
<td>4.68</td>
<td>1.3</td>
<td></td>
<td>23</td>
<td>21.0</td>
<td>24.1</td>
<td>18.3-29</td>
<td>23.0</td>
<td>3.05</td>
<td>0.62</td>
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<td>Nulliparous</td>
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<td></td>
<td></td>
<td></td>
<td>16</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parous</td>
<td></td>
<td>13 (100%)</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>7</td>
<td>30.4%</td>
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</tbody>
</table>
5.5.5 Study 2: Measurement of metabolites in women who had previously suffered from AFLP (n=13) v non-AFLP women (n=23) following 24 hours of fasting and including 6 hours of fat-burning exercise.

i. β-hydroxybutyrate (5HB)

After 24 hours of fasting and 6 hours of fat-burning exercise, women who had previously suffered from AFLP had a similar plasma level of β-hydroxybutyrate (mean 0.87 +/- SEM 0.15 mmol/L) as non-AFLP women (mean 0.74 +/- SEM 0.08 mmol/L), p=0.43. See Table 5-16 and Figure 5-11.

Figure 5-11 Study 2: Changes in serum β-hydroxybutyrate after 24 hours of fasting and throughout 6 further hours of fat-burning exercise in women who had previously suffered from AFLP (n= 13) and healthy non-AFLP women (n=23); (95% CI (-0.45, 0.2)) p= 0.43.

When applying the mixed model analysis, 5HB increased over time in all participants. However, there was no differential effect of time on 5HB levels in AFLP women over non-AFLP women (95% CI (-0.03 to 0.11)), p=0.27. See
Table 5-10 Study 2: Parsimonious final model with random intercept and slope for Beta-hydroxybutyrate mmol/L.

A mixed model analysis of plasma β-hydroxybutyrate (mmol/L) levels in women who had previously suffered from AFLP (n=13) vs non-AFLP women (n=28 where the length of fast has been adjusted for in the analysis) following 6 hours of fat-burning exercise was performed. This showed during fat-burning exercise women who had previously suffered from AFLP produce 0.06 mmol/L more 5HB compared with non-AFLP women, however this result was not significant (95%CI (-0.04, 0.15); p = 0.25).

<table>
<thead>
<tr>
<th></th>
<th>Coefficients</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
<td>0.18</td>
<td>(0.14, 0.21)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Fast</td>
<td>-0.01</td>
<td>(-0.13, 0.11)</td>
<td>0.85</td>
</tr>
<tr>
<td>AFLP vs Non-AFLP (mmol/L)</td>
<td>0.06</td>
<td>(-0.04, 0.15)</td>
<td>0.25</td>
</tr>
<tr>
<td>Interaction with time</td>
<td>0.04</td>
<td>(-0.03 to 0.11)</td>
<td>0.27</td>
</tr>
<tr>
<td>AFLP vs Non-AFLP</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Random effects

<p>| | | |</p>
<table>
<thead>
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<th></th>
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</thead>
<tbody>
<tr>
<td>SD slope</td>
<td>0.10</td>
<td>(0.07, 0.12)</td>
</tr>
<tr>
<td>SD intercept</td>
<td>0.11</td>
<td>(0.08, 0.15)</td>
</tr>
<tr>
<td>Correlation</td>
<td>0.99</td>
<td>(-1,1)</td>
</tr>
</tbody>
</table>

ii. Non-esterified Fatty acids (NEFA)

After 24 hours of fasting and 6 hours of fat-burning exercise, women who had previously suffered from AFLP had a similar plasma level of NEFA (mean 1.76 +/-
SEM 0.18 mmol/L) after 24 hours of fasting and 6 hours of fat-burning exercise as non-AFLP women (mean 1.55 +/- SEM 0.13 mmol/L), p=0.34. See Table 5-16 and Figure 5-12.

Figure 5-12 Study 2: Changes in serum NEFA after 24 hours of fasting and throughout 6 further hours of fat-burning exercise in women who had previously suffered from AFLP (n= 13) and healthy non-AFLP women (n=23); (95% CI ([-0.66, 0.23]) p= 0.34).

When applying the mixed model analysis NEFA levels increased in all participants, but there were no differences between the 2 groups over time (95% CI ([-0.06 to 0.18]), p=0.35). See
Table 5-11 Study 2: Parsimonious final model with random intercept and slope for NEFA mmol/L.

A mixed model analysis of serum NEFA (mmol/L) levels in women who had previously suffered from AFLP (n=13) v non-AFLP women (n=28 where the length of fast has been adjusted for in the analysis) following 6 hours of fat-burning exercise was performed. This showed during fat-burning exercise women who had previously suffered from AFLP produced 0.08 mmol/L more NEFA compared with non-pregnant women. However, this was not a significant finding 95%CI (-0.04, 0.21); p = 0.19).
### Table 5-16

<table>
<thead>
<tr>
<th></th>
<th>Coefficients</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Time</strong></td>
<td>0.31</td>
<td>(0.24, 0.38)</td>
<td><strong>0.0001</strong></td>
</tr>
<tr>
<td><strong>Fast</strong></td>
<td>-0.02</td>
<td>(-0.19, 0.15)</td>
<td>0.81</td>
</tr>
<tr>
<td><strong>AFLP vs Non-AFLP</strong></td>
<td>0.08</td>
<td>(-0.04, 0.21)</td>
<td>0.19</td>
</tr>
<tr>
<td><strong>Interaction with time</strong></td>
<td>0.06</td>
<td>(-0.06 to 0.18)</td>
<td>0.35</td>
</tr>
</tbody>
</table>

#### Random effects

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<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td><strong>SD slope</strong></td>
<td>0.16</td>
<td>(0.13, 0.21)</td>
</tr>
<tr>
<td><strong>SD intercept</strong></td>
<td>0.10</td>
<td>(0.05, 0.2)</td>
</tr>
<tr>
<td><strong>Correlation</strong></td>
<td>0.69</td>
<td>(-0.50, 0.98)</td>
</tr>
</tbody>
</table>

### Glucose

After 24 hours of fasting and 6 hours of fat-burning exercise, women who had previously suffered from AFLP had similar glucose levels (mean 4.20 +/- SEM 0.12 mmol/L) compared with non-AFLP women (mean 4.10 +/- SEM 0.11 mmol/L), p=0.45 See Table 5-16 and Figure 5-13.

**Figure 5-13 Study 2:** Changes in serum glucose levels after 24 hours of fasting and throughout 6 further hours of fat-burning exercise in women who had previously suffered from AFLP (n= 13) and healthy non-AFLP women (n=23); (95% CI (-0.47, 0.21)) p= 0.45.
When applying the mixed model analysis glucose levels decreased in both groups. However, there were no statistical differences in glucose between the groups and there was no differential effect of time on glucose levels in AFLP women over non-AFLP women (95% CI (-0.01, 0.17), p=0.08). See Table 5-12.
Table 5-12 Study 2: Parsimonious final model with random intercept and slope for Glucose mmol/L.

A mixed model analysis of serum Glucose (mmol/L) levels in women who had previously suffered from AFLP (n=13) v non-AFLP women (n=28 where the length of fast has been adjusted for in the analysis) following 6 hours of fat-burning exercise was performed. This showed during fat-burning exercise women who had previously suffered from AFLP produced 0.16 mmol/L less glucose compared with non-AFLP women. However, this was not a statistically significant result (95%CI (-0.44, 0.12); p = 0.27).
### Coefficients

<table>
<thead>
<tr>
<th></th>
<th>Coefficients</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
<td>-0.28</td>
<td>(-0.34, -0.23)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Fast</td>
<td>-0.14</td>
<td>(-0.50, 0.22)</td>
<td>0.43</td>
</tr>
<tr>
<td>AFLP vs Non-AFLP</td>
<td>-0.16</td>
<td>(-0.44, 0.12)</td>
<td>0.27</td>
</tr>
<tr>
<td>Interaction with time AFLP vs Non-AFLP</td>
<td>0.08</td>
<td>(-0.01, 0.17)</td>
<td>0.08</td>
</tr>
</tbody>
</table>

#### Random effects

<table>
<thead>
<tr>
<th></th>
<th>SD slope</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.12</td>
<td>(0.09, 0.16)</td>
</tr>
<tr>
<td>SD intercept</td>
<td>0.39</td>
<td>(0.31, 0.49)</td>
</tr>
<tr>
<td>Correlation</td>
<td>-0.37</td>
<td>(-0.62, -0.05)</td>
</tr>
</tbody>
</table>

#### iv. Insulin

After 24 hours of fasting and 6 hours of fat-burning exercise, women who had previously suffered from AFLP produced greater levels of insulin (mean 3.93 +/- SEM 0.93 mIU/L) compared to non-AFLP women (mean 2.70 +/- SEM 0.33 mIU/L), p=0.14, but this did not reach statistical significance. See Table 5-16 and Figure 5-14.

**Figure 5-14 Study 2:** Changes in plasma Insulin levels after 24 hours of fasting and throughout 6 further hours of fat-burning exercise in women who had previously suffered from AFLP (n= 13) and healthy non-AFLP women (n=23); (95% CI (-2.9, 0.43)) p= 0.14.
When the mixed model analysis was applied insulin levels decreased in both groups. However, there was no overall difference in insulin between the two groups and there was no differential effect of time on insulin levels in AFLP women over non-AFLP women (95% CI (-0.66, 0.91), p=0.76). See Table 5-13.
Table 5-13 Study 2: Parsimonious final model with random intercept and slope for Insulin mmol/L.

A mixed model analysis of serum insulin (mmol/L) levels in women who had previously suffered from AFLP (n=13) v non-AFLP women (n=28 where the length of fast has been adjusted for in the analysis) following 6 hours of fat-burning exercise was performed. This showed during fat-burning exercise women who had previously suffered from AFLP women produced 0.3 mmol/L more insulin compared with non-AFLP women with time. However, this was not statistically significant (95%CI (-2.6, 3.14); p = 0.84).
v. **Cholesterol**

After 24 hours of fasting and 6 hours of fat-burning exercise, women who had previously suffered from AFLP had greater levels of cholesterol (mean 4.93 +/- SEM 0.31 mmol/L) than non-AFLP women (mean 4.50 +/- SEM 0.18 mmol/L), p=0.21, but this did not reach statistical significance. See Table 5-16 and Figure 5-15.

**Figure 5-15 Study 1**: Changes in serum cholesterol levels after 24 hours of fasting and throughout 6 further hours of fat-burning exercise in women who had previously suffered from AFLP (n= 13) and healthy non-AFLP women (n=23); (95% CI ((-1.11, 0.25)) p= 0.21).
When applying the mixed model analysis there was no difference in cholesterol levels between women who had previously suffered from AFLP and non-AFLP women. However, there was a differential effect of time on cholesterol levels in AFLP women over non-AFLP women (95% CI (-0.17, 0.00), p=0.05). See Table 5-14.
Table 5-14 Study 2: Parsimonious final model with random intercept and slope for Cholesterol mmol/L.

A mixed model analysis of serum Cholesterol (mmol/L) levels in women who had previously suffered from AFLP (n=13) v non-AFLP women (n=28) where the length of fast has been adjusted for in the analysis) following 6 hours of fat-burning exercise was performed. This showed during fat-burning exercise women who had previously suffered from AFLP produced 0.41 mmol/L more cholesterol compared with non-AFLP women. However, this was not a significant result (95%CI (-0.35, 1.17); p = 0.29). There was an interaction with time between the 2 groups (95%CI (-0.17, 0.00009); p = 0.05).
Coeficients	95% CI	�-value

<table>
<thead>
<tr>
<th>Time</th>
<th>-0.005</th>
<th>(-0.05, 0.04)</th>
<th>0.85</th>
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</thead>
<tbody>
<tr>
<td>Fast</td>
<td>-0.08</td>
<td>(-1.13, 0.98)</td>
<td>0.89</td>
</tr>
<tr>
<td>AFLP vs Non-AFLP (mmol/L)</td>
<td>0.41</td>
<td>(-0.35, 1.17)</td>
<td>0.29</td>
</tr>
<tr>
<td>Interaction with time AFLP vs Non-AFLP</td>
<td>-0.09</td>
<td>(-0.17, 0.00)</td>
<td><strong>0.05</strong></td>
</tr>
</tbody>
</table>

Random effects

| SD slope | 0.06 | (0.02, 0.21) |
| SD intercept | 1.09 | (0.9, 1.34) |
| Correlation | -0.17 | (-0.65, 0.41) |

vi. **Triglyceride**

After 24 hours of fasting and 6 hours of fat-burning exercise, women who had previously suffered from AFLP produced greater levels of triglyceride (mean 1.00 +/- SEM 0.19 mmol/L) compared to non-AFLP women (mean 0.77 +/- SEM 0.04 mmol/L), p=0.001. See Table 5-16 and Figure 5-16.

**Figure 5-16** Study 1: Changes in serum triglyceride levels after 24 hours of fasting and throughout 6 further hours of fat-burning exercise in women who had previously suffered from AFLP (n= 13) and healthy non-AFLP women (n=23); (95% CI ([-0.53, 0.07]) p= 0.13).
When the mixed model analysis there was no difference in levels between the women who had previously suffered from AFLP and non-AFLP women. There was also no differential effect of time on triglyceride levels in women who had previously suffered from AFLP over non-AFLP women (95% CI (-0.06, 0.05)) p=0.83). See

Table 5-15.
A mixed model analysis of serum triglyceride (mmol/L) levels in women who had previously suffered from AFLP (n=13) v non-AFLP women (n=28) where the length of fast has been adjusted for in the analysis following 6 hours of fat-burning exercise was performed. This showed during fat-burning exercise women who had previously suffered from AFLP had Triglyceride levels that were 0.17 mmol/L higher compared with non-AFLP women. However, this was not a statistically significant result (95%CI (-0.23, 0.56); p = 0.41).
<table>
<thead>
<tr>
<th></th>
<th>Coefficients</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
<td>-0.04</td>
<td>(-0.07, -0.004)</td>
<td>0.03</td>
</tr>
<tr>
<td>Fast</td>
<td>-0.14</td>
<td>(-0.64, 0.36)</td>
<td>0.58</td>
</tr>
<tr>
<td>AFLP vs Non-AFLP (mmol/L)</td>
<td>0.17</td>
<td>(-0.23, 0.56)</td>
<td>0.41</td>
</tr>
<tr>
<td>Interaction with time AFLP vs Non-AFLP</td>
<td>-0.01</td>
<td>(-0.06, 0.05)</td>
<td>0.83</td>
</tr>
</tbody>
</table>

**Random effects**

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>SD slope</td>
<td>0.08</td>
<td>(0.06, 0.1)</td>
</tr>
<tr>
<td>SD intercept</td>
<td>0.58</td>
<td>(0.47, 0.7)</td>
</tr>
<tr>
<td>Correlation</td>
<td>-0.47</td>
<td>(-0.67, -0.2)</td>
</tr>
</tbody>
</table>

vii. **HDL**

After 24 hours of fasting and 6 hours of fat-burning exercise, women who had previously suffered from AFLP produced lower levels of HDL (mean 1.64 +/- SEM 0.14 mmol/L) compared to non-AFLP women (mean 0.92 +/- SEM 0.09 mmol/L), p=0.09. However, this difference was not statistically significant. See Table 5-16 and Figure 5-17.

**Figure 5-17 Study 2: Changes in serum HDL levels after 24 hours of fasting and throughout 6 further hours of fat-burning exercise in women who had previously suffered from AFLP (n= 13) and healthy non-AFLP women (n=23); (95% CI ((-0.6, 0.05)) p= 0.09).**
After 24 hours of fasting and 6 hours of fat-burning exercise, women who had previously suffered from AFLP produced greater levels of triglyceride (mean 2.76 +/- SEM 0.29 mmol/L) compared to non-AFLP women (mean 1.87 +/- SEM 0.09 mmol/L), p=0.0006. See Table 5-16 and Figure 5-18.

Figure 5-18 Study 2: Changes in serum HDL levels after 24 hours of fasting and throughout 6 further hours of fat-burning exercise in women who had previously suffered from AFLP (n=13) and healthy non-AFLP women (n=23); (95% CI (-0.6, 0.05)) p= 0.13.
Table 5-16 Study 2: Final concentration of each metabolites at the end of a 24-hour fast and 6 hours of fat burning exercise.

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>AFLP Cases n=13</th>
<th>Control n=23</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
<td>25th</td>
<td>75th</td>
<td>Range</td>
</tr>
<tr>
<td>β-hydroxybutyrate (mmol/L)</td>
<td>0.7</td>
<td>0.43</td>
<td>1.21</td>
<td>0.25-2.05</td>
</tr>
<tr>
<td>NEFA mmol/L</td>
<td>1.52</td>
<td>1.23</td>
<td>2.43</td>
<td>1.03-2.93</td>
</tr>
<tr>
<td>NEFA/βHB</td>
<td>2.33</td>
<td>1.67</td>
<td>3.43</td>
<td>0.7-4.68</td>
</tr>
<tr>
<td>Glucose mmol/L</td>
<td>4.2</td>
<td>3.8</td>
<td>4.5</td>
<td>3.6-5.1</td>
</tr>
<tr>
<td>Insulin mIU/L</td>
<td>2.7</td>
<td>2.25</td>
<td>6.5</td>
<td>0.79-11.5</td>
</tr>
<tr>
<td>Cholesterol mmol/L</td>
<td>4.8</td>
<td>4.1</td>
<td>4.9</td>
<td>2.5-6.7</td>
</tr>
<tr>
<td>Triglyceride mmol/L</td>
<td>0.9</td>
<td>0.7</td>
<td>0.98</td>
<td>0.5-3</td>
</tr>
<tr>
<td>HDL mmol/L</td>
<td>1.65</td>
<td>1.43</td>
<td>1.98</td>
<td>0.7-2.6</td>
</tr>
<tr>
<td>LDL mmol/L</td>
<td>2.6</td>
<td>2.1</td>
<td>3.0</td>
<td>1.4-4.9</td>
</tr>
</tbody>
</table>
5.6 Discussion

5.6.1 Study 1

My novel fat-burning exercise study has confirmed that during the third trimester, pregnant women develop hypoglycaemia and increased ketone body production more readily compared with non-pregnant women. This phenomenon is known as “accelerated starvation” (74). It is thought to occur to preserve glucose for fetal and placental growth, leaving fatty acid metabolism for maternal energy production.

The rate of lipolysis increases throughout gestation (226). One study demonstrated increased ketogenesis following 84 hours of starvation in the second trimester (227). In a separate study, women in the third trimester of pregnancy who had fasted for 14-18 hours had similar plasma ketone levels as women in the second trimester after 84 hours of starvation (74, 227). This lipolytic effect may be due to the hormonal action of human placental lactogen (HPL) which activates the direct release of fatty acids from adipose tissue in vitro (228). The free fatty acids are an energy source for skeletal and cardiac muscle and can also be used as a substrate for ketogenesis especially when glucose levels are low (229).

