The Effects of Maternal Obesity on Neonatal Anthropometry and Placental Regulation of Cytokine Production.

This thesis is submitted in accordance with the requirements of the University of Liverpool for the degree of Master of Philosophy.

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Abstract

Introduction: The prevalence of obesity is rising and maternal obesity has been implicated as a perpetuating factor. Maternal obesity has been linked to the development of macrosomic neonates who are at increased risk of becoming obese themselves. Current understanding of the mechanisms instigating this are unknown, however the pro-inflammatory status associated with obesity has been suggested.

Method: Anthropometric estimation of fat mass and percentage body fat was conducted on neonates of lean and obese women and their placentae analysed for morphological changes by light microscopy. Placental explants from both cohorts of women were incubated in order to determine the regulatory role of glucose, leptin, tumour-necrosis factor-α (TNF-α), and insulin on the production of interleukin-1 (IL-1β), interleukin-6 (IL-6), and TNF-α.

Results: Neonates of obese mothers were found to have increased birth weight, fat mass, fat free mass, and percentage body fat in comparison to neonates of lean mothers. The rise in birth weight, fat mass, fat free mass, and percentage body fat were not shown to significantly correlate to maternal booking BMI on univariate analysis. Placentae of obese women were shown to produce more IL-6 under basal conditions and in response to TNF-α stimulation. Glucose was shown to reduce placental production of IL-1β, IL-6, and TNF-α in both cohorts, and leptin and insulin stimulation had no effect on cytokine production. There was no evidence of placental inflammation on examination by light microscopy.

Conclusion: Maternal obesity is associated with neonates of increased birth weight and adiposity, and increased production of inflammatory mediators by the placenta which is not evidenced in overall placental appearance. It is hoped that by elucidating the mechanisms by which obesity may be perpetuated, via the in-utero environment for

example, foundations for future research can be established which are aimed at tackling the epidemic of obesity.

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Chapter 1: Introduction

1.1. Obesity:

1.1.1. Defintion:

Obesity develops when the energy intake of an individual is greater than their energy expenditure and, in adults, is defined by a body mass index (BMI) of greater than, or equal to, thirty[1]. In children, obesity is defined as having a BMI of greater than the 95th percentile based on UK age-specific reference population charts[2]. BMI is a simple weight-for-height index and is the most commonly used calculation for determining if an individual's weight is ideal. BMI is calculated by dividing weight in kilograms (kg) by height in metres squared (m^2). The classification system for BMI is listed in the table below[3]:

Calculated BMI (kg/m^2)	Classification
>18.5	Underweight
18.5-24.9	Ideal Weight
25-29.9	Overweight
30-39.9	Obese
>40	Morbidly Obese

Table 1.0 – Classification of BMI

These classifications are benchmarks for individual assessment and are not a direct correlation of overall adiposity. Two individuals with the same BMI are unlikely to have the same degree of fat mass. BMI does, however, provide a useful measure of obesity within the population as the same calculation is used for both sexes and all ages.

1.1.2. How Big a Problem is Obesity?

In 2008, the World Health Organisation (WHO) estimated that over 500 million adults worldwide were obese[4]. More worryingly, in 2010 the WHO estimated that at least 43 million children under the age of five years

were overweight. Their projections for the future are also ominous, with an estimated 700 million obese adults worldwide by 2015. Originally obesity was confined to high-income, developed countries however it is now becoming an increasing problem in low-income developing countries[4].

The picture for the United Kingdom (UK) is no better. In 2007, the Health Survey for England reported that amongst adults aged 16 years and over, 24% of men and women were obese, a rise of 11%, and 8%, respectively[5]. And, among boys and girls aged 2-15, 17% of boys were obese and 16% of girls were obese. By 2050, it is predicted that 60% of adult men, 50% of adult women and 25% of children will be obese. This trend is mirrored in other EU countries, but the UK rates are some of the highest[5].