Previous studies have shown that by the third trimester, plasma cholesterol and triglyceride levels increase by 25-50% and 200-300% respectively compared with non-pregnant women (230). The results of my study are comparable to these findings. In the third trimester lipolysis is increased resulting in greater NEFA production. NEFA is transferred to the liver, where it is either re-esterified and released into circulation as VLDL or used for mitochondrial beta oxidation and ketone production. A gestational reduction in Lipoprotein Lipase (LPL) activity in adipose tissue also results in increased VLDL levels by reducing its removal from circulation (226). These mechanisms contribute to raised lipid levels in the third trimester of pregnancy.
In this study, I expected pregnant women to have higher plasma NEFA levels following a prolonged fast. During pregnancy, “accelerated starvation” should result in increased rates of lipolysis, resulting in more NEFA being released into circulation. My results do not replicate these findings, which may be a result of the small sample size and difference in baseline characteristics in that the non-pregnant group were younger and actually had greater insulin resistance.

The results of this study support the observation that pregnant women utilize fatty acid oxidation with increased ketogenesis earlier in a fast than do non-pregnant women (Table 5-8).

5.6.2 Study 2

It was my hypothesis that women who had AFLP have a subclinical defect in FAO, which is unmasked by the reliance on FAO in the third trimester of pregnancy and exaggerated by fasting. However, the fasting and exercise study did not unmask a defect in ketone body production in women who had previously had a pregnancy affected by AFLP. Interestingly, there was a difference in cholesterol levels where AFLP women have greater cholesterol levels over time compared to non-AFLP women (p=0.05). This could be a result of the age difference between the groups as women who had previously experienced AFLP were approximately 9 years older than non-AFLP women. This would need further investigation by studying more women who were appropriately age-matched with women who had AFLP.

These results suggest that the defect of FAO in AFLP occurs only during pregnancy and resolves postpartum. In favour of this hypothesis is the fact that these women almost always make a full recovery from AFLP. Interestingly, they do not develop AFLP again in a subsequent pregnancy, which suggests that a period of fasting (nausea and vomiting) is also necessary to develop AFLP.
An alternative explanation is that a 24 hour fast outside of pregnancy was not a great enough stress-test of the fatty acid pathway to demonstrate impaired ketogenesis.

To investigate this further one option would be to increase the length of the fast prior to commencing the fat burning exercise. Another alternative would be to study women with active AFLP, by collecting plasma and serum and measuring key metabolites involved in FAO. This second option would be very difficult as AFLP is so rare and immediate childbirth is the cure. I would not see more than 1-2 cases out of approximately 6500 deliveries at UCLH a year.

During this study, we have not studied the affected offspring or father of the pregnancies. It may be that AFLP follows a defect in FAO in the offspring. This has been seen in families affected by a deficiency in the LCHAD enzyme involved in FAO. Another example is an imprinted gene only inherited from the father, which expresses a protein called Delta-like homolog 1 (DLK1) in the fetus (231). Absence of DLK1 derived from the fetus reduces maternal ketone production during fasting (231). However, pregnancies associated with reduced DLK-1 production tend to be growth restricted rather than of normal weight. The offspring of AFLP cases in our cohort and in general tend to be of normal weight.

### 5.6.3 Limitations of my study

i. This study aimed to induce a substantial stress on women who had been affected by AFLP to replicate the metabolic conditions of their pregnancy. During pregnancy, women affected by AFLP were starved through anorexia or vomiting and in an insulin resistant state of the third trimester, which favors lipolysis. My study protocol induced fat burning, however it is possible that the length of the fast was not sufficient to demonstrate impaired lipolysis and FAO.
However, on studying these women following an 18 and 24 hour fast and fat burning exercise, all women were fatigued and therefore it is unlikely that they would tolerate a longer fast.

ii  
Over 60 years ago, gestational Insulin resistance was recognized as a feature of the second half of pregnancy (232, 233). Hyperglycaemia and hyperinsulinaemia follow each meal during pregnancy (234). Several studies went on to try and quantify insulin secretion and response especially in the third trimester (235). It was in the 1980’s where euglycaemic hyperinsulinaemic clamp studies were first used to accurately measure insulin sensitivity and resistance. (236) If I were to repeat the fat-burning exercise study it may be an option to induce insulin resistance using a euglycaemic clamp and dexamethasone, which is a technique that has been used to cause insulin resistance in humans (237).

iii  
The non-pregnant group were much younger than the pregnant group which may have had an impact on the differences identified in the various metabolites. Age and parity predominantly affect metabolism. Any future studies would need to include a better matched control group.

iv  
The Mixed family model is the most appropriate statistical test to analyze the data as it analyses study data where repeated measurements are taken. My study involved 4 measures of metabolites in the last 6 hours of the study. However, the random intercept and slope model which allows correlations to be made within individual samples and between individual samples could be overfitting the data. Overfitting is where a complex model has been used to assess the data but there are too many parameters in relation to the number of observations. This limitation of the statistical analysis needs to be considered when interpreting the results as minor changes in the data may have a greater statistical result. To overcome this I would need a larger sample size.
5.7 Key Findings

Following an 18-hour fast and fat burning exercise, women in the third trimester of pregnancy

i. produce a higher concentration of 5-hydroxybutyrate (5-HB) compared with healthy non-pregnant women.

ii. have lower plasma glucose levels compared with non-pregnant women.

iii. have higher cholesterol and triglyceride levels compared with non-pregnant women.

Following a 24-hour fast and fat burning exercise, women who have previously had AFLP;

i. produce a similar concentration of ketones (5HB) as women who have not had AFLP.

ii. have higher cholesterol levels compared with women who have not had AFLP.

5.8 Summary

In this novel study of fasting and fat-burning exercise, I have shown

i. Women in the third trimester of pregnancy demonstrate the phenomenon of accelerated starvation with rapid ketone body production.

ii. Women who have had AFLP and who are no longer pregnant do not appear to have a sub-clinical defect in ketone body production.

I next explored whether women who had AFLP had other undiscovered metabolic defects that might predispose them to AFLP using Proteomics and next generation gene sequencing.
CHAPTER 6

PROTEOMICS
Chapter 6  Proteomics

6.1  Introduction

6.1.1  Abstract

Aims:

To investigate whether women who have had AFLP have a sub-clinical defect in energy metabolism that can be identified through proteomic analysis of their plasma, following 24 hours of fasting and 6 hours of fat-burning exercise.

Methods:

I studied 13 non-pregnant women who had a pregnancy affected by AFLP and 13 women who had not had AFLP. Proteomic analysis was carried out in 2 batches. Batch 1 contained 6 women who had AFLP v 6 non-parous controls and Batch 2 contained 7 women who had AFLP v 7 parous controls. All subjects were fasted for 24 hours and underwent 6 hours of fat-burning exercise, as described in Chapter 5. Plasma samples were taken from all participants after 18 hours of fasting and then again after 24 hours of fasting and 6 hours of fat-burning exercise. Plasma samples were stored until ready for proteomic analysis, using the SomaLogic platform based at the institute of child health (ICH).

Results:

i  In batch 1, following a 24-hour fast and 6 hours of fat-burning exercise, plasma proteomic analysis showed that non-pregnant women who previously had AFLP (n=6) had higher ferritin and hepcidin levels and lower plasma hemojuevelin levels compared with 6 younger, non-parous women.

ii  In batch 2, following a 24-hour fast and 6 hours of fat-burning exercise, plasma proteomic analysis showed that non-pregnant women who previously had AFLP (n=7) had higher Glypican-2 and Galectin-3 levels compared with 7 age-matched parous women who did not have AFLP.
Conclusions:

Women who previously had AFLP have differences in iron metabolism proteins (Hemojuvelin, hepcidin and ferritin) associated with underlying fatty liver, however these differences were not replicated with age and parity matched controls.

Comparison of women who previously had AFLP with non-AFLP women of similar age and parity detected elevated levels of Glypican 2 and Galectin 3. Further analysis to determine the importance of these observations in the pathogenesis of AFLP is necessary.

6.1.2 Background

In order to further determine the pathogenesis of AFLP, I used a proteomic platform to discover whether the concentration of 1300 circulating proteins was different after 24 hours fasting and 6 hours of fat-burning exercise in women who had AFLP compared with women who had not had AFLP.

Proteins are essential in providing cell structure as well as being key in several biological activities including cell regulation, signalling, metabolism, transport and storage. They also have the ability to move between cells to carry out their specific function (238).

Advances in technology have opened up the fields of genomics, metabolomics and proteomics have provided an opportunity to generate and process large biological data sets, leading to a better understanding of health and disease.
6.1.3 Genomics

Genomics is the study of the whole genome of an organism. However, less than 2% of the human genome is made up of 20,000 protein-coding genes (239). The rest of the genome is made of regulatory Deoxyribonucleic Acid (DNA) sequences, non-coding Ribonucleic Acid (RNA) and other sequences for which function has yet to be determined. Although genomics has improved our knowledge of disease processes, in particular single gene disorders, the complex interactions of multiple proteins in polygenic disorders requires a fuller assessment of protein elaboration.

6.1.4 Metabolomics

The Metabolome describes all endogenous low-molecular-weight components (<1 kDa) in a biological sample such as urine or serum. Each cell type and biological fluid has a characteristic set of metabolites that reflects the organism under a particular set of environmental conditions and that fluctuates according to physiological demands.

Nuclear Magnetic Resonance (NMR) spectroscopy is an analytical chemistry technique used in metabolomics due to its ability to determine molecular structures (240). In NMR, nuclei in a magnetic field absorb and re-emit electromagnetic radiation. The strength of the magnetic field and the magnetic properties of the isotopes of the atoms determine the specific frequency of the energy emitted.

NMR spectroscopy entails interpretation of the chemical shifts and splitting patterns due to indirect nuclear interactions (J-couplings). Pattern recognition and related multivariate statistical approaches can be used to discern significant patterns in complex data sets aimed at identifying molecular structure (241).

In metabolomics, the main aim is to identify and quantify as many metabolites as possible (242).
The phenotype of an organism is linked to its metabolites and therefore metabolomics can be useful in a number of applications from phenotyping genetically modified organisms to ascertaining gene function of an organism (243).

However, extracting relevant data and interpretation of large datasets remains an immense challenge. Samples from the AFLP cases I have studied are currently being analyzed by collaborators. Although results are eagerly awaited, they will not be available in time for inclusion in my thesis.

6.1.5 Proteomics

Proteins are key to an individual’s phenotype. As products of gene expression, proteins are involved in multiple metabolic, immune and vascular processes within healthy and diseased cells. When proteins are released into the circulation, their identification and quantification has the potential to serve as a biomarker for the diagnosis and management of pathological conditions. In obstetrics, proteomics has been useful to assist in diagnosis, management and prognosis of obstetric conditions including pre-eclampsia, intra-uterine growth restriction, premature rupture of membranes and ectopic pregnancy. Several studies have shown specific proteins in plasma, urine, placental tissue and trophoblastic cells associated with the development of pre-eclampsia (244-246).

The term Proteomics was first characterised in 1995 to describe the large-scale study of the structure, function and interaction of proteins within a cell or organism (247). Although, proteomic analysis using 2D gels has been performed since the 1970's (248). We now know that although the genome of an organism remains constant, gene expression and consequent protein elaboration varies with time and with changes to the surrounding environment (249).

The proteome of a cell or organism is the entire number of proteins present during a specific period or phase of development. Proteomics is the study of the proteome which can be performed using various techniques.
There are three main types of proteomics:

- **Expression Proteomics**

  This form of proteomics quantifies protein expression between different samples under different conditions (e.g. before and after stress) and therefore protein expression between the separate samples can be compared. Techniques employed to perform this type of proteomics include 2D gel electrophoresis and mass spectrometry (MS). This approach, in particular, has been shown to be useful in recognising proteins involved in signalling and proteins specific to health disorders and diseases (250).

- **Structural Proteomics**

  The aim of this type of proteomics is to facilitate complete mapping of the structure of protein complexes. Firstly, all proteins are identified and located, followed by characterisation of all protein-protein interactions. This type of proteomics is useful in understanding the 3D shape, structure and function of protein complexes. NMR spectroscopy and X-ray crystallography are techniques used to determine the structure (251).

- **Functional Proteomics**

  This is where a more detailed and directed approach to proteomics can take place. For example, certain proteins or protein complexes can be isolated and further analysed in greater detail. This direct approach is useful in learning about specific pathways such as drug interactions with proteins or protein signalling especially in disease (252).

There are several different technologies used to carry out proteomics. The traditional methods of enzyme-linked immunosorbent assay (ELISA) and mass spectrometry (MS) are successful in quantifying numerous proteins in biological samples (253). However, both techniques have limitations. ELISA involves a lengthy technique and requires the production of antibodies for each and every protein of interest.
To improve the binding of targeted proteins the ELISA technique uses two different types of antibodies; a capture and detection antibody. However, ELISA is not the method of choice in large scale proteomic studies mainly due to non-specific binding of these antibodies. Mass spectrometry processes are influenced by protein concentrations producing biases towards proteins with a higher relative concentration. It is also known to have poor reproducibility abilities on a large scale (254).

Newer technologies have tried to overcome the limitations associated with ELISA and MS. In this study, I have used a new aptamer based proteomic methodology which has been developed by the group SomaLogic Inc. This method allows the measurement of both large numbers of abundant proteins and small numbers of less abundant proteins.

Aptamers are classified as nucleic acid molecules and are single stranded oligonucleotides. They have the ability to transform into structures that can bind to proteins (255). SomaLogic have been successful in creating a new aptamer called Slow Off Rate Modified Aptamer (SOMAmer), where synthetic DNA-based molecules in the reagent bind to proteins with high affinity and specificity. This protein capture reagent replaces the need for antibodies. The SOMAscan concurrently measures over 1000 protein analytes in the samples (256).

By accurately measuring the concentration of serum proteins in my cohort of women with a previous history of AFLP under precisely controlled conditions, I hoped to identify key pathways that may be defective or overactive in the pathogenesis of AFLP.
6.2 Hypothesis

Women previously affected by AFLP have a different proteome following a 24-hour fast and fat burning exercise compared with healthy women who did not have AFLP.

6.3 Specific aims

i. To determine whether women who have had AFLP have any differences in protein concentration, which might explain a gestational defect in energy metabolism, specifically fatty acid oxidation.

6.4 Methodology

6.4.1 Study design

Plasma samples before and after 6 hours of fat burning exercise were sent for proteomic analysis in women who have previously had AFLP and non-AFLP women. See Chapter 5 for the full exercise protocol.

6.4.2 Study Samples

Plasma samples were sent to SomaLogic (and were analysed) in 2 batches:

Batch 1: These participants were chosen because I had studied them at the time SomaLogic were looking to obtain samples from pregnancy related studies.

- Women who previously suffered from AFLP (n=6)
- Women who had never been pregnant (n=6, non-parous)
Batch 2: Later in my study, I recruited better matched controls and therefore I decided to analyse a further 7 samples where the groups were appropriately matched.

- Women who previously suffered from AFLP (n=7)
- Women who had a healthy pregnancy (n=7, parous)

### 6.4.3 Study Protocol

Plasma samples (collected in K2 EDTA tubes) were transferred on dry ice to ICH for proteomic analysis before and after the exercise study. In the laboratory, the plasma samples were processed and transformed into raw proteomic data which are essentially binary files that measures the total mass of a peptide or protein which enable protein identification based on the molecular weights (see section 3.7.1 for detailed methodology).

Processing of plasma samples was performed by Dr. Yasmin Panchbhaya and Dr. Jutta Palmen. The raw proteomic output data was then sent to SomaLogic. The first step involved a Multiplexed SOMAmer affinity assay, which is described in detail in section 3.7. To reduce intra-sample bias, normalization was performed, which involves placement of hybridization control sequences into the assay eluate before hybridization takes place. This is then measured for each sample array correcting for systematic effects. The process of normalization was followed by calibration processes which reduces variability between samples. These quality control procedures subsequently enabled analysis of the data produced.
6.4.4 Enzyme-linked immunosorbent assay (ELISA)

Somalogic proteomics discovered a strongly significant difference ($P < 10^{-6}$) in many proteins between the AFLP and non-AFLP women (See section 6.5.2). By way of verification, I used a targeted ELISA assay to measure the plasma concentration of the 3 proteins that were most significantly different between the groups; Peptide YY ($P<0.001$), Glucagon Like peptide-1 and ferritin ($P<0.001$). All assays were performed with the assistance of the same experienced lab technician, Jenny Jones.

Total serum Peptide YY (PYY) concentrations were measured using the commercially available ELISA kit (Catalogue #EZHPYYT-66K, EMD Millipore Corporation, Darmstadt, Germany). Manufacturer instructions were followed and the intra-assay and inter-assay coefficients of variation were <3% and <7%, respectively (Appendix 3 S1).

Total serum Glucagon Like peptide-1 (GLP-1) concentrations were measured using another commercially available ELISA kit (Catalogue #EZGLP1T-36K, EMD Millipore Corporation, Darmstadt, Germany). The intra-assay and inter-assay coefficients of variation were <2% and <12%, respectively (Appendix 3 S2).

Total serum ferritin concentrations were measured using the commercially available ELISA kit (Catalogue #AB108837, ABCAM PLC, Cambridge, UK). The intra-assay and inter-assay coefficients of variation were <4% and <8%, respectively (Appendix 3 S3).
6.4.5 Statistical analysis

The raw data from the proteomic assessment was analyzed by a bioinformatics expert Jorge Garcia-Hernandez at the Farr Institute, UCL. However, the Bonferroni post-hoc comparison was used to adjust for multiple comparisons. In my study regression analysis and ANCOVA analysis was performed. This study included cases (women who had previously had AFLP) and controls (non-AFLP women) as well a stress factor in the form of exercise, therefore potential changes in protein levels before and at the end of exercise could be measured. As a result, time was adjusted to accurately analyze any difference between cases and controls.

This was achieved through two analogous statistical tests.

- A two-sample t-test, comparing the mean change in protein from the baseline in both groups.
- Covariance ANCOVA analysis, to test whether the mean after exercise in cases is equal to the mean after exercise in controls. Adjustments were made for observed baseline mean differences between cases and controls.

All results have been transposed using the natural log transformation. This was the most appropriate transformation to employ to reduce outliers and interpret the data more easily. The R statistical software package, version 3.3.3 was used for the analysis. The ELISA results were analyzed using the statistical paired t-tests in Graph Pad Prism.
6.5 Results

6.5.1 Baseline Characteristics

In Batch 1, women who had AFLP were of similar height, weight and BMI as women who had not had AFLP. However, in Batch 1, women previously affected by AFLP were on average 12 years older than non-AFLP women and all non-AFLP women were nulliparous (See Table 6-1).

<table>
<thead>
<tr>
<th>Participants</th>
<th>AFLP women (n=6)</th>
<th>Non-AFLP women (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Range</td>
</tr>
<tr>
<td>Age (Years)</td>
<td>37.7</td>
<td>32-47</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>161.3</td>
<td>156-169</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>58.4</td>
<td>46-66</td>
</tr>
<tr>
<td>BMI (kg/m^2)</td>
<td>22.5</td>
<td>19-26</td>
</tr>
<tr>
<td>Nulliparous</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>Parous</td>
<td>6 (100%)</td>
<td></td>
</tr>
</tbody>
</table>

I subsequently recruited 7 more controls who were all parous and of similar age to 7 further women who had AFLP (Batch 2). Women who had AFLP were also of similar BMI as non-AFLP women (Table 6-2).
Table 6-2 Baseline characteristics for all Batch 2 study participants.

<table>
<thead>
<tr>
<th>Participants</th>
<th>AFLP women (n=7)</th>
<th>Non-AFLP women (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Range</td>
</tr>
<tr>
<td>Age (Years)</td>
<td>37.4</td>
<td>31-45</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>163.6</td>
<td>152-176</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>66.2</td>
<td>48-109</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>24.6</td>
<td>19-35</td>
</tr>
<tr>
<td>Nulliparous</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>Parous</td>
<td>7 (100%)</td>
<td></td>
</tr>
</tbody>
</table>

6.5.2 Analysis 1: Batch 1; 6 women with previous AFLP vs 6 non-parous women

In Batch 1, several proteins were significantly different between AFLP women and non-parous women after 24-hours of fasting and 6-hours of fat-burning exercise, adjusting for time with the Bonferroni correction (Figure 6-1a).

i The following proteins were significantly lower in women who had AFLP compared with non-AFLP women: Hemojuvelin ($P=5.19\times10^{-6}$), Leptin receptor ($P=8.6\times10^{-6}$), Alpha-2-macroglobulin ($P=2.03\times10^{-3}$) and Peptide YY ($P<0.001$).

ii The following proteins were significantly higher in women who had AFLP compared with non-AFLP women: Ferritin and Hepcidin (both $P<0.001$).

After adjusting for the protein levels at baseline, (after 18-hours of fasting), comparison between AFLP and non-AFLP women showed no statistical difference in protein levels at $p<0.001$ after 6 further hours of fat-burning exercise (Figure 6-1b). However, out of all proteins that were initially different between the groups after
adjusting for time \((p<0.001)\), serum Hemojuvelin levels did not rise as much as in women who had AFLP \((p=0.01)\) (Figure 6-1a).

6.5.3 Analysis 2: Batch 2; 7 women who had AFLP vs 7 parous women who did not have AFLP.

The most significantly different proteins between AFLP and non-AFLP women after adjusting for time with the Bonferroni correction were Glypican-2 \((P=2.72 \times 10^{-7})\) and Galectin-3 \((P=2.71 \times 10^{-5})\). Both proteins were higher in women who had previous AFLP compared with non-AFLP women.

After adjusting for the levels of these 2 proteins at baseline (after 18-hours of fasting), there was no further significant difference in these proteins between AFLP and non-AFLP women after a further 6-hours of fat-burning exercise (See Figure 6-2b). However, after adjusting for baseline protein levels, several other proteins were significantly different between the groups.

Most pertinently, Glucagon levels were lower \((p=0.001)\) and Peroxisomal targeting signal 1 receptor \((p=0.01)\), insulin like growth factor binding protein 1 \((p=0.01)\), TNF superfamily 1B \((p=0.01)\) and EGF-containing fibulin-like extracellular matrix protein 1 \((p=0.0001)\) were all higher.

The proteomic findings between women who had AFLP and non-parous women (Batch 1) were not replicated in Batch 2.
Figure 6-1 Volcano plots showing the difference between AFLP cases and controls: Batch 1 (AFLP n=6, non-AFLP, non-parous n=6)

The X axis refers to the log mean difference between cases and controls. Positive difference refers to cases > controls on log mean. Y axis refers to the statistical significance of the difference of the protein. a) Protein log mean difference adjusting for time. b) Protein difference adjusting for the protein levels at baseline.
Figure 6-2 Volcano plots showing the difference between AFLP cases and controls: Batch 2 (AFLP n=7, non-AFLP, parous n=7)

The X axis refers to the log mean difference between cases and controls. Positive difference refers to cases > controls on log mean. Y axis refers to the statistical significance of the difference of the protein. a) Protein log mean difference adjusting for time. b) Protein difference adjusting for the protein levels at baseline.
6.5.4 ELISA analysis.

In Batch 1, proteomic analysis found that Peptide YY concentration was significantly lower in AFLP women compared with non-parous women. As women who had AFLP have a prodromal illness of nausea and vomiting, I decided to validate the result of the Peptide YY level with an ELISA measure of all 13 women who had AFLP and 13 women who had not had AFLP. As GLP-1 has a close relationship with Peptide YY and despite its absence in any proteomic results, I also measured the GLP-1 level by ELISA.

Hormones released from the gut have a significant role in controlling appetite. The main hormones involved are Peptide tyrosine tyrosine (Peptide YY), Glucagon-like peptide 1 (GLP-1), Oxyntomodulin, Glucagon, Cholecystokinin, Pancreatic polypeptide amylin. Peptide YY and GLP-1 are anorectic gut hormones that are both released simultaneously following a meal. They act to induce post-prandial satiety (257-259).

6.5.4.1 Peptide YY (pg/ml)

Peptide YY levels decrease after fasting and exercise. I measured the level of PYY after 18-hours of fasting (baseline) and then after a further 6-hours of fasting and fat-burning exercise. Non-parous women (n=6) showed the expected fall in peptide YY levels following a prolonged fast (111.3 vs 83.0 pg/mL, but the decrease was not statistically significant (p=0.19, 95%CI (-16.3, 72.9)). However, women who had AFLP (n=13) (mean 99.9 +/- SEM 12pg/ml) and healthy parous controls (n=7) (mean 97.3 +/- SEM 13 pg/ml) both showed no change in PYY level (p=0.67, 95% CI ((-32.4, 49.3)) and 95%CI ((-32.4, 49.3)) respectively). When comparing the 2 groups there was no difference between the AFLP women and non-AFLP women, before (mean 99.9 +/- SEM 12 pg/ml and mean 103.8 +/- SEM 9.8 pg/ml respectively), (p=0.8, 95% CI ((-35.8, 28.0)) or at the end of the study (mean 91.4 +/- SEM 15.8 pg/ml and mean 95.7 +/- SEM 10.4 pg/ml respectively), (p=0.82, 95% CI ((-43.2, 34.7)).
See Table 6-3 and Figure 6-3. Parity did not have any effect on the results (See Figure 6-4).

Table 6-3 Peptide YY analysis in AFLP (n=13) and non-AFLP women (n=13)

The ‘baseline’ measurement of Peptide YY levels occurred after 18-hours of fasting and prior to 6-hours of fat-burning exercise. The ‘end’ measurement of Peptide YY levels occurred after 24-hours of fasting and 6 hours of fat-burning exercise.

<table>
<thead>
<tr>
<th></th>
<th>Mean (pg/ml)</th>
<th>SD</th>
<th>SEM</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFLP- Baseline (n=13)</td>
<td>99.9</td>
<td>43.2</td>
<td>12.0</td>
<td>(73.8, 126)</td>
</tr>
<tr>
<td>AFLP- End (n=13)</td>
<td>91.4</td>
<td>56.8</td>
<td>15.8</td>
<td>(57.1, 125.8)</td>
</tr>
<tr>
<td>All Controls- Baseline (n=13)</td>
<td>103.8</td>
<td>35.2</td>
<td>9.8</td>
<td>(82.5, 125)</td>
</tr>
<tr>
<td>All Controls- End (n=13)</td>
<td>95.7</td>
<td>37.5</td>
<td>10.4</td>
<td>(73.0, 118.3)</td>
</tr>
<tr>
<td>Controls Batch 1- Baseline (n=6)</td>
<td>111.3</td>
<td>38.1</td>
<td>15.7</td>
<td>(71.3,151.3)</td>
</tr>
<tr>
<td>Controls Batch 1-End (n=6)</td>
<td>82.95</td>
<td>30.89</td>
<td>12.6</td>
<td>(50.5,115.4)</td>
</tr>
<tr>
<td>Controls Batch 2- Baseline (n=7)</td>
<td>97.3</td>
<td>34.1</td>
<td>12.9</td>
<td>(65.9,128.8)</td>
</tr>
<tr>
<td>Controls Batch 2-End (n=7)</td>
<td>106.6</td>
<td>41.5</td>
<td>15.7</td>
<td>(68.2,144.9)</td>
</tr>
</tbody>
</table>
Figure 6-3 Peptide YY levels after 18 hours fasting and again after fat burning exercise and a further 6 hours of fasting.

‘Baseline’ is the measurement of Peptide YY levels at 18 hours of fasting and prior to commencing the fat burning exercise. End levels are measurement of Peptide YY levels at 24 hours of fasting and 6 hours of fat burning exercise. Analysis includes all controls (Batch 1 and 2).
Figure 6-4 Peptide YY levels after 18 hours fasting and again after fat burning exercise and a further 6 hours of fasting.

‘Baseline’ is the measurement of Peptide YY levels at 18 hours of fasting and prior to commencing the fat burning exercise. End levels are measurement of Peptide YY levels at 24 hours of fasting and 6 hours of fat burning exercise.

a. AFLP women (n=13), Non-parous non-AFLP women (n=6) b. AFLP women (n=13), parous non-AFLP women (n=7)

6.5.4.2 Glucagon-Like Peptide-1 (pg/ml)

Following a 24 hour fast and fat burning exercise, Glucagon-Like Peptide-1 (GLP-1) levels unexpectedly rise in all groups. GLP-1 significantly increases in the AFLP women (34.7 to 62.9 pg/mL, 95% CI (-46.9, -9.5) p= 0.005) and non-AFLP women (31.2 to 56.3 pg/mL, 95% CI (-42.7, -7.5) p= 0.007), by the end of the study. However, when comparing the 2 groups to each other there is no significant difference between GLP-1 levels between the AFLP and non-AFLP women (95% CI (-16.9, 30.2), p=0.57) at the end of the study. See Table 6-4 and Figure 6-5. Differences in parity did not have any effect on the results. After 18 hours of fasting, but before exercise AFLP women mean plasma GLP-1 levels 34.7 +/- SEM 4.2 pg/ml compared to Non-parous non-AFLP women (mean 33.7 +/- SEM 4.7 pg/ml) did not show a statistically significant difference (p= 0.88 (95% CI (-13.55, 15.72)).
There was also no difference at the end of exercise between AFLP women (mean 62.9 +/- SEM 8.0 pg/ml) and Non-parous non-AFLP women (mean 65.0 +/- SEM 14.1 pg/ml) p=0.89 (95% CI (-34.13, 29.91)). There was no difference between AFLP women (mean 34.7 +/- SEM 4.2 pg/ml) and parous non-AFLP women (mean 29.1 +/- SEM 2.9 pg/ml), p= 0.37 (95% CI (-7.18, 18.5)) before exercise and there was no significant difference between AFLP women (mean 62.9 +/- SEM 8.0 pg/ml) and parous non-AFLP women (mean 48.3 +/- SEM 9.0 pg/ml) p=0.29 (95% CI (-12.83, 41.13)) after exercise. (See Figure 6-6).

Table 6-4 Glucagon-Like peptide-1 analysis in AFLP and non-AFLP women

‘Baseline’ is the measurement of Glucagon-Like peptide-1 levels at 18 hours of fasting and prior to commencing the fat burning exercise. End levels are measurement of Peptide YY levels at 24 hours of fasting and 6 hours of fat burning exercise.

<table>
<thead>
<tr>
<th></th>
<th>Mean (pg/ml)</th>
<th>SD</th>
<th>SEM</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AFLP-Baseline</strong></td>
<td>34.7</td>
<td>15.0</td>
<td>4.2</td>
<td>(25.7, 43.8)</td>
</tr>
<tr>
<td>(n=13)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>AFLP-End</strong></td>
<td>62.9</td>
<td>29.0</td>
<td>8.0</td>
<td>(45.4, 80.4)</td>
</tr>
<tr>
<td>(n=13)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>All Controls-Baseline</strong></td>
<td>31.2</td>
<td>9.4</td>
<td>2.6</td>
<td>(25.5, 36.9)</td>
</tr>
<tr>
<td>(n=13)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>All Controls-End</strong></td>
<td>56.3</td>
<td>29.3</td>
<td>8.1</td>
<td>(38.6, 73.9)</td>
</tr>
<tr>
<td>(n=13)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Controls Batch 1-</strong></td>
<td>33.7</td>
<td>11.4</td>
<td>4.7</td>
<td>(21.7, 45.6)</td>
</tr>
<tr>
<td><strong>Baseline</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Controls Batch 1-</strong></td>
<td>65.0</td>
<td>34.6</td>
<td>14.1</td>
<td>(28.7, 101.3)</td>
</tr>
<tr>
<td><strong>End</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>(n=6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Controls Batch 2-</strong></td>
<td>29.1</td>
<td>7.6</td>
<td>2.9</td>
<td>(22.0, 36.1)</td>
</tr>
<tr>
<td><strong>Baseline</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Controls Batch 2-</strong></td>
<td>48.3</td>
<td>23.9</td>
<td>9.0</td>
<td>(26.7, 70.9)</td>
</tr>
<tr>
<td><strong>End</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 6-5 Glucagon-Like peptide-1 levels after 18 hours fasting and again after fat burning exercise and a further 6 hours of fasting.

‘Baseline’ is the measurement of Glucagon-Like peptide-1 levels at 18 hours of fasting and prior to commencing the fat burning exercise. End levels are measurement of Peptide YY levels at 24 hours of fasting and 6 hours of fat burning exercise.
Figure 6-6 Glucagon-Like peptide-1 levels after 18 hours fasting and again after fat burning exercise and a further 6 hours of fasting.

a. AFLP women (n=13), Non-parous, non-AFLP women (n=6)  
b. AFLP women (n=13), parous, non-AFLP women (n=7)

6.5.4.3 Ferritin (ng/ml)

Ferritin is an intracellular protein that stores iron and it is the liver where most iron is deposited. Raised iron levels have also been identified in over a third of adults with non-alcoholic fatty liver disease (NAFLD) (260, 261). My proteomic analysis in batch 1 shows higher ferritin levels in AFLP women when compared to non-AFLP women. Iron metabolism, in particular the regulatory pathway involving Hemojuvelin, Ferritin and Hepciden may be key in understanding the pathogenesis of AFLP, which is why I felt it was important to re-measure ferritin protein levels using ELISA.

Baseline levels of ferritin were higher in AFLP women (mean 45.7 +/- SEM 3.9 ng/ml) when compared to non-parous non-AFLP women (mean 30 +/- SEM 5.8 ng/ml), (p=0.04, 95% CI (-0.89, 29.53)), confirming what was seen in the proteomic analysis.
However, there is no difference in ferritin levels between AFLP women and parous, non-AFLP women before and after fasting and exercise (Before exercise AFLP women (mean 45.7 +/- SEM 3.9 ng/ml), Parous Non-AFLP women (mean 52.5 +/- SEM 3.2 ng/ml), p=0.30, 95% CI (-19.18, 5.64), after exercise AFLP women (mean 47.0 +/- SEM 4.1 ng/ml), Parous Non-AFLP women (mean 53.2 +/- SEM 3.1 ng/ml), p=0.32, 95% CI (-18.74, 6.50)).

Ferritin levels were similar before and after exercise in the AFLP women (before exercise mean Ferritin level 45.7 +/- SEM 3.9 ng/ml, after exercise, mean ferritin level 47.0 +/- SEM 4.1 ng/ml), (p=0.81, 95% CI (-13.0, 10.3)). When combining all of the non-AFLP women (batch 1 and 2), there was no difference before or after exercise (before exercise mean 42.1 +/- SEM 4.4 ng/ml, after mean 43.7 +/- SEM 4.2 ng/ml), (p=0.79, 95% CI (-14.3, 11.1)) before or after the fat burning exercise study.

There was no difference in plasma ferritin levels between the AFLP and non-AFLP women (batch 1 and 2) before exercise (45.7 +/- SEM 3.9 ng/ml v 42.1 +/- SEM 4.4 ng/ml, p=0.55, 95% CI (-8.67, 15.85) and after exercise 47.0 +/- SEM 4.1 ng/ml v 43.7 +/- SEM 4.2 ng/ml, p=0.58, 95% CI (-8.80, 15.42)). See Table 6-5 and Figure 6-7.

However, before exercise non-Parous, non-AFLP women have significantly less ferritin than parous women (30.0 +/- SEM 5.8 ng/ml v 52.5 +/- SEM 3.2 ng/ml p=0.005, 95% CI (8.36, 36.54)). This may be explained by the fact that parous women were on average 12 years older than the non-parous group. (See Figure 6-8).
Table 6-5 Ferritin analysis in AFLP and non-AFLP women.

‘Baseline’ is the measurement of Ferritin levels at 18 hours of fasting and prior to commencing the fat burning exercise. End levels are measurement of Peptide YY levels at 24 hours of fasting and 6 hours of fat burning exercise.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SD</th>
<th>SEM</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AFLP- Baseline (n=13)</strong></td>
<td>45.7</td>
<td>14.2</td>
<td>3.9</td>
<td>(37.1, 54.3)</td>
</tr>
<tr>
<td><strong>AFLP- End (n=13)</strong></td>
<td>47.0</td>
<td>14.6</td>
<td>4.1</td>
<td>(38.2, 55.9)</td>
</tr>
<tr>
<td><strong>All Controls- Baseline (n=13)</strong></td>
<td>42.1</td>
<td>16.0</td>
<td>4.4</td>
<td>(32.4, 51.8)</td>
</tr>
<tr>
<td><strong>All Controls- End (n=13)</strong></td>
<td>43.7</td>
<td>15.3</td>
<td>4.2</td>
<td>(34.5, 53.0)</td>
</tr>
<tr>
<td><strong>Controls Batch 1- Baseline (n=6)</strong></td>
<td>30.0</td>
<td>14.3</td>
<td>5.8</td>
<td>(15.0, 45.0)</td>
</tr>
<tr>
<td><strong>Controls Batch 1-End (n=6)</strong></td>
<td>32.7</td>
<td>14.6</td>
<td>6.0</td>
<td>(17.4, 48.1)</td>
</tr>
<tr>
<td><strong>Controls Batch 2- Baseline (n=7)</strong></td>
<td>52.5</td>
<td>8.5</td>
<td>3.2</td>
<td>(44.6, 60.4)</td>
</tr>
<tr>
<td><strong>Controls Batch 2-End (n=7)</strong></td>
<td>53.2</td>
<td>8.1</td>
<td>3.1</td>
<td>(45.7, 60.7)</td>
</tr>
</tbody>
</table>
Figure 6-7 Ferritin levels before and after 18 hours fasting and again after fat burning exercise and a further 6 hours of fasting.