The principal problem with obesity is the multitude of morbidities that are associated with it, and the list appears to be ever increasing, including such ailments as cardiovascular disease, diabetes mellitus, hypertension, stroke, and certain cancers[6].
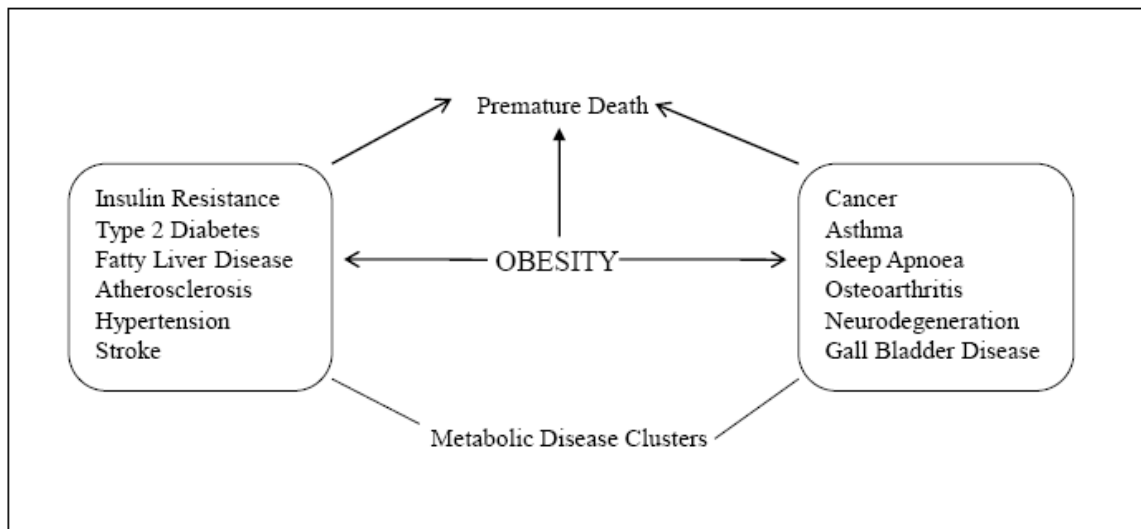

Figure 1-1 - Schematic diagram illustrating the clustering of morbidities associated with obesity[7].

It is believed that; 58% of type 2 diabetes mellitus; 21% of coronary heart disease; and between 8% and 42% of certain cancers (mainly endometrial, colon, and breast); are attributable to excess body fat[8]. Not only are there severe health implications associated with obesity but

there are many social disadvantages too. Many obese people are stigmatised and bullied, resulting in depression and low self-esteem[2].

As a result of the associated morbidities, obesity has been shown to reduce life expectancy by, on average, 9 years[9]. Each year in the UK, approximately 9000 premature deaths occur as a result of obesity, far exceeding the mortality rates of any other common disease[9]. Some recent data even suggests that, in the UK, obesity is responsible for up to seven percent of all morbidity and mortality[10].

In order to prevent increasing obesity-related mortality, the treatment of the associated morbidities falls to the NHS. In 2007, the direct cost of obese and overweight patients on the NHS was calculated at £3.23 billion, representing 5% of the total NHS budget[11]. This figure is set to double by the year 2050. The cost to the economy as a whole, including money lost through sick days or disability allowance, was £16 billion in 2007, and that is also set to rise to a staggering £50 million by 2050[11].

Obesity rates are rising worldwide, and currently, no country has managed to reverse this trend. In the modern world, fuelled by high-fat, high-sugar convenience food, our ability to store surplus energy, which was evolutionary developed to help withstand famine, has undoubtedly led to one of the biggest epidemics to ever face this planet[7]. Obesity threatens to jeopardise, not only the health and well-being of millions of people worldwide, but also the economic stability of their countries. It is imperative that the determinants of obesity and its associated morbidities are understood. Without doing so, therapeutic modalities to target obesity and prevent further escalation will be unable to be developed.