![Graph showing ferritin levels before and after fasting and exercise](image)

Figure 6-8 Ferritin levels before and after 18 hours fasting and again after fat burning exercise and a further 6 hours of fasting.

a. AFLP women (n=13), Non-parous, non-AFLP women (n=6)  b. AFLP women (n=13), parous, non-AFLP women (n=7).
6.6 Discussion

6.6.1 Proteomic and ELISA results

During this study, I was particularly interested to discover whether women who had AFLP had novel differences in serum proteins following a 24 hour fast and fat burning exercise (maximal metabolic stress) compared with women who had not had AFLP. My initial proteomics comparison (batch 1) showed significant differences in members of the iron pathway. After 24 hours of fasting and fat burning exercise, women with a history of AFLP had reduced hemojuvelin \( (P=x10^{-6}) \) and erythropoietin levels \( (P=x10^{-4}) \), elevated ferritin \( (P=0.001) \) and hepcidin levels \( (p=0.001) \). This pattern of iron disturbance has been seen in men and women with non-alcoholic fatty liver disease (260, 262, 263).

As the non-AFLP women (aged 26 +/- 1.74 years) in batch 1 were significantly younger and nulliparous compared with women who had AFLP (aged 38 years +/- 2.35 years), I recruited 7 further healthy women who were older and parous to be compared against 7 other women who had AFLP. The second proteomic analysis did not confirm the original differences in the iron metabolism found in batch 1, suggesting that the original differences in proteins associated with iron metabolism were indeed related to differences in age and parity of controls. However, this can only be concluded if proteomic analysis is repeated with all the non-AFLP women analyzed at the same time.

i. Iron metabolism

The liver is the predominant site for iron metabolism. Iron homeostasis is controlled by several proteins including ferritin, transferrin, hemojuvelin and hepcidin. Ferritin and transferrin are the main transporters of iron in the blood, whereas iron regulatory peptide proteins such as hepcidin determine iron regulation within the body.
Initially, dietary iron is reduced from its ferric form to its ferrous form in the small intestine. Ferrous iron is then transported by divalent metal transporter (DMT1) in enterocytes. Iron can now either be stored as ferritin in enterocytes or be transferred to plasma by the transport protein ferroportin 1 (264, 265). Hepcidin, peptide hormone is the primary iron regulator within the body and is derived by liver hepatocytes (266). Other cells express lower levels of hepciden and include syncytiotrophoblast cells of the placenta (267). Increased hepcidin expression leads to a decrease in iron absorption and preventing its release from cells, resulting in a reduction of circulating iron. The mechanism of action of hepcidin is not fully understood. One likely explanation is that hepcidin binds to ferroportin forming a complex through which hepcidin is internalized and degraded by proteasomes in the lysosomes and iron is maintained with the cell; in particular hepatocytes, enterocytes and macrophages (268). This inhibits the transport of iron and results in lower serum iron levels due to hepatocyte iron trapping and decreased iron absorption from the gut. Increased expression of hepcidin leads to chronic anemia and low hepcidin levels is seen in hereditary haemachromatosis (and subsequent iron accumulation in body organs).

Hemojuvelin is a soluble, membrane bound protein encoded by the Haemochromatosis Type 2 (HFE2) gene (269). It is a key regulator of hepciden secretion. Recent investigations into signal transduction pathways, regulating iron homeostasis have shown that hemojuvelin interacts with another protein call bone morphogenetic protein (BMP e.g. BMP6) which signals via the SMAD pathway and regulates hepcidin expression (270).

Liver hepatocytes have the ability to detect the amount of iron circulating and as a result regulate hepcidin production. In cases of iron deficiency, hemojuvelin is cleaved to produce a soluble extracellular fragment (sHJV). Soluble hemojuvelin competes with membrane-bound hemojuvelin for BMP6; therefore, it attempts to inhibit hepcidin expression (271). However, raised ferritin levels inhibit hemojuvelin cleavage resulting in increased hepciden production (272, 273).
Increased iron deposits have been found in people with chronic liver diseases such as non-alcoholic fatty liver disease (NAFLD). Raised hepcidin levels have also been found in patients with NAFLD (263, 274). One study showed lower serum hemojuvelin and hepciden levels in NAFLD patients. However, in those patients with iron overload hemojuvelin was lower and hepciden levels were raised when compared to patients without iron overload (261).

These findings were replicated in my proteomic analysis of batch 1 where AFLP women had significantly lower hemojuvelin levels and higher hepciden levels compared with non-AFLP women. The findings in the study suggested that the gradual change in hemojuvelin and hepciden levels were a result of the physiological response of iron accumulation in the liver.

ELISA analysis showed no difference in ferritin levels between the AFLP and non-AFLP women, however parity did have a significant effect on the ferritin levels. Non-Parous women have significantly less ferritin than parous women. The most likely explanation for these findings is that the non-AFLP women in batch 1 were significantly younger and nulliparous and not appropriately matched to the women who had previously suffered with AFLP. Therefore, the higher ferritin levels may be a result of inflammatory processes secondary to age related changes and parity. Although, there have been no studies to date investigating iron levels with increasing age. These discrepancies in phenotype would possibly explain these results, however to confirm this I would need to repeat the analysis with all samples analysed in 1 batch or increase the sample size to include more AFLP and non-AFLP women.

ii. Leptin Receptor

My proteomic analysis showed significantly lower levels of the Leptin receptor ($P=8.6\times10^{-6}$) after fasting and exercise in women with AFLP compared with non-AFLP women.
Leptin (adipocytokine) is the product of the \textit{ob} gene and is mainly but not exclusively produced by adipocytes in white adipose tissue and acts on its receptor in the midbrain to inhibit hunger and stimulate satiety (278). Studies have shown mice that are homozygous for the \textit{ob} gene mutation have a defect in leptin expression and as a result do not sense hunger and become obese (278). When injected with recombinant leptin these mice lose weight (279). Despite these findings in mice, obese people are thought to be insensitive to leptin (280).

In pregnancy leptin levels rise significantly during pregnancy. By 12 weeks’ gestation leptin levels are 30\% higher, however they return back to pre-pregnancy levels immediately after delivery (281).

The Leptin receptor (\textit{OB-R}) is part of the class I cytokine receptor family and so far 4 different isoforms have been identified in humans (\textit{OB-Rf1}, \textit{OB-R219.1}, \textit{OB-R219.2} and \textit{OB-R219.3}) (282). The action of leptin is facilitated through the leptin receptor and it is the soluble leptin receptor that is the main binding protein for leptin in humans (283).

Studies have shown an inverse relationship between the soluble leptin receptor and total serum leptin levels and BMI (284). In obese patients, leptin receptor levels are low and serum leptin is found predominantly in the free form (285). One study showed an increase in leptin receptor levels following weight loss after gastric surgery (286).

Leptin is known to increase fatty acid oxidation through initial activation of AMP-activated protein kinase (AMPK). The activated AMPK induces phosphorylation of acetyl coenzyme A carboxylase (ACC) which leads to a reduction in the enzyme malonyl-CoA (Figure 6-9). Low levels of malonyl CoA activate CPT1 which is an essential step in the oxidation of fatty acids within mitochondria (287, 288).
**Figure 6-9 Leptin and fatty acid oxidation.**

AMPK is activated by Leptin and the activated AMPK induces phosphorylation of ACC leading to a reduction in Malonyl CoA production. Low levels of malonyl CoA activates CPT1 and subsequently increases fatty acid oxidation within mitochondrion.

AMPK (Activated protein kinase), ACC (Acetyl coenzyme A carboxylase), CPT1 (Carnitine palmitoyltransferase 1), FAO (Fatty acid oxidation)

Low levels of soluble leptin receptor would reduce activation of this pathway, with a consequent reduction in fatty acid oxidation. A defect in the leptin pathway is therefore consistent with my hypothesis that AFLP is a disorder of FAO.
This finding will need further investigation as it was not identified in the proteomic analysis of batch 2 and may represent differences in participant phenotype.

Leptin also has an important role in inducing the expression of genes involved in FAO, in particular peroxisome proliferator activated receptor (PPAR) (288). Following activation of AMPK, PPAR gene expression is increased in muscle cells. However, the precise molecular mechanisms as to how leptin activates FAO through AMPK and PPAR gene expression is yet to be fully determined. Nevertheless, this pathway could be important in the pathogenesis of AFLP.

iii. Glypican-2

Glypican-2 (GPC2) also known as cerebroglycan, is a member of the Glypican family of heparan sulfate proteoglycans. It is also known to be associated with motile behaviors of developing neurons (299).

The proteomic analysis in batch 2 shows significantly greater levels of GPC2 in AFLP cases compared with controls \((P=2.72\times10^{-7})\). This is an unusual observation. GPC2 appears to be restricted to the nervous system. Unless it has interfered with neurodevelopment in the hypothalamus that controls eating, it is hard to understand why it should be elevated after fasting in women with a history of AFLP. Another Glypican GPC4, is associated with insulin resistance and fatty liver (300). Studies have shown GPC4 levels correlate with insulin sensitivity in humans. It has been hypothesized that GPC4 is a adipokine that interacts directly with insulin receptors to regulate their activation. The same group went on to identify greater levels of GPC4 in men compared to women and documented an association with cardiovascular risk factors and NAFLD (300). Furthermore, Glypican-3 is expressed by hepatocellular carcinomas, but not in other liver diseases (301). It is possible that the proteomic analysis confused these similar proteins.
iv. Galectin-3

The proteomic results in batch 2 show greater levels of Galectin-3 in AFLP women compared with non-AFLP women.

Galectin-3, a member of the lectin family is encoded by the LGALS3 gene (302). Galectin-3 has been associated with a variety of biological processes including cell adhesion and activation, cell growth and differentiation and apoptosis (302). Clinically, Galectin-3 has been linked to cancer, heart disease, fibrosis and inflammation (303, 304).

Studies have shown Galectin-3 knockout mice to develop severe fatty change, although its relevance in NAFLD in humans remains unclear (305).

My results identified raised levels of Galectin-3 in women with AFLP, which does not explain why these women develop AFLP from what is known about Galectin-3. More recently there is emerging evidence that Galectin-3 plays an important role in the pathogenesis of NAFLD. In an initial animal study, Galectin 3 was ablated which resulted in NAFLD (305).

However, in humans Galectin-3 was found to be present in liver disease, namely cirrhosis (306, 307). A recent study investigating the association of Galectin-3 in biopsy confirmed NAFLD patients did not show a significant difference in levels of Galectin-3 in NAFLD patients and controls (308). The findings of several studies are conflicting, therefore further investigation is warranted.
6.7 Conclusion

In order to discover the pathogenesis of AFLP, I investigated whether women previously affected by AFLP have a different proteome after a 24-hour fast and fat burning exercise compared with healthy women.

My results showed clear differences in the proteins (Haemajuvelin, Peptide YY, Alpha-2-macroglobulin, Ferritin and Hepciden) identified in batch 1 between AFLP and non-AFLP women, yet these proteins are not identified in batch 2. One key explanation could be due to differences in age and parity. The control group in batch 1 were non-parous and younger than women who had AFLP, whereas the control group in batch 2 were much better matched (parous and age-matched) to AFLP women. The proteins associated with iron metabolism and regulation (hemojuvelin, hepcidin and ferritin) are associated with NAFLD, but could be associated with age. Furthermore, the ELISA studies did corroborate the proteomic results, however when both parous and non-parous groups were combined the only differences identified was a raised ferritin levels in parous non-AFLP women compared to non-parous women.

Galectin 3 and Glypican 2 levels were raised in AFLP women compared to non-AFLP women. These controls were more appropriately age matched and were parous women and therefore it would be prudent to investigate these differences further.

Having completed this study it was clear that my study was not sufficiently powered and therefore my results are not overall significant. It is often easy to over interpret the data to find an association with AFLP, however it is important to avoid this in order to prevent inaccurate conclusions.

To fully assess the proteomic results and ELISA findings I would have liked to re-run the proteomics analysis with both batches together and include more women who have had AFLP and matched non-AFLP controls. I would also have liked to measure hemojuvelin, glypican 2 and galectin 3 using the ELISA technique, as these proteins were shown to be significantly different in the better matched controls.
6.8 Study Limitations

i The proteomic study was performed in 2 batches. Ideally this study should have been performed in one batch to avoid any variability.

ii The batch 1 control group was not phenotypically matched to the AFLP cases. Batch 1 controls were younger and non-parous. This could have created differences in the proteins identified.

iii The new aptamer-based proteomic technology employed in this study allows for rapid and multiple measures of plasma proteins compared with ELISA. Therefore, to assess reproducibility the analysis should have been repeated with appropriately matched parous controls.

Summary of findings:

i The results of the proteomics analysis were validated by two ELISA studies (Peptide YY and Ferritin).

ii Following a 24 hour fast and 6 hours of fat-burning exercise, plasma proteomic analysis showed that women who had AFLP (n=6) had higher ferritin and hepcidin levels and lower plasma hemojuvelin levels compared with 6 younger, non-parous women. A subsequent comparison of plasma proteomics between 7 other women who had AFLP and 7 age-matched parous women showed no such differences. Initial differences in iron metabolism may be explained by age-related changes in iron metabolism, but do fit with a propensity to non-alcoholic steatosis in the women who had AFLP.

iii Following a 24 hour fast and 6 hours of fat-burning exercise, women who had AFLP had lower plasma peptide YY levels compared with non-parous younger women. A further comparison with older parous women showed no such difference.
In batch 2, with better matched controls, Glypican-2 and Galectin-3 levels were higher in women who had previous AFLP compared to non-AFLP women. The significance of these differences needs to be checked with an ELISA assay of these 2 proteins.

Non-parous, non-AFLP women (n=6) showed the expected fall in peptide YY levels following a prolonged fast, however the decrease was not significant. There was no overall difference in Peptide YY levels between AFLP and non-AFLP women, before or at the end of the study.

Glucagon-Like Peptide-1 levels significantly increase in the AFLP and non-AFLP women by the end of the study. However, there is no significant difference between GLP-1 levels between the 2 groups.

Non-Parous, non-AFLP women have significantly less Ferritin than parous, non-AFLP women. Taken together, there was no difference in Ferritin levels between the AFLP and non-AFLP women before and after the study.

6.9 Summary

I hypothesised that women previously affected by AFLP have a different proteome before and after 24 hours of fasting and 6 hours of fat burning exercise compared with healthy, non-AFLP women. My findings did show differences in protein levels between women with AFLP and non-AFLP women.

I initially discovered that women who had AFLP had differences in iron metabolism proteins (Haemajuvelin, hepcidin and ferritin) associated with underlying fatty liver, but when I compared women who had AFLP with those of a similar age and parity, I could not detect these differences. It is possible that initial differences in iron metabolites were secondary to differences in age and parity, but could reflect a propensity for women who have AFLP to non-alcoholic steatosis.

Comparison of women who had AFLP with those of similar age and parity detected elevated levels of Glypican 2 and Galectin 3.
This observation deserves further investigation to determine the importance of other members of the Glypican family known to be associated with liver disease. The role of low plasma levels of Galectin-3 in the pathogenesis of fatty liver outside of pregnancy appear to be contrary to my observation of high Galectin-3 levels in women who had AFLP.
CHAPTER 7
NEXT GENERATION SEQUENCING OF FAMILIES WITH AFLP
Chapter 7  Next Generation Sequencing of AFLP

7.1  Introduction

7.1.1  Gene variants known to be associated with Acute Fatty Liver of Pregnancy

From my phenotype study (Chapter 4), it is clear that the clinical and biochemical presentation of AFLP has much in common with inherited disorders of fatty acid oxidation (FAO). Defects in FAO result in energy failure and multi-organ dysfunction as seen in AFLP (32, 309, 310).

Further support for AFLP being a disorder of FAO comes from the discovery that a small number of women with AFLP have a deficiency in enzymes required for normal fatty acid oxidation, specifically long chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) and carnitine palmitoyltransferase 1 (CPT1) (4, 309-312). Women who are obligate heterozygotes for an autosomal recessive mutation (G1528C) of the LCHAD gene and who have a fetus homozygous for the same gene variant have developed AFLP.

7.1.2  Next generation DNA sequencing

The human genome is composed of approximately three billion base pairs which consists of both coding and non-coding sequences. Approximately 1-2% (30 million base pairs) of the genome are protein coding sequences, commonly referred to as the exome (184, 313). It is within the protein coding region of the gene where 85% of Mendelian disorders are found (185).

It was only in the 1970’s when Sanger published findings on the chain termination method for DNA sequencing, which now bears his name (314). Since then advances in next generation sequencing (NGS) platforms mean that DNA sequencing can be performed faster, with less manpower and more cheaply. NGS platforms are a high throughput DNA sequencing technology that can sequence millions of fragments of DNA from a single sample in parallel.
Hence it is often referred to as “massively parallel sequencing”. NGS primarily includes whole genome sequencing (WGS) and whole exome sequencing (WES).

NGS platforms have principally taken over from Sanger sequencing as the first choice of DNA sequencing for research. Over the next few years it is probable that NGS will become a part of routine clinical practice (315, 316).

Fatty acid oxidation (FAO) disorders present with hypoketotic hypoglycaemia and can lead to hepatic failure, cardiomyopathy, skeletal myopathy, encephalopathy, coma and death (29). An example of how WES was used to successfully identify a disorder of FAO involves the identification of ACAD9 deficiency in a patient who initially presented with an atypical presentation for this disorder. ACAD9 is part of the acyl-CoA dehydrogenase family and is an enzyme involved in the first step of the electron transport system and oxidative phosphorylation. ACAD9 deficiency was not considered in the patient until a gene defect was discovered through WES (317-319).

Another recent study performed WES of 85 individuals who presented to a metabolic center with predominantly neurological disabilities. A diagnosis had not been reached with conventional investigations. WES provided a diagnosis in over 50% of patients, of which 16% of patients had an inborn error of metabolism (320). Inborn errors of metabolism (IEM) are rare genetic disorders that are heterogeneous, where individuals present with non-specific clinical phenotypes, making them extremely difficult conditions to diagnose, like AFLP. WES has significantly improved the ability to rapidly diagnose IEMs (321).

Introducing NGS into regular clinical practice to assist clinicians with diagnosis of unknown genetic conditions is an exciting prospect. Initially it might be sensible to focus on specific human genes in which knowledge of pathways is already known enabling faster diagnosis. However, there are some challenges that need to be addressed including ethics surrounding the genetic data produced (for example should we inform participants of all gene variants that have implications in the future?), as well as creating simple, straightforward and user-friendly software for prompt data analysis.
7.1.3 Whole Exome Vs Whole Genome sequencing

Scientists and clinicians often deliberate over whether to use WGS or WES as their primary choice for sequencing. WGS attempts to sequence the entire genome. Due to the challenges in sequencing certain parts of the genome, it is only 95-98% of the genome that is sequenced, which is a vast amount of data. WES only sequences the protein coding regions of the genome. WES has many advantages over WGS, the costs are significantly less and it is faster to perform. The data produced from WES would require approximately 5-6Gb of storage compared to 90Gb with WGS, also leading to faster analysis of the data. If a gene variant is identified in the protein coding region where the majority of Mendelian disorders identified to date occur, interpretation of the variant data is more straightforward as we can functionally characterize coding variants more precisely than non-coding variants (184, 185).

WGS does not require an enrichment stage (unlike WES), which can introduce technical artefacts such as poor coverage of regions with a high G/C content. Both WES and WGS read lengths are the same and are dependent on the sequencer used, typically 100-150bp. WGS offers the advantage of uniform coverage across the whole genome which is preferential in copy number variant (CNV) analysis as it allows the breakpoints of CNVs to be mapped accurately in comparison WES only offers a punctate coverage and therefore rarely covers the actual breakpoints. Also, due to the variable efficiency of the target enrichment stage of WES the read-depth data shows far greater variability than seen in WGS (184, 316).

Research into AFLP is currently limited as it only occurs in 1:15,000 pregnancies and as soon as it is diagnosed, the baby must be delivered. From current evidence, AFLP is a heterogeneous disorder which is difficult to diagnose. With new genetic techniques such as WES and WGS relatively small numbers of families are needed to identify novel gene variants. This makes it feasible to identify potential causes of the condition with relatively few participants.
Identifying a novel gene variant that is associated with AFLP, could be used as a diagnostic test for AFLP and predict future health problems for the mother. It may also identify a metabolic disorder associated with AFLP, which would allow early targeted treatment for the mother and her offspring, improving both their quality of life.

In this chapter, I investigated the genotype of 7 families affected by AFLP using NGS. I attempted to identify novel gene variants as well as an inheritance pattern that might explain the development of AFLP. As explained in chapter 3, the DNA from 3 families were sent for WES and the DNA from 4 families were sent for WGS. The exome region was analyzed in all families despite having WGS data. Gosgene had changed its protocol and only sent samples for WGS which is why 4 families underwent WGS and not WES. The sequencing protocols differed slightly, however the analysis remained the same for all families, where only the protein coding region (exome) of the genome was analyzed.

7.2 Hypothesis

i. Mothers who have suffered from AFLP have a novel gene variant associated with energy metabolism, particularly fatty acid oxidation which will predispose them to energy failure during periods of fasting.

7.3 Specific aim

i. To discover whether families affected by AFLP (mother, father, affected and unaffected offspring) have a gene variant identified by NGS, related to abnormal energy metabolism.
7.4 Methodology

7.4.1 Study Design

I recruited 7 well phenotyped families that had been affected by AFLP. Study 1 comprised of 3 families where DNA was extracted and sent for WES and study 2 involved performing WGS of 4 families affected by AFLP. Both WES and WGS required 1.0µg of DNA extracted from whole blood. The process of DNA extraction is described in the methodology section 3.8.

For WES, DNA samples were prepared with an enrichment phase and samples were run on an illumina platform HiSeq2500 analyser. DNA for WGS sequencing was prepared in the same way as DNA for WES, however an enrichment phase was not required and samples were run on an Illumina HiSeq X Ten sequencer. Sequenced reads of data were aligned against the human reference genome as described in section 3.8.2.3. Filter pipelines were used to analyse the data using the Ingenuity Variant Analysis Software from Ingenuity Systems (See section 3.8.2.4).

7.5 Results

7.5.1 Baseline characteristics of the families affected by AFLP

7.5.1.1 Study 1

Three families were initially identified for WES. All mothers from each of the 3 families met the clinical criteria for a diagnosis of AFLP. DNA was collected 7 years after the mother had AFLP in family 1, after 4 years in family 2 and 8 years in family 3 (See Table 7-1). The offspring in family 2 were twin boys that were both affected by AFLP.
Chapter 7 Next Generation Sequencing of Families with AFLP

7.5.1.2 Study 2

DNA was extracted for WGS from 4 families affected by AFLP. Family 4 were a trio where there only child had been affected by AFLP. The offspring of family 5 and 7 were twin girls. Family 6 had an affected offspring and unaffected sibling (See Table 7-2).

Table 7-1 Families taking part in WES

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<th>Relation</th>
<th>Age (years)</th>
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<th>Birth Weight (g)</th>
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## Table 7-2 Families taking part in WGS

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<td>AFLP019</td>
<td>Unaffected sibling</td>
<td>4 months</td>
<td>Female</td>
<td>3430</td>
<td>75&lt;sup&gt;th&lt;/sup&gt;</td>
</tr>
<tr>
<td>7</td>
<td>White British</td>
<td>AFLP020</td>
<td>Mother</td>
<td>37</td>
<td>Female</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AFLP021</td>
<td>Father</td>
<td>34</td>
<td>Male</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AFLP022</td>
<td>Affected Child</td>
<td>2</td>
<td>Female</td>
<td>1900</td>
<td>5&lt;sup&gt;th&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AFLP023</td>
<td>Affected Child</td>
<td>2</td>
<td>Female</td>
<td>2240</td>
<td>10&lt;sup&gt;th&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

All affected offspring in both studies were well grown for their gestational age at the time of delivery. Currently, they are well without any ongoing co-morbidities.
Chapter 7 Next Generation Sequencing of Families with AFLP

7.5.2 Quality Control (QC) of the DNA samples

All DNA samples were prepared and passed the QC checks. The concentration of DNA required was also achieved. See Table 7-3.

Table 7-3 DNA QC and Concentration

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Volume (µl)</th>
<th>Concentration (ng/µl)</th>
<th>Total ng</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALM</td>
<td>33</td>
<td>120</td>
<td>3960</td>
</tr>
<tr>
<td>ALD</td>
<td>33</td>
<td>75</td>
<td>2482</td>
</tr>
<tr>
<td>ALG</td>
<td>33</td>
<td>109</td>
<td>3597</td>
</tr>
<tr>
<td>ALB</td>
<td>33</td>
<td>104</td>
<td>3432</td>
</tr>
<tr>
<td>AFLP001</td>
<td>150</td>
<td>19</td>
<td>2850</td>
</tr>
<tr>
<td>AFLP002</td>
<td>95</td>
<td>40</td>
<td>3829</td>
</tr>
<tr>
<td>AFLP003</td>
<td>150</td>
<td>26</td>
<td>3900</td>
</tr>
<tr>
<td>AFLP004</td>
<td>160</td>
<td>23</td>
<td>3680</td>
</tr>
<tr>
<td>AFLP005</td>
<td>120</td>
<td>20</td>
<td>2436</td>
</tr>
<tr>
<td>AFLP006</td>
<td>120</td>
<td>20</td>
<td>2352</td>
</tr>
<tr>
<td>AFLP007</td>
<td>120</td>
<td>15</td>
<td>1752</td>
</tr>
<tr>
<td>AFLP008</td>
<td>160</td>
<td>25</td>
<td>3936</td>
</tr>
<tr>
<td>AFLP009</td>
<td>200</td>
<td>15</td>
<td>3020</td>
</tr>
<tr>
<td>AFLP010</td>
<td>200</td>
<td>19</td>
<td>3820</td>
</tr>
<tr>
<td>AFLP011</td>
<td>200</td>
<td>22</td>
<td>4420</td>
</tr>
<tr>
<td>AFLP012</td>
<td>200</td>
<td>24</td>
<td>4720</td>
</tr>
<tr>
<td>AFLP013</td>
<td>200</td>
<td>16</td>
<td>3120</td>
</tr>
<tr>
<td>AFLP014</td>
<td>200</td>
<td>62</td>
<td>12460</td>
</tr>
</tbody>
</table>
7.6 Gene Variant Analysis

7.6.1 Ingenuity variant analysis using biological filters

Sequenced data for each family member was uploaded to Ingenuity variant analysis software in the form of ‘vcf’ files. Biological filters were applied to identify variants that could have a significant biological or clinical effect and as a result be associated with the development of AFLP. Candidate gene lists were compiled with genes already known to cause FAO disorders. This was obtained from published literature and genetic sites such as “OMIM” which were used to help with identification.
7.6.1.1 Study 1: Analysis 1; Affected child (case) and mother, father and sibling (s) (control).

AFLP has been associated with fetal fatty acid oxidation disorders namely LCHAD deficiency. The Mendelian inheritance for FAO disorders is an autosomal recessive pattern. Previous studies have shown maternal and paternal heterozygosity for LCHAD deficiency and fetal homozygosity for the gene mutation resulting in maternal AFLP (4). Therefore, I decided to use this model of inheritance to first analyse each family using ingenuity.

For analysis 1 the affected child was set as the case and the mother, father and unaffected sibling if applicable were set as the controls.

On upload of all files there were initially many thousands of variants identified in each family before biological filters were applied. See Table 7-4. The data filter pipelines were then employed. The first filter; confidence, established a call quality log score of at least 20 and a read depth of at least 10 in both cases and controls. This ensured that the variants were confidently called in the sample and were not a result of sequencing errors.

Next, the common variants filter excluded variants with an allele frequency of >0.5% in the 1000 genomes project, >0.5% of all in the ExAC (The Exome Aggregation Consortium) and >0.5% of all NHLBI ESP (exome sequencing project) exomes.

The predicted deleterious filter allowed variants to be identified if they were associated with loss of function of a gene, such as a frameshift and nonsense change.
Table 7-4 Number of Gene variants identified following application of confidence and predicted deleterious biological filters.

<table>
<thead>
<tr>
<th>Family</th>
<th>Confidence</th>
<th>Predicted deleterious</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>171059</td>
<td>973</td>
</tr>
<tr>
<td>2</td>
<td>181817</td>
<td>2346</td>
</tr>
<tr>
<td>3</td>
<td>180227</td>
<td>2328</td>
</tr>
</tbody>
</table>

The genetic filter allowed me to apply an inheritance model to the cases and control. In analysis 1, I only wanted variants that were homozygous in the case and heterozygous in the controls to be called. A final biological filter was applied to the variants that remained.

Here I used 3 biological terms; Acute fatty liver of pregnancy, fatty acid oxidation defect and ketogenesis (pathway) as a variant filter. I also added candidate gene lists associated with energy metabolism to this filter as described in the methods section 3.8.2.4. This filter also provided the ability to generate a list of genes that were ‘1 hop’ away or towards the genes associated with the 3 biological terms used. If this feature was not employed the list of genes identified were directly associated with the biological terms entered.

My results for Family 1, 2 and 3 showed 3, 1 and 2 variants respectively once the filters had been applied (See Figure 7-1).
Figure 7-1 Analysis 1: gene variants identified in each family

AFLP Family 1: Gene variants

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Position</th>
<th>Sample Allele</th>
<th>Gene Region</th>
<th>Gene Symbol</th>
<th>Protein Variant</th>
<th>Variant Findings</th>
<th>Case Samples</th>
<th>Control Samples</th>
<th>Translation Impact</th>
<th>CADDS S.</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>11244067</td>
<td>TT</td>
<td>Exonic, Intronic</td>
<td>PRH1, PRH1-TASSR14, p.S2549*27</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td>frameshift</td>
<td>10.500</td>
</tr>
<tr>
<td>12</td>
<td>11244091</td>
<td>T</td>
<td>Exonic, Intronic</td>
<td>PRH1, PRH1-TASSR14, p.M248V</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td>missense</td>
<td>&lt; 10</td>
</tr>
</tbody>
</table>

There were no gene variants identified when applying the biological filter. Therefore, the genes analysed were the ones called following application of the genetic filter. The 3 gene variants identified are shown above.

Proline rich protein HaellII subfamily 1 (PRH1), is a gene encoding proline-rich salivary glycoproteins that are involved in the production of saliva. When analysing the BAM there was a deletion in the affected child, however this appeared to be an artefact.

AFLP Family 2: Gene variants

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Position</th>
<th>Sample Allele</th>
<th>Gene Region</th>
<th>Gene Symbol</th>
<th>Protein Variant</th>
<th>Variant Findings</th>
<th>Case Samples</th>
<th>Control Samples</th>
<th>Translation Impact</th>
<th>CADDS S.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>48707808</td>
<td>AGGOCAGCAGA</td>
<td>Exonic</td>
<td>TMEM247</td>
<td>p.R129_E141delp</td>
<td></td>
<td></td>
<td></td>
<td>in-frame</td>
<td>13.370</td>
</tr>
</tbody>
</table>

Like Family 1, there were no gene variants identified when applying the biological filter in family 2. Therefore, the genes analyzed were the ones called following application of the genetic filter.

Transmembrane protein (TMEM247) is a member of the transmembrane protein family and is found in eukaryotes. TMEM247 is found in the placenta and testis, but its function is unknown (322).
AFLP Family 3: Gene variants

There were 2 gene variants called when applying the genetic filter. When the biological filter was applied one gene variant (ARMCX5) was called. Armadillo repeat containing, x-linked 5 (ARMCX5) has been associated with primary aldosterism and adrenal hyperplasia (323, 324). However, its function is not fully understood.

Figure 7-2 BAM file for analysis of Family 3: ARM CX5

This BAM file clearly shows, the homozygous variant in the affected child, a heterozygous variant in the mother, wildtype variant in the father and a heterozygous variant in the unaffected sibling.

The variants listed in Figure 7-1 were analyzed in greater detail using BAM files (binary format of stored sequenced data) as shown, where I viewed the actual coverage of the gene variant in each family member. An example is shown in the analysis above for family 3 (Figure 7-2). Dr Hywel Williams supervised the analysis of the data. Using BAM files and a combined annotation dependent depletion (CADD) score of less than 15, the variants identified were not predicted to be deleterious and not associated with energy metabolism, fatty liver or AFLP.
7.6.1.2 Study 1: Analysis 2; Mother (case), father and children (controls)

An identical filtering cascade for analysis was performed as described in analysis 1, however I decided to set the analysis with the mother as the case and the father and children as the controls. I set the genetic filter for mothers to be homozygous for any variants and the controls could be either homozygous, heterozygous or reference. The other filters were applied in the same format as above. See Figure 7-3.

This mode of inheritance has not been associated with AFLP, however I did not find any pathological variants when the affected child was selected as the homozygous case. In fact, it is plausible that the mother is the case as she is the one who develops AFLP.

Error! Reference source not found.

Figure 7-3 Analysis 2: gene variants identified in each family

<table>
<thead>
<tr>
<th>AFLP Family 1: Gene variants</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chromosome</strong></td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td>11</td>
</tr>
<tr>
<td>17</td>
</tr>
<tr>
<td>20</td>
</tr>
</tbody>
</table>

In family 1, there were 12 gene variants called when all filters were applied. The biological filter included variants 1 hop upstream and downstream of the genes included in the biological filter terms (AFLP, FAO and Ketogenesis). The BAM files are shown in Appendix 4.
AFLP Family 2: Gene variants

In family 2, there were 9 gene variants called when all filters were applied. The biological filter included variants 1 hop upstream and downstream of the genes included in the biological filter terms.

AFLP Family 3: Gene variants

In family 3, 9 gene variants were called when all filters were applied. The biological filter included variants 1 hop upstream and downstream of the genes included in the biological filter terms.

There was no evidence in the literature or analysis to suggest any of the gene variants were disease causing or associated with a perturbation in energy metabolism.

7.6.1.3 Study 1: Analysis 3; Mother (case), father and children (Not included)

In analysis 2 the mother is assumed to have inherited the causative variants in an autosomal recessive homozygous model from her parents. It is possible that the mother has a loss of function variant associated with fat metabolism irrespective of the father and children.
Therefore, I applied a similar filtering cascade as for my previous analysis, however I set the genetic filter to look for gene variants in the mother only (where the father and children were excluded). The other filters were applied in the same format as above (See Figure 7-4).

**Figure 7-4 Analysis 3: gene variants identified in each family**

**AFLP Family 1: Gene variants**

<table>
<thead>
<tr>
<th>Chromosome Position</th>
<th>Sample Allele</th>
<th>Gene Region</th>
<th>Gene Symbol</th>
<th>Protein Variant</th>
<th>Variant Findings</th>
<th>Case Samples</th>
<th>Control Samples</th>
<th>Translation Impact</th>
<th>CADD Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>Exonic</td>
<td>KCNN3</td>
<td>p.G777dup</td>
<td>-</td>
<td>9</td>
<td>-</td>
<td>in-frame</td>
<td>7.332</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>Intronic</td>
<td>KIF1A</td>
<td>p.E917fs*6</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>frameshift</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>Exonic, Intronic</td>
<td>FOXP1</td>
<td>p.H178fs*7</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>in-frame</td>
<td>16.209</td>
</tr>
<tr>
<td>17</td>
<td></td>
<td>Intronic</td>
<td>FBLN3</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>4.595</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>Intronic</td>
<td>SGK2</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>X</td>
<td>156720970</td>
<td>Intronic</td>
<td>HAUS7</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>1.398</td>
</tr>
</tbody>
</table>

In family 1, there were 13 gene variants called when all filters were applied. The biological filter included variants 1 hop upstream and downstream of the genes included in the biological filter terms (AFLP, FAO and Ketogenesis).

**AFLP Family 2: Gene variants**

<table>
<thead>
<tr>
<th>Chromosome Position</th>
<th>Sample Allele</th>
<th>Gene Region</th>
<th>Gene Symbol</th>
<th>Protein Variant</th>
<th>Variant Findings</th>
<th>Case Samples</th>
<th>Control Samples</th>
<th>Translation Impact</th>
<th>CADD Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>A</td>
<td>Intronic</td>
<td>ZNF117</td>
<td>p.A437, p.A937</td>
<td>-</td>
<td>4</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>T</td>
<td>Exonic</td>
<td>ZNF117</td>
<td>p.A437, p.A937</td>
<td>-</td>
<td>4</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>Intronic</td>
<td>HLTF</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>G</td>
<td>Exonic, Intronic</td>
<td>SRA1</td>
<td>p.Y110C</td>
<td>-</td>
<td>10</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>D309G4:G455G</td>
<td>Exonic</td>
<td>FANSCAN</td>
<td>p.G36:G455del</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>Intronic, Intronic</td>
<td>FOXP1</td>
<td>p.H78fs*7</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>in-frame</td>
<td>16.209</td>
</tr>
<tr>
<td>13</td>
<td></td>
<td>Exonic</td>
<td>ZNF117</td>
<td>p.A987fs*21</td>
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<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td></td>
<td>Intronic</td>
<td>SLAN1</td>
<td>p.L795fs*23</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>7.025</td>
</tr>
<tr>
<td>16</td>
<td>C</td>
<td>Exonic</td>
<td>ZP7M1</td>
<td>p.E4444</td>
<td>-</td>
<td>5</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td></td>
<td>Exonic</td>
<td>ZP7M1</td>
<td>p.E4444</td>
<td>-</td>
<td>4</td>
<td>-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In family 2, there were 10 gene variants called when all filters were applied. The biological filter included variants 1 hop upstream and downstream of the genes included in the biological filter terms.
AFLP Family 3: Gene variants

In family 3, there were 11 gene variants called when all filters were applied. The biological filter included variants 1 hop upstream and downstream of the genes included in the biological filter terms.

All gene variants were benign except a possible mitochondrial gene variant which will be discussed below.

7.6.1.4 Study 1: Analysis 4; 3 mothers (case) and 10 random female controls

In this final analysis of study 1, I decided to analyse the 3 mothers in each family affected by AFLP compared to 10 healthy female controls provided by the GOSgene database. The 10 controls were all female and part of another study which did not have any association with metabolism, AFLP or fatty acid oxidation. All of the samples were prepared and sequenced in the same batch, therefore any differences should not represent systematic technical artefacts.
There are no variants that overlap and are present in all 3 women. See Figure 7-5. Case 1 (Mother of family 1) appeared to have a potential significant gene variant in the mitochondrial genes *MT-ND1*, *MT-ATP6* and *MTND5*.

However, whole exome sequencing is not a reliable tool to investigate mitochondrial DNA, as a result the mother’s DNA was sent to Dr. Cathy Woodward, clinical scientist at Queen Square who sequenced the mitochondrial DNA.

### Table 7-5 Potential mitochondrial gene variants

<table>
<thead>
<tr>
<th>Chromosome Position</th>
<th>Reference Allele</th>
<th>Sample Allele</th>
<th>Gene Region</th>
<th>Gene Symbol</th>
<th>Protein Variant</th>
<th>Translational Impact</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>8557G</td>
<td>A</td>
<td>Exonic</td>
<td>MT-ND1</td>
<td>MT-ATP6, MT-ATP8</td>
<td>p.A11T, p.L64L</td>
</tr>
<tr>
<td>M</td>
<td>13879T</td>
<td>C</td>
<td>Exonic</td>
<td>MT-ND5</td>
<td>p.S515P</td>
<td>Missense</td>
</tr>
</tbody>
</table>

Mitochondrial sequencing identified the m.1555A>G change, which is a deafness mutation and has been described with deafness due to aminoglycoside exposure. See Figure 7-6.
The change does have implications in that certain antibiotics need to be avoided during any medical treatment. This patient was referred to Prof Maria Bitner-Glindzicz at Family Genetics Clinic at GOSH. This was an incidental finding and no other pathogenic mutations of mtDNA were detected.

7.6.1.5 Study 2: Analysis 1; Affect child (case) and mother, father and siblings (control)

Whole genome sequenced data for the 4 families studied were uploaded to the ingenuity variant analysis software program as described in study 1. Despite having more sequenced data the principle of analysis remained the same as study 1. Identical biological filters were applied to all analyses (confidence, common variants, predicted deleterious, genetic analysis and biological filter). The first analysis was set for the affected child to be the case (homozygous) and the mother, father and siblings to act as the controls (heterozygous). See Figure 7-7.
Chapter 7 Next Generation Sequencing of Families with AFLP

Figure 7-7 Gene variants in the 4 families.

The biological filter included genes ‘1 hop’ away or towards the genes associated with the biological terms AFLP, fatty acid oxidation disease and ketogenesis.

AFLP Family 4: Gene variants

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Position</th>
<th>Sample Allele</th>
<th>Gene Region</th>
<th>Symbol</th>
<th>Protein Variant</th>
<th>Variant Findings</th>
<th>Case Samples</th>
<th>Control Samples</th>
<th>Translation Impact</th>
<th>CADD Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>7302618</td>
<td>TG</td>
<td>Splice Site</td>
<td>RECQL5</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>38482355</td>
<td>Exonic</td>
<td>BAAP2L2</td>
<td></td>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>26.700</td>
</tr>
</tbody>
</table>

In family 4, there were 2 gene variants called when applying the genetic filter. When the biological filter was applied one gene variant (RECQL5) was called. RECQL5 is a splice variant with a high CADD score.

Polymorphisms in RecQ like helicase 5 (RECQL5) have been associated with cancers including breast and colorectal cancer (325). The BAM file is shown in Figure 7-8. There is no association with energy or fat metabolism.

AFLP Family 5: Gene variants

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Position</th>
<th>Sample Allele</th>
<th>Gene Region</th>
<th>Symbol</th>
<th>Protein Variant</th>
<th>Variant Findings</th>
<th>Case Samples</th>
<th>Control Samples</th>
<th>Translation Impact</th>
<th>CADD Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1865571</td>
<td>Exonic</td>
<td>FAM51B</td>
<td></td>
<td>p.R725V</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>4919190</td>
<td>A</td>
<td>Exonic, Promoter</td>
<td>LAMB2, USP19</td>
<td>p.G1676R</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>18.410</td>
</tr>
<tr>
<td>3</td>
<td>4919191</td>
<td>T</td>
<td>Exonic, Promoter</td>
<td>LAMB2, USP19</td>
<td>p.G1676R</td>
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</table>

In family 5, there were 5 gene variants called when applying the genetic filter. When the biological filter was applied 2 gene variants (in the gene LAMB2) were called.

Laminin subunit beta 2 (LAMB2) gene mutations have been shown to result in congenital nephrotic syndrome (326).
AFLP Family 6: Gene variants

In family 6, there were 3 gene variants called when applying the genetic filter. When the biological filter was applied 2 gene variants (in genes SKA3 and ACSF3) were called.

Spindle and kinetochore associated complex subunit 3 (SKA3) gene regulates microtubule action during mitosis and cell division. SKA3 gene variants are not known to be disease causing. The BAM file is shown in Figure 7-9.

Acyl-CoA synthetase family member 3 (ACSF3) gene encodes a protein that allows long chain fatty acids to be transformed in fatty acyl-CoA esters important in fatty acid oxidation. On initial analysis, this gene appeared to be interesting as it is associated with FAO. However, further analysis by Dr Hywel Williams at Gosgene, showed this gene variant to be an artefact due to misalignment of the genes during sequencing. When samples were sent for WGS they were sent in batches of 30-50 samples and all underwent identical sequencing processes. The same gene ACSF3 variant was also identified in these separate samples, therefore proving that this gene variant is in fact an artefact.

AFLP Family 7: Gene variants

In family 7, 1 gene variant (SKA3) was called when applying the genetic filter and biological filter. This gene was also seen in family 6 and has been described above.

All variants identified were explored further using BAM files and gene databases are described in Study 1. No gene variants were found to be pathological as they did not
have any association with FAO and energy metabolism and/or they were not true variants when analyzed closely with BAM files.

**Figure 7-8 BAM file for analysis of Family 4 (RECQL5)**

This gene variant is a false positive as it has been poorly mapped. This is identified by the Poly G stretch of single nucleotides.

**Figure 7-9 BAM file for analysis of Family 4 (SKA3)**

This gene variant is a false positive as it has also been poorly mapped. This is identified by the Poly A stretch of single nucleotides.
7.6.1.6 Study 2: Analysis 2; Affected mother (case), father and children (controls)

I decided to expand the analysis of the sequenced data by labelling the affected mother as the case and the father and children as the controls in each family (Figure 7-10).

**Figure 7-10 Analysis 2: gene variants identified in each family**

**AFLP Family 4: Gene variants**

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Following application of all of the filters 11 gene variants were identified in family 4. BAM files are shown in Appendix 4.

**AFLP Family 5: Gene variants**

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In family 5, there were 11 gene variants identified once all filters were applied.
### Chapter 7 Next Generation Sequencing of Families with AFLP

**AFLP Family 6: Gene variants**

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Eleven gene variants were identified in family 6.

**AFLP Family 7: Gene variants**

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</table>

Ten gene variants were identified in family 7.

Further evaluation of the gene variants did not identify any novel variants associated with energy metabolism, fatty acid oxidation or AFLP in any of the families.

#### 7.6.1.7 Study 2: Analysis 3; Mother (case), father and children (Not included)

This analysis was performed to identify any loss of function gene variants in the mother only irrespective of the father and children. I applied a similar filtering cascade as for my previous analysis, however I set the genetic filter to look for variants in only the mother affected by AFLP. The other filters were applied in the same format as above. See Figure 7-11.
There were 8 gene variants called when all filters were applied. The biological filter included variants 1 hop upstream and downstream of the genes included in the biological filter terms (AFLP, FAO and Ketogenesis).

Ten gene variants were identified in family 5 following application of the biological filters.
Chapter 7 Next Generation Sequencing of Families with AFLP

AFLP Family 6: Gene variants

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<th>Chromosome</th>
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Following application of all of the filters 14 gene variants were identified in family 6.

AFLP Family 7: Gene variants

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<th>Sample Allele</th>
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<th>Protein Variant</th>
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Nine gene variants were identified in family 7 following application of the biological filters.

Further evaluation of the gene variants using BAM files did not identify any novel variants associated with energy metabolism, fatty acid oxidation or AFLP in any of the families.
7.7 Discussion

Next generation sequencing of 7 families affected by AFLP did not identify any known or novel gene variants associated with energy metabolism, fatty acid oxidation or AFLP. Some of the gene variants that produce proteins, do not have a known function. It is beyond the scope of my thesis to investigate the function of these proteins.

7.7.1 WES of 3 families

To date, the only known gene variants associated with AFLP are associated with a disorder of fetal fatty acid oxidation (4). Under these circumstances, the fetus is homozygous and the parents are heterozygous for the gene defect (LCHAD deficiency). WES of the 3 families affected by AFLP did not identify a similar gene variant. All gene variants identified were either benign and did not have a clear association with a known fatty acid oxidation disorder.

When I analyzed the data again using the mother of each family as the case, examination of the variants using BAM files helped to determine that the variants were not known to be disease causing.

Analyzing the sequenced data for the mothers affected by AFLP against healthy females selected from another cohort at random, did not identify any gene variants that were shared with all AFLP affected women. Those variants that were identified were not associated with known disease when examined in more detail.

These results show no known or novel gene variants in these 3 AFLP affected families, however we know that one of the limitations of WES is that the exome coverage is not as optimal as WGS (327). The whole exome occupies less than 2% of the entire human genome and it may be that variants associated with the development of AFLP may lie in the non-coding region of the genome.
It is well known that WES can detect approximately 12,000 gene variants in the protein coding region of the genome and there are accessible databases that include 90% of these variants (313, 328). Conversely, WGS can identify approximately 5 million gene variants (328).

One way to proceed would be to either recruit more families or perform WGS in these 3 families.

### 7.7.2 WGS of 4 families

I proceeded to perform WGS on 4 families affected by AFLP. The gene variants identified in all analyses were not known to be pathological. The analyses were performed similarly to WES, in that the biological filters and the candidate genes used to identify variants were identical. The gene variants identified were individually assessed and the genetic inheritance of the families were evaluated. It was clear that there were no novel gene variants in any of the families. Gene variants were further assessed using BAM files and literature databases (e.g. pubmed), which failed to confirm any correlation between the gene variants identified and energy metabolism or AFLP.

### 7.7.3 Challenges of NGS in diagnosing AFLP

WES and WGS are validated sequencing tools that when used appropriately can identify novel gene variants. I aimed to discover gene variants in families affected by AFLP that are associated with energy metabolism, using both WES and WGS. The results of this genetic investigation did not identify any novel gene candidates, nor any gene variation that is implicated in energy metabolism, including LCHAD gene variants and the development of AFLP.
Currently, the only known inheritance model for the development of AFLP in a pregnant woman is the LCHAD inheritance model. However, I did not replicate this model in the families I studied.

AFLP is a rare disorder of the third trimester of pregnancy. Generally rare disease studies are restricted to analysis of exonic variants, where approximately 85% of known disease-related variants are found (329). It is possible that the gene defect involved in AFLP is present in the non-coding region of the genome. To investigate this further non-coding regions of the genome would need to be evaluated in the future. Furthermore, as a heterogeneous disorder, AFLP affects each family uniquely. As a result, a single gene variant is unlikely to be responsible for all cases of AFLP.

During the process of fatty acid transport and beta oxidation there are approximately 20 inherited gene defects that are known to date (330). These gene defects were included in the biological filter for all analyses in my study. I did not find any novel variants associated with fatty acid oxidation, however potentially there may be other undiscovered gene defects associated with FAO and energy metabolism which may have a role in AFLP.

7.7.4 Limitations of my study

My study comprised only 7 families, which may be too small to identify any important gene variants. However, NGS has been successful in identifying gene variants to diagnose rare diseases in small patient numbers. In addition, the development of AFLP is most likely influenced by external or environmental factors, which could impact the expression of genes. This may represent the difficulty I have experienced with this study.
Chapter 7 Next Generation Sequencing of Families with AFLP

7.8 Summary

I did not discover any coding gene variants through NGS of 7 families previously affected by AFLP. This is not to say that genes do not play an important role in the pathogenesis of AFLP. There is clear evidence to show genes such as LCHAD, MCAD and CPT1 are implicated in the development of AFLP. Furthermore, it is possible that AFLP develops in a normally well mother with a mild sub-clinical defect in FAO, which is unmasked during pregnancy when a placental derived factor expressed by genes from the father inhibits her limited capacity for FAO, especially when required in the fasted state. AFLP may therefore follow from a complex interaction between all members of the family and environmental influences.

7.8.1 Future work

Future work to investigate the genetics of AFLP should involve:

i. **WGS:** It would be reasonable to perform WGS on the families that underwent WES in this study. Furthermore, with WGS data from the 4 AFLP families, it would be possible to explore non-coding variants in regulatory regions of the genome. This may be possible in the future as databases are becoming available but our ability to interpret this data is still in its infancy.

ii. **Epigenetics:** If genetic variance cannot explain the pathophysiology of AFLP, it would be worth exploring epigenetic differences that might influence gene regulation associated with energy metabolism, especially during fasting.

iii. **MicroRNA:** It is possible that fetal microRNA are transferred into maternal circulation through the placenta, disrupting maternal hepatic protein expression, resulting in AFLP. MicroRNAs are small noncoding RNAs (18-22 nucleotides in length) that are involved in the expression of protein coding genes. The function of microRNAs in pregnancy has been investigated over the last 20 years and we have yet to understand their role in pregnancy (331, 332).

These future studies may provide a detailed insight into the pathogenesis of AFLP.
CHAPTER 8

DISCUSSION AND FUTURE WORK
Chapter 8  Discussion and Future Work

8.1  Research Study Key Findings and Conclusions

 Phenotypic clues from women who develop AFLP have led to the hypothesis that AFLP is a disorder of fatty acid oxidation (FAO) (4, 144). These features have led to my study on AFLP. Before I started this PhD, only a few cases of AFLP were explained by the enzyme defect long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) which is an enzyme involved in mitochondrial fatty acid oxidation. There are only a small number of cases associated with an LCHAD gene defect (4, 9). However, this gene defect does not explain all cases of AFLP.

During this study, I applied several methodologies to investigate the aetiology of AFLP.

8.1.1  Phenotype study.

My work has identified key characteristics that more precisely define the phenotype of AFLP as a defect in fatty acid oxidation. The absence of ketonuria, despite prolonged nausea and vomiting in the third trimester of pregnancy is suggestive of a defect in FAO. Hypocholesterolaemia and hypotriglyceridaemia is likely another feature attributable to synthetic liver failure. Women with AFLP also have offspring with a normal birth weight for gestational age, unlike babies born to mothers with pre-eclampsia and HELLP syndrome, which are growth restricted. None of these features have previously been associated with AFLP. I therefore propose an update to the diagnostic criteria for AFLP to include the absence of ketonuria despite nausea and vomiting in the third trimester, hypolipidaemia and normally grown babies.
My study and its phenotypic findings strongly indicate that AFLP is a metabolic disorder, where fasting in the insulin resistant state of the third trimester unmasks a subclinical defect in energy metabolism leading to multi-organ failure. A simple cure would be for early admission to hospital and parenteral glucose administration.

8.1.2 Fasting and fat burning exercise study

My work firstly showed that following an 18-hour fast and fat burning exercise, women in the third trimester of pregnancy produce a higher concentration of β-hydroxybutyrate (5HB) compared with healthy non-pregnant women. This outcome supports the already well known phenomenon of ‘accelerated starvation’ (74).

However, following a 24-hour fast and fat burning exercise, women who have previously had AFLP produced a similar concentration of 5HB as women who have not had AFLP, demonstrating that they do not appear to have a sub-clinical defect in ketone body production outside of pregnancy. This key study finding as well as the phenotypic features provide evidence that women with AFLP having the ability to generate energy from ketone bodies outside of pregnancy yet not during pregnancy, which is reinforced by the fact that these women recover with supportive treatment once the fetus has been delivered.

In this study, I have not studied the father or the affected offspring. It is conceivable that AFLP is a result of a defect in FAO in the offspring, as suggested by published studies where women developed AFLP whilst carrying fetuses that were LCHAD deficient (4). The next steps in this study would be to study the father and offspring. I plan to look at the imprinted gene only inherited from the father, which expresses a protein called Delta-like homolog 1 (DLK1) in the fetus (231). Studies have shown absence of DLK1 derived from the fetus reduces maternal ketone production during fasting (231).
8.1.3 Proteomic Study

I initially discovered that women who had AFLP had differences in proteins involved in iron metabolism that have also been associated with non-alcoholic fatty liver. However, these differences may be explained by the fact that women who had AFLP were significantly older and parous compared with controls. When controlling for age and parity in a second comparison of the proteome, I found that women who had AFLP had elevated levels of Glypican 2 and Galectin 3.

My study findings were not validated when 2 cohorts of women with AFLP were compared with two different control groups. However, as the initial findings were compatible with AFLP heralding NASH in later life, it would be prudent to repeat this study with well-matched controls. Furthermore, Glypican 2 and Galectin 3 may also be integral to AFLP as both are known to be associated with liver disease.

As proteomic technology improves, we may learn more about the pathogenesis of AFLP.

8.1.4 Genetic Study

It is evident from what we know about AFLP that genes involved in FAO (LCHAD, MCAD, CPT1) are also involved in the development of AFLP. However, my study of 7 different families with AFLP did not identify any novel gene defects associated with energy metabolism or FAO using whole exome and genome sequencing (WES and WGS). AFLP is a heterogeneous disorder and it is likely to affect each family in a distinctive way. This could explain why a single gene variant was not identified in all families. It is more than plausible that a single gene variant is unlikely to be responsible for all cases of AFLP. Additionally, there are many gene variants that produce proteins that do not have a known function. Therefore, investigating the function of these proteins may help us in our understanding of AFLP.
AFLP could develop in a previously well women with a mild sub-clinical defect in FAO. This defect in FAO is unmasked only during pregnancy when a placental derived factor expressed by genes from the father inhibits her already limited capacity for FAO, especially when required in the fasted state. As a result, AFLP may occur from a complex interaction between all family members and importantly environmental influences. This would explain why WES and WGS did not identify any single gene variants associated with energy metabolism.

8.2 Future Work

This thesis has generated several paths for further investigation into the aetiology of AFLP. We are well aware of the devastating consequences of untreated AFLP to both the mother and offspring, yet until we identify how it develops we will not be able to diagnose, treat and counsel families appropriately.

There is strong evidence that genes involved in FAO are important in the pathogenesis of AFLP. It would be important to perform WGS on more families affected by AFLP to discover other gene variants associated with a defect in FAO and ketogenesis. Exploring non-coding variants in regulatory regions of the genome is currently in its initial phase. Once established interpretation of this data from AFLP families would be invaluable.

Alternatively, it would be appropriate to consider exploring epigenetic differences that might influence gene regulation associated with FAO, especially if genetic variance is unable to explain the aetiology of AFLP.

MicroRNAs are small noncoding RNAs (18-22 nucleotides in length) that are involved in the expression of proteins (331, 332). It is possible that fetal microRNA are transferred into maternal circulation through the placenta, disrupting maternal hepatic protein expression, resulting in AFLP. This theory could be investigated by extracting MicroRNA in whole blood prior to and following delivery in selected patients and analyzed using mass spectrometry or microRNA sequencing.
To complete the phenotypic profile of AFLP and to understand its aetiology it would be important to investigate the metabolome of women who have had AFLP compared to unaffected women. The metabolome includes all metabolites within a cell, tissue or biological samples at a given time point. Serum samples collected from the fast and fat burning exercise study have been sent for metabolomics screening.

These studies are underway, yet would provide a comprehensive and detailed insight into the pathogenesis of AFLP.

8.3 Summary

This thesis provides evidence that women who present with AFLP do not have ketonuria despite a prolonged fast in the third trimester of pregnancy. This observation suggests AFLP is associated with a defect in fatty acid oxidation. As these women produce ketones normally after an affected pregnancy, it is clear that AFLP is a gestational-specific syndrome. Placental, fetal and paternal factors may play a role in the development of AFLP.

I did not identify any gene variant in any family affected by AFLP that is known to be associated with energy metabolism. Whole genome sequencing of more families affected by AFLP might identify the pathogenesis of what appears to be a heterogeneous gestational syndrome specific to individual offspring within each family.

The evidence from this thesis points towards AFLP as being a disorder of energy metabolism. Future studies into the pathogenesis of AFLP are focusing on integrated metabolic and genetic features of all members of an affected family.
APPENDICES
Appendices

Appendix 1: Scout and graph for 6 volunteers.

a. Volunteer 1

Low PCr- indicates low chemical shift artefact

Decoupling particularly effective for PME/PDE

PEP-Phosphoenolpyruvate, NADPH- Nicotamide adenine dinucleotide phosphate, UDPG- Uridine diphosphoglucose

Using PI- $\alpha$-ATP chemical shift

Intra-cellular pH = 7.47 ± 0.02

b. Volunteer 2
c. Volunteer 3

d. Volunteer 4

e. Volunteer 5
f. Volunteer 6
Appendices

Appendix 2 Exercise and Fasting Study Supplementary information

S1 Patient Information Leaflet

INFORMATION LEAFLET FOR PARTICIPANTS (Control group)

An investigation into the cause of Acute Fatty Liver of Pregnancy:

Exercise and Fasting Study
(Student study version 1 10.07.15)

We would like to invite you to take part in our research study. Before you decide we would like you to understand our study and why the research is being done. A member of the research team will go through the leaflet with you and answer any questions you have.

What is the purpose of the study?
Our research group is investigating the cause of a rare complication of pregnancy Acute Fatty Liver of Pregnancy (AFLP). AFLP affects approximately 1 in 10000 pregnant women a year in the UK. It is a serious complication of pregnancy that can result in the death or profound disability of a mother and her child. AFLP resembles a disorder of Lipid (fat) metabolism. Known defects in lipid metabolism result in reduced energy production leading to multi-organ failure.

In the first half of normal pregnancy there is significant accumulation of fat. During the second half of pregnancy fat is metabolised to produce energy for the mother. However, little is known about how lipid metabolism alters during healthy pregnancy.

By investigating lipid (fat) metabolism in healthy pregnancies we will gain a better understanding of how accumulated fat might expose a lipid disorder in a vulnerable mother, which could lead to AFLP.

Dr Mandeep Kaler is the Clinical Researcher for this project. She is an obstetric registrar with 4 years clinical experience at University College Hospital and other London hospitals and is working under the supervision of Dr Williams, consultant in Obstetric medicine. She will be carrying out this research as part of her PhD at the institute for women’s health, University College London.

Why have I been invited?
In order to understand what goes wrong in women who have had AFLP, we not only have to study pregnant women but we also need to study healthy non-pregnant volunteers to compare our findings. You have been invited to take part in this study because you are healthy and not pregnant.

Do I have to take part?
It is up to you to decide to join the study. We will describe the study and go through this information sheet. If you agree to take part, we will then ask you to sign a consent form. You are free to withdraw at any time, without giving a reason. This would not affect the standard of care you receive.
What will happen to me if I take part?

If you wish to take part in the study we will first ask for your consent. You will then see the clinical researcher once on the day of the study.

There are several ways to measure fat metabolism and we would like to study your metabolism in these different ways.

1. **Fast and Exercise test:** We will ask you to fast from 2.30pm after you have had your lunch at 2pm. After 2.30pm you will not be allowed to eat anything. Water will be the only drink that you can consume freely. The next morning we will ask you to attend the Institute of Sport, Exercise and health, 170 Tottenham court road, UCL at 8.30am. Dr Kaler will meet you there and for the next 6 hours you will participate in a supervised exercise test. The exercise involves sitting on an exercise bike with a full seat and a back to the seat (see photo). You will then pedal gently at a very low intensity for 30 minutes and then rest for another 30 minutes. This cycle will be repeated over the next 5 hours. Whilst on the exercise bike you will wear a light facemask, which collects breath-by-breath gas measurements. Whilst on the exercise bike you will be able to read a magazine and listen to music. During the study Dr Kaler will monitor your blood pressure and heart rate every 2 hours. Food and drink will be provided at the end of the study.

   Fast and Exercise studies have been performed a number of times in the past with no ill effect to volunteers. However, we are asking you to fast for a further 6 hours at home. This length of a fast has not been studied before with exercise.

2. **Blood test:** We will take 20mL (approximately 1 tablespoon) of blood from you at 18 hours, 21 hours and 24 hours of fasting and exercising. The blood will be used to measure proteins, lipids and other metabolic factors.

3. **Urine specimen:** We will collect a mid-stream urine specimen from you at 18 hours and 24 hours of fasting and exercising. The urine will be used to measure proteins, lipids and other metabolic factors.

What will happen to the samples I give?

Dr Mandeep Kaler at the Clinical Research Facility, Elizabeth Garrett Anderson Obstetric Hospital, 235 Euston Road, London, NW1 2DU will process, store and dispose of blood, urine and placenta in accordance with all applicable legal and regulatory requirements, including the Human Tissue Act 2004 and any amendments thereto.
If you agree, once initial investigations are complete, any sample(s) you provide will be stored for research purposes. The stored material may be used for several other purposes:

- Future investigations that may be useful to refine diagnosis, treatment or screening.
- Ongoing research approved by the Research Ethics Committee.
- Future research projects that have similarly received scientific and ethical review. In this case, the researcher may have access to information concerning the diagnosis, but no information of a personal nature that could link back to you or your family. Similarly, the results of the research project will not be traceable to you or your family.

Expenses and payments

Participating in this research will involve you travelling to UCL. The research team will compensate you for any travel expenses once they have received a receipt (Petrol 40 pence/mile or standard coach/rail ticket).

What do I have to do?

If you take part in the study, Dr Kaler will arrange all the appointments necessary to see you. She will explain each test and ask for your consent. She will provide you with information including the location of where you will need to attend.

What are the possible disadvantages and risks of taking part?

- **Fast:** We do not anticipate any significant risks during the initial unsupervised fast (18 hours). The last 6 hours of the fast will be supervised (Total fast 24 hours). There is a small risk of feeling lightheaded and faint. Water can be consumed throughout the study to prevent dehydration. If you do feel lightheaded the first thing you should do is lie down. If you continue to feel unwell, end the study and have something to eat. If you feel unwell the study can end at any time.

- **Exercise:** The exercise test will be supervised at all times. We do not anticipate any significant risks as the exercise will occur for a short period of time, at a low intensity and you will be sitting in a comfortable position. There are facilities to lie down during the rest periods if necessary. During this time if you feel unwell the study can end at any time. Food and drink will be provided.

- **Blood and urine collection:** There may be minor discomfort whilst taking the blood tests. Dr Kaler is appropriately trained to take blood. If your blood or urine tests show a significant problem we will inform your GP.

- In the unlikely event of complications Dr Kaler is trained and qualified in Advanced Life Support.

- The next day Dr Kaler will give you a call to see how you are feeling. In the unlikely event that you continue to feel unwell make an appointment to see your GP. If this is not possible go to your local accident and emergency department.
What are the potential benefits of taking part?

There are no direct benefits to you, but we hope to improve our understanding of fat metabolism in healthy pregnancy, which will help to identify the cause of AFLP. This may improve our ability to diagnose and treat this condition and may have benefits to the offspring from an AFLP pregnancy.

What if there is a problem?

If you wish to complain, or have any concerns about any aspect of the way you have been approached or treated by members of staff you may have experienced due to your participation in the research, National Health Service, UCL complaints mechanisms and a Patient advice and liaison services (PALS) are available to you. Please ask your research doctor if you would like more information on this.

In the unlikely event that you are harmed by taking part in this study, compensation may be available. If you suspect that the harm is the result of the Sponsor’s (University College London) or the hospital’s negligence then you may be able to claim compensation. After discussing with your research doctor, please make the claim in writing to the Dr David Williams who is the Chief Investigator for the research and is based at Institute for women’s health, UCL. The Chief Investigator will then pass the claim to the Sponsor’s Insurers, via the Sponsor’s office.

Will taking part in this study be kept confidential?

Only the researchers and authorised persons from UCL will have access to parts of your medical records and the data collected during this study. They may also be looked at by authorised people to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and we will do our best to meet this duty. The use of some types of personal information is safeguarded by the Data Protection Act 1998 (DPA). The DPA places an obligation on those who record or use personal information, but also gives rights to people about whom information is held.

What will happen to the results of the research study?

They will be stored in a computer accessible only with a personal password owned by the researchers. Clinical members of the study group will code all data for recognition. A full copy of the final research will be available to you at the end of the study, unless you request not to want a copy of the research.

Who has reviewed the study?

All research in the NHS is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favorable opinion by London (Bromley) Research Ethics Committee.

Contact for Further Information:

Dr Mandeep Kaler
Clinical Research Fellow
Tel: 07958617445
Email: m.kaler@ucl.ac.uk

Dr David Williams (Principal Investigator)
Consultant Obstetric Physician
Email: d.j.williams@ucl.ac.uk
Appendices

S2 Consent

University College London Hospitals

Consent Form for Female Adults
Participating in Research Studies
(Non-pregnant, healthy women)

Title: An investigation into the cause of Acute Fatty Liver of Pregnancy (Student study)

Name of Researchers:
Dr David Williams, Consultant Obstetric Medicine
Dr Mandeep Kaler, Clinical Research Fellow

Notes for Female Adults

1. You have been asked to take part in a research study. The person organising that study is responsible for explaining the project to you before you give consent.
2. Please ask the researcher any questions you may have about this project, before you decide whether you wish to participate.
3. If you decide, now or at any stage, that you do not wish to participate in the research project, that is entirely your right, and if you are a patient it will not in any way prejudice any present or future treatment.
4. You will be given an information sheet, which describes the research project. This information sheet is for you to keep and refer to. Please read it carefully.
5. You can agree to as much or as little of the following requests.

Please tick boxes

1. I agree to take part in this study (Title above).

2. I confirm that I have read and understand the information sheet dated................ (version............) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.

3. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.

4. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by individuals from UCL, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.

5. I agree to the research team informing my GP of my participation in this study.

Consent: An Investigation into the cause of AFLP
Non-pregnant women with previous healthy pregnancy
1 for patient, 1 copy for researcher, 1 to be kept with hospital notes

Version 3.0 01.01.16
Appendices

University College London Hospitals

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<td>I agree to take part in the Exercise study, which involves blood tests and urine specimen collection.</td>
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<td>I agree for my samples to be gifted to UCLH and stored for future studies. This would require future ethics committee approval.</td>
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<td>8</td>
<td>I agree to my GP being informed of any clinically significant results.</td>
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<td>I would like to receive details regarding the results of this study once it has been completed and published.</td>
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<td>I agree to be contacted after this study has finished in order to be asked if I will consider being involved in any follow-up studies. This would require future ethics committee approval.</td>
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CONSENT for Female Adults

I ____________________________, agree that the Research Project named above has been explained to me to my satisfaction, and I give consent to take part in this study.

I have read both the notes written above and the Information Sheet provided, and understand what the research study involves.

__________________________________  ____________  __________
Name of Participant  Signature  Date

c____________________________  _________  _________
Name of Researcher  Signature  Date

taking consent

Consent: An Investigation into the cause of AFLP
Non-pregnant women with previous healthy pregnancy
1 for patient, 1 copy for researcher, 1 to be kept with hospital notes

Version 3.0 01.01.16

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MEDICAL SCREENING QUESTIONNAIRE FOR THE EXERCISE STUDY

Thank you for taking the time to fill out this questionnaire. We need to know details of your medical background to assess your suitability and ensure your safety for participating in The Exercise Study. The information you provide will be kept strictly confidential.

The research team will review this form and if these doctors have any concerns, they discuss further issues raised on the medical form, which may mean that it is not safe for you to take part in the study. The information will not be shared with other doctors and it will not be used as part of the scientific research project without your permission.

If you would like to discuss any medical concerns related to the Exercise Study please speak to Dr Mandeep Kaler. She can be contacted in confidence by email or telephone:

e-mail: m.kaler@ucl.ac.uk
Tel: 07958617445
PERSONAL DETAILS

Name:
Date of Birth:
Address:

Contact telephone number:
e-mail address:
Next of Kin Name:
Relationship:
Contact Details:
PARTICIPANT NAME:

PAST MEDICAL HISTORY

Have you visited your GP or hospital in the last 12 months for anything except a check-up? Please give details: Y/N

In the past year have you attended hospital or had an operation? Are you currently waiting to see a hospital doctor/specialist for any reason? Please give details. Y/N

Are you pregnant/trying to fall pregnant (Women only, please give details) Y/N

Do you suffer from/have you ever had any form of heart disease? Y/N

Do you suffer from/have you ever had any form of kidney disease? Y/N

Do you suffer from/have you ever had any form of liver disease? Y/N

Do you suffer from/have you ever had high blood pressure/hypertension? Y/N

Do you suffer from/have you ever had any lung disease? Y/N

Do you suffer from/have you ever had asthma? Y/N

Have you ever suffered from a epilepsy/blackouts/fits/funny turns Y/N

Do you suffer from diabetes? Y/N

Do you suffer from any allergies? Y/N

If the answer to any of the questions above is yes, please give details below
## MEDICATION HISTORY

<table>
<thead>
<tr>
<th>Question</th>
<th>Y/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Are you taking any medication on a regular basis including the oral contraceptive pill? (Please list)</td>
<td>Y/N</td>
</tr>
<tr>
<td>Have you ever been on any long-term medication including psychiatric drugs? Please give details.</td>
<td>Y/N</td>
</tr>
<tr>
<td>Are you allergic to any medications?</td>
<td>Y/N</td>
</tr>
<tr>
<td>Are you taking any herbal remedies or alternative medicines? (Please list)</td>
<td>Y/N</td>
</tr>
<tr>
<td>In the last year have you used any recreational drugs? If so please give details below</td>
<td>Y/N</td>
</tr>
<tr>
<td>Are you taking any vitamin or food supplements? (Please list)</td>
<td>Y/N</td>
</tr>
<tr>
<td>Do you smoke?</td>
<td>Y/N</td>
</tr>
<tr>
<td>How many cigarettes per day/week? For how many years?</td>
<td></td>
</tr>
<tr>
<td>How many cigars per day/week? For how many years?</td>
<td></td>
</tr>
<tr>
<td>How much pipe tobacco per week? For how many years?</td>
<td></td>
</tr>
<tr>
<td>How much hand-rolling tobacco per week? For how many years?</td>
<td></td>
</tr>
<tr>
<td>Do you drink alcohol?</td>
<td>Y/N</td>
</tr>
<tr>
<td>On average, how much do you drink a week?</td>
<td></td>
</tr>
<tr>
<td>Do you drink coffee regularly   Approximately how many cups a day?</td>
<td>Y/N</td>
</tr>
</tbody>
</table>
I declare that the information provided on this form is accurate at the time of completion. I declare that I know of nothing relating to my health or fitness, which might prohibit me from taking part in this study.

Name: ____________________________ Date: __________

Signature: ____________________________
Appendix 3 ELISA Preparations

S1: Total serum Peptide YY (PYY) manufacturer instructions

VIII. REAGENT PREPARATION

A. Preparation of Capture and Detection Antibody Mixture

Prior to use, measure and combine equal amounts of the Human PYY (Total) Capture Antibody (3mL) and Human PYY Detection Antibody (3mL). Invert to mix thoroughly. If the total volume of antibody mixture needed for the assay is less than 6 mL, mix the two antibody solutions at equal volume and keep the rest separated for next assay. Prepare mixture immediately prior to use. Discard unused remaining mixture after use.

B. STANDARD AND QUALITY CONTROLS PREPARATION

PYY Standard Preparation

Use care in opening the lyophilized Standard vial. Using a pipette, reconstitute the PYY Standard with 0.5 mL distilled or deionized water into the vial to give a concentration prescribed in the analysis sheet. Invert and mix gently, let sit for five minutes or until completely dissolved then mix well.

Label six tubes 1, 2, 3, 4, 5, and 6. Add 0.2 mL Assay Buffer to each of the six tubes. Prepare serial dilutions by adding 0.2 mL of the reconstituted standard to tube 1, mix well and transfer 0.2 mL of tube 1 to tube 2, mix well and transfer 0.2 mL of tube 2 to tube 3, mix well and transfer 0.2 mL of tube 3 to tube 4, mix well and transfer 0.2 mL of tube 4 to tube 5, mix well and transfer 0.2 mL of tube 5 to tube 6, mix well.

Note: Do not use a Repeater pipette. Change tip for every dilution. Wet tip with Standard before dispensing. Unused portions of reconstituted standard should be stored at ≤-20°C. Avoid multiple freeze/thaw cycles.

<table>
<thead>
<tr>
<th>Tube #</th>
<th>Standard Concentration pg/mL</th>
<th>Volume of Assay Buffer to Add</th>
<th>Volume of Standard to Add</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>X/2</td>
<td>0.2 mL</td>
<td>0.2 mL of reconstituted standard</td>
</tr>
<tr>
<td>2</td>
<td>X/4</td>
<td>0.2 mL</td>
<td>0.2 mL of tube 1</td>
</tr>
<tr>
<td>3</td>
<td>X/8</td>
<td>0.2 mL</td>
<td>0.2 mL of tube 2</td>
</tr>
<tr>
<td>4</td>
<td>X/16</td>
<td>0.2 mL</td>
<td>0.2 mL of tube 3</td>
</tr>
<tr>
<td>5</td>
<td>X/32</td>
<td>0.2 mL</td>
<td>0.2 mL of tube 4</td>
</tr>
<tr>
<td>6</td>
<td>X/64</td>
<td>0.2 mL</td>
<td>0.2 mL of tube 5</td>
</tr>
</tbody>
</table>
B. STANDARD AND QUALITY CONTROLS PREPARATION (continued)

PYY Quality Control 1 and 2 Preparation

Use care in opening the lyophilized Quality Control vials. Using a pipette, reconstitute each of the PYY Quality Control 1 and Quality Control 2 with 0.5 mL distilled or deionized water into the vials. Invert and mix gently, let sit for five minutes or until completely dissolved then mix well.

Note: For exact concentration of Quality Control 1 and 2, refer to Analysis Sheet. Unused portions of Quality Controls should be stored at ≤ -20°C. Avoid multiple freeze/thaw cycles.

IX. ASSAY PROCEDURE

Pre-warm all reagents to room temperature immediately before setting up the assay. Thaw frozen reagents in lukewarm water if necessary.

1. Dilute the 10X HRP wash buffer concentrate 10 fold by mixing the entire contents of both buffer bottles with 900 mL de-ionized or glass distilled water.

2. Remove the required number of strips from the Microtiter Assay Plate. Assemble the strips in an empty plate holder and fill each well with 300 µL diluted (1X) Wash Buffer. Decant wash buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times. Wash assay plate using this procedure 3 times. Do not let wells dry before proceeding to the next step. If an automated machine is used for the assay, follow the manufacturer’s instructions for all washing steps described in this protocol.

3. Add 20 µL Matrix Solution to Blank, Standard, and Quality Control wells (refer to X for suggested well orientations).

4. Add 20 µL Assay Buffer to each of the Blank and sample wells.

5. Add in duplicate 20 µL human PYY standards in order of ascending concentration to the appropriate wells.

6. Add in duplicate 20 µL QC1 and 20 µL QC2 to the appropriate wells.

7. Add sequentially 20 µL of the unknown samples in duplicate to the remaining wells.

8. Add 20 µL Blocking Solution to each well. Cover the plate with plate sealer and incubate at room temperature for 30 min on an orbital microtiter plate shaker set to rotate at moderate speed (approximately 400 to 500 rpm).
IX. ASSAY PROCEDURE (continued)

9. Remove plate sealer [CAUTION: Do Not Decant At This Step] and add 50 µL of the 1:1 mixture of capture and detection antibodies with a multi-channel pipette. Re-cover plate with sealer and incubate at room temperature for 1.5 hours on an orbital microtiter plate shaker set to rotate at moderate speed (approximately 400 to 500 rpm).

10. Remove plate sealer and decant solution from the plate. Tap as before to remove residual solution in the wells. Wash wells 3 times with 1X HRP wash buffer, 300 µl per well per wash. Decant and tap firmly after each wash to remove residual buffer.

11. Add 100 µl Enzyme Solution to each well with a multi-channel pipette. Cover the plate with sealer and incubate with moderate shaking at room temperature for 30 minutes on the microtiter plate shaker.

12. Remove sealer, decant solutions from the plate, and tap plate to remove the residual fluid. Wash wells 6 times with 1X HRP wash buffer, 300 µl per well per wash. Decant and tap firmly after each wash to remove residual buffer.

13. Add 100 µL of Substrate Solution to each well, cover plate with sealer and shake in the plate shaker for 5 - 20 minutes. Blue color should be formed in wells of reference standards with intensity proportional to increasing concentrations of PYY.

Note: Please be aware that the color may develop more quickly or more slowly than the recommended incubation time depending on the localized room temperature. Please visually monitor the color development to optimize the incubation time.

Remove sealer and add 100 µL stop Solution [CAUTION: CORROSIVE SOLUTION] and shake plate by hand to ensure complete mixing of solution in all wells. The blue color should turn into yellow after acidification. Wipe the bottom of the microtiter plate to remove any residue prior to reading on plate reader.

Read absorbance at 450 nm and 590 nm in a plate reader within 5 minutes and ensure that there is no air bubbles in any well.
XI. CALCULATIONS

Graph a reference curve by plotting the absorbance unit of 450nm, Less unit at 590nm, on the Y-axis against the concentrations of PYY standard on the X-axis The dose-response curve of this assay fits best to a sigmoidal 4- or 5-parameter Logistic equation. The results of unknown samples can be calculated with any computer program having a 4- or 5-parameter Logistic function.

Note: When sample volumes assayed differ from 20 μL, an appropriate mathematical adjustment must be made to accommodate for the dilution factor (e.g., if 10 μL of sample is used, then calculated data must be multiplied by 2). When sample volume assayed is Less than 20 μL, compensate the volume deficit with either matrix solution or assay buffer, whichever is appropriate.

XII. INTERPRETATION

1. The assay should be rejected if one of the two QCs falls outside of 2 standard deviations of the applicable mean. See the supervisor.
2. If the difference between duplicate results of a sample is >15% CV, repeat the sample.
3. The theoretical minimal detecting concentration of this assay is 6.5 pg/mL human PYY (20 μL sample size).
4. The dynamic range of this assay is 14 pg/mL to 1,800 pg/mL human PYY (20 μL sample size). Any result greater than 1,800 pg/mL in a 20 μL sample should be diluted using matrix solution or assay buffer as diluent, whichever is appropriate, and the assay repeated until the results fall within range.
S2: Total serum Glucagon Like peptide-1 (GLP-1) manufacturer instructions

VIII. REAGENT PREPARATION

A. GLP-1 Standard Preparation

1. Use care in opening the lyophilized Standard vial. Using a pipette, reconstitute the GLP-1 Standard with 0.5 mL distilled or de-ionized water to give a concentration described on the analysis sheet. Invert and mix gently until completely in solution.

2. Label five tubes as 1, 2, 3, 4, and 5. Add 200 µL Assay Buffer to each of the five tubes. Perform 3 times serial dilutions by adding 100 µL of the reconstituted standard to Tube 5, mix well and transfer 100 µL from Tube 5 to Tube 4, mix well and transfer 100 µL from Tube 4 to Tube 3, mix well and transfer 100 µL from Tube 3 to Tube 2, mix well and transfer 100 µL from Tube 2 to Tube 1. Mix well.

   Note: Change tip for every dilution. Wet tip with standard before dispensing. Unused portions of reconstituted standard should be stored in small aliquots at ≤ -20°C. Avoid multiple freeze/thaw cycles.

<table>
<thead>
<tr>
<th>Volume of Deionized Water to Add</th>
<th>Volume of Standard to Add</th>
<th>Standard Stock Concentration (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 mL</td>
<td>0</td>
<td>X (refer to analysis sheet for exact concentration)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tube #</th>
<th>Volume of Assay Buffer to Add</th>
<th>Volume of Standard to Add</th>
<th>Standard Concentration (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tube 5</td>
<td>0.2 mL</td>
<td>0.1 mL of reconstituted standard</td>
<td>X/3</td>
</tr>
<tr>
<td>Tube 4</td>
<td>0.2 mL</td>
<td>0.1 mL of Tube 5</td>
<td>X/9</td>
</tr>
<tr>
<td>Tube 3</td>
<td>0.2 mL</td>
<td>0.1 mL of Tube 4</td>
<td>X/27</td>
</tr>
<tr>
<td>Tube 2</td>
<td>0.2 mL</td>
<td>0.1 mL of Tube 3</td>
<td>X/81</td>
</tr>
<tr>
<td>Tube 1</td>
<td>0.2 mL</td>
<td>0.1 mL of Tube 2</td>
<td>X/243</td>
</tr>
</tbody>
</table>

B. GLP-1 Quality Control 1 and 2 Preparation

Use care in opening the lyophilized Quality Control vials. Reconstitute each GLP-1 Quality Control 1 and Quality Control 2 with 0.50 mL distilled or de-ionized water and gently invert to ensure complete hydration. Unused portions of the reconstituted Quality Controls should be stored in small aliquots at ≤ -20°C. Avoid further freeze/thaw cycles.
IX. GLP-1 TOTAL ELISA ASSAY PROCEDURE

Pre-warm all reagents to room temperature immediately before setting up the assay.

1. Dilute the 10X concentrated HRP wash buffer 10 fold by mixing the entire contents of both buffer bottles with 900 ml de-ionized or glass distilled water.

2. Remove the required number of strips from the Microtiter Assay Plate. Unused strips should be resealed in the foil pouch and stored at 2-8°C. Assemble the strips in an empty plate holder and fill each well with 300 µl diluted Wash Buffer. Decant wash buffer and remove the residual amount by inverting the plate and tapping it smartly onto absorbent towels several times. Wash assay plate using this procedure 2 additional times. Do not let wells dry before proceeding to the next step. If an automated machine is used for the assay, follow the manufacturer’s instructions for all washing steps described in this protocol.

3. Add in duplicate 50 µL Matrix Solution to Blank, Standards and Quality Control (refer to Section X for suggested sample order placement).

4. Add in duplicate 50 µL assay buffer to each of the Blank and sample wells.

5. Add in duplicate 50 µL GLP-1 Standards in the order of ascending concentrations to the appropriate wells. Add in duplicate 50 µL QC1 and 50 µL QC2 to the appropriate wells. Add sequentially 50 µL of the sample to the remaining wells.

Note: For mouse and rat samples use 20 µL of sample with 30 µL of assay buffer. Sample values will need to be multiplied by 2.5 for final concentration.

6. Cover the plate with plate sealer and incubate at room temperature for 1.5 hours on an orbital microtiter plate shaker set to rotate at moderate speed, about 400 to 500 rpm.

7. Remove plate sealer and decant solutions from the plate. Tap as before to remove residual solutions in well. Wash wells 3 times with diluted Wash Buffer, 300 µL per well per wash. Decant and tap after each wash to remove residual buffer.

8. Add 100 µL Detection Antibody Solution to each well. Re-cover plate with sealer and incubate at room temperature for 1 hour on an orbital microtiter plate shaker set to rotate at moderate speed, approximately 400-500 rpm.
IX. GLP-1 TOTAL ELISA ASSAY PROCEDURE (continued)

9. Remove plate sealer and decant solutions from the plate. Tap as before to remove residual solutions in well. Wash wells 3 times with diluted Wash Buffer, 300 µL per well per wash. Decant and tap after each wash to remove residual buffer.

10. Add 100 µL Enzyme Solution to each well. Cover plate with sealer and incubate with moderate shaking at room temperature for 30 min on the micro-titer plate shaker.

11. Remove sealer, decant solutions from the plate and tap plate to remove the residual fluid. Wash wells 3 times with diluted Wash Buffer, 300 µL per well per wash. Decant and tap after each wash to remove residual buffer.

12. Add 100 µL of Substrate Solution to each well, cover plate with sealer and shake on the plate shaker for **approximately** 5 to 20 minutes. Blue color should be formed in wells of the GLP-1 standards with intensity proportional to increasing concentrations of GLP-1 Total.

   **Note:** Please be aware that the color may develop more quickly or more slowly than the recommended incubation time depending on the localized room temperature. Please visually monitor the color development to optimize the incubation time.

13. Remove sealer and add 100 µL Stop Solution [CAUTION: CORROSIVE SOLUTION] and shake plate by hand to ensure complete mixing of solution in all wells. The blue color should turn to yellow after acidification. Wipe the bottom of the microtiter plate to remove any residue prior to reading on plate reader. Read absorbance at 450 nm and 590 nm in a plate reader within 5 minutes and ensure that there are no air bubbles in any well. Record the difference of absorbance units.
XI. CALCULATIONS

The dose-response curve of this assay fits best to a sigmoidal 4- or 5-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a 4- or 5-parameter logistic function.

Note: When sample volumes assayed differ from 50 µL, an appropriate mathematical adjustment must be made to accommodate for the dilution factor (e.g., if 25 µL of sample is used, then calculated data must be multiplied by 2). When sample volume assayed is less than 50 µL, compensate the volume deficit with Matrix Solution with the exception of mouse and rat samples. When using 20 µL of mouse and rat samples, compensate with 30 µL of assay buffer.

XII. INTERPRETATION

1. The assay will be considered accepted when all Quality Control values fall within the calculated QC range. If any QC s fall outside of the control range, review results with a supervisor.
2. If the difference between duplicate results of a sample is >15% CV, repeat the sample.
3. The limit of sensitivity of this assay is 1.5 pM GLP-1 Total (50 µl sample size).
4. The approximate range of this assay is 4.1 pM to 1000 pM GLP-1 Total (50 µl sample size). Any result greater than 1000 pM in a 50 µl sample should be diluted using Matrix Solution and the assay repeated until the results fall within range.
S3: Total serum ferritin concentrations manufacturer instructions

13. ASSAY PROCEDURE

- Equilibrate all materials and prepared reagents to room temperature (18 - 25°C) prior to use.
- It is recommended to assay all standards, controls and samples in duplicate.

13.1 Prepare all reagents, working standards and samples as instructed. Equilibrate reagents to room temperature before use. The assay is performed at room temperature (18-25°C).

13.2 Remove excess microplate strips from the plate frame and return them immediately to the foil pouch with desiccant inside. Reseal the pouch securely to minimize exposure to water vapor and store in a vacuum desiccator.

13.3 Add 50 μL of Ferritin Standard or sample per well. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed. Cover wells with a sealing tape and incubate for two hours. Start the timer after the last sample addition.

13.4 Wash five times with 200 μL of 1X Wash Buffer manually. Invert the plate each time and decant the contents; tap it 4-5 times on absorbent paper towel to completely remove the liquid. If using a machine wash six times with 300 μL of 1X Wash Buffer and then invert the plate, decant the contents; tap it 4-5 times on absorbent paper towel to completely remove the liquid.

13.5 Add 50 μL of 1X Biotinylated Ferritin Antibody to each well. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed. Cover wells with a sealing tape and incubate for one hour.

13.6 Wash microplate as described above.

13.7 Add 50 μL of 1X SP Conjugate to each well. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed. Cover wells with a sealing tape and
incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.

13.8 Wash microplate as described above.

13.9 Add 50 μL of Chromogen Substrate per well. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed. Incubate for 10 minutes or till the optimal blue colour density develops.

13.10 Add 50 μL of Stop Solution to each well. The color will change from blue to yellow. Gently tap plate to ensure thorough mixing. Break any bubbles that may have formed.

13.11 Read the absorbance on a microplate reader at a wavelength of 450 nm immediately. If wavelength correction is available, subtract readings at 570 nm from those at 450 nm to correct optical imperfections. Otherwise, read the plate at 450 nm only. Please note that some unstable black particles may be generated at high concentration points after stopping the reaction for about 10 minutes, which will reduce the readings.

14. **CALCULATIONS**

Calculate the mean value of the triplicate readings for each standard and sample. To generate a Standard Curve, plot the graph using the standard concentrations on the x-axis and the corresponding mean 450 nm absorbance on the y-axis. The best-fit line can be determined by regression analysis using log-log or four-parameter logistic curve-fit. Determine the unknown sample concentration from the Standard Curve and multiply the value by the dilution factor.
Appendices

Appendix 4 BAM files

All BAM files have the same layout as to the ones annotated in the main body of the chapter.

Analysis 1: Offspring (Homzygous), Mother, Father and Siblings (Heterozygous)

Family 1:
Family 3

Appendices

Human hgl9  chX  chX:101,857,518-101,857,578

101,857,518 bp  101,857,556 bp  101,857,593 bp  101,857,630 bp

BG1-825 hGam Coverage
BG1-826 hGam
BG1-826 hGam
BG1-827 hGam Coverage
BG1-827 hGam
BG1-827 hGam Coverage
BG1-827 hGam

Sequence: A G C A T G T G G A T G A A A T C G A G T C A T G A G G A T G A A A A A A A A

101,857,506

Human hgl9  chX  chX:131,219,946-131,219,986

131,219,946 bp  131,219,983 bp

BG1-825 hGam Coverage
BG1-826 hGam
BG1-826 hGam
BG1-827 hGam Coverage
BG1-827 hGam
BG1-827 hGam Coverage
BG1-827 hGam

Sequence: A G C A T G T G G A T G A A A T C G A G T C A T G A G G A T G A A A A A A A A

131,219,905

299
Appendices

Analysis 2: Mother (Heterozygous), Father and offspring

Family 1:
### Appendices

#### Human hg19

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<th>ChrM</th>
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</table>

#### Human hg19

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<thead>
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<th>chrM 8.840–8.880</th>
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</thead>
</table>

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**Sequence**

```
A A C G T T C C T C T E A T T G A C T C G T G C
```

**Strand loaded**

chrM.8.540

---

**Sequence**

```
G C C A T C C C C T T A T G A O C G O C C C C A G T O A T T A T A O C C T T C G
```

**Strand loaded**

chrM.8.540
Family 2:
Appendices
Family 3:
Study 2

Analysis 1: Offspring (Homozygous), Mother and Father (Heterozygous)

Family 4
Family 6:
Family 7

Analysis 2: Mother (Homozygous), Father and Siblings

Family 4
Family 6
REFERENCES

28. Shekhawat PS, Matern D, Strauss AW. Fetal fatty acid oxidation disorders, their effect on maternal health and neonatal outcome: impact of expanded newborn screening on their diagnosis and management. Pediatric research. 2005;57(5 Pt 2):78r-86r.