To elucidate all the determinants of the metabolic pathologies associated with obesity is beyond the scope of this thesis. Primarily, the literature review will focus on inflammation as a key mediator in the development of certain metabolic pathologies. Focus will then be paid on how this inflammatory state could potentially be conveyed to the in-utero environment, and how this may alter foetal growth.

1.1.3. Adipose tissue:

In humans, there are two forms adipose tissue, white and brown[12]. Brown adipose tissue is found mainly within the thorax and derives its' colour from rich vascularisation and densely packed mitochondria. Brown adipose tissue is of particular importance in neonates for body temperature regulation by thermogenesis[12].

Most excess energy is stored as triglycerides in white adipose tissue (WAT). The triglycerides are stored as a buffer for when energy intake does not match energy output and can be released as free fatty acids and glycerol[12]. Storing energy as triglycerides is an efficient storage of energy as one gram of fat can generate 9 kilocalories compared to one gram of carbohydrate or protein which only generates 4 kilocalories[13].

Until recent years, it was assumed that the only role of WAT was energy storage, and thermal and mechanical insulation[14]. However, WAT has now been recognised as a highly active endocrine organ, secreting several major hormones, principally leptin and adiponectin, and a range of other protein factors, which are now collectively known as 'adipokines'[14]. Currently, over fifty adipokines have been recorded, and their functions have been described by categorising them into groups:

Category / Function of Adipokine	Examples of Adipokine within each Category
Classical cytokines	Tumour necrosis factor-α (TNF-α), Interleukin-6 (IL-6), Interleukin-8 (IL-8)
Growth factors	Transforming growth factor-β
Alternative complement system	Adipsin, Acylation-stimulating protein
Vascular haemostasis	Plasminogen activator inhibitor-1 (PAI-1), Tissue factor
Regulation of blood pressure	Angiotensinogen
Lipid metabolism	Retinol-binding protein
Glucose homeostasis	Leptin, Adiponectin, Resistin
Angiogenesis	Vascular endothelial growth factor (VEGF)
Acute-phase and stress responses	Haptoglobin, Metallothionein

Table 1.1 - Table to show categories and functions of adipokines secreted by WAT[14].

Even without specifying any detail, it is clear by just looking at Table 1.1 that WAT secretion of adipokines has widespread effects. Therefore, it is not surprising that when there is relentless nutritional overload, and excess WAT is accumulated, many physiological processes become asynchronous, disrupting homeostasis and resulting in metabolic abnormalities. Obesity is characterised by the following physiological changes:

1.1.4. Hyperleptinaemia:

Due to the excessive volume of adipose tissue, obesity is associated with hyperleptinaemia and leptin resistance[15]. Leptin is a 16-kDa polypeptide hormone, and is primarily synthesised by adipose tissue and is coded for by the obese (*ob*) gene[15]. The production and secretion of leptin is positively correlated with adipocyte volume and number, such that larger adipocytes produce more leptin than smaller adipocytes, explaining why leptin levels are elevated in obesity[16].

Leptin exerts is biological actions through interaction with its specific surface receptor, OB-R[15]. The OB-R receptor mediates leptin action by activation of a multitude of divergent signal transduction pathways and transcription factors, including mitogen activated protein kinase (MAPK), nuclear factor-κB (NF-κB), and peroxisomal proliferator-activated receptor-γ (PPARγ)[15].

The primary function of leptin is to act locally, and centrally, co-ordinating metabolism and nutrient availability[17]. When nutrients are in excess and adipocytes are saturated, leptin acts as a satiety factor in the hypothalamus to suppress appetite and increase energy expenditure[18]. In order for leptin to exert its effects on the hypothalamus, it must first be transported across the blood-brain barrier by a transporter[19]. When high leptin plasma concentrations are reached, this transporter becomes saturated and its ability to transport leptin becomes impeded[19]. This leads to decreased suppression of appetite, which subsequently leads to excessive food intake[18]. This then increases the volume of adipose tissue, leading to increasing hyperleptinaemia, escalating the problem further. Thus, hyperleptinaemia is associated with a decreased energy homeostasis[18].

Leptin has also been found to interact with insulin, in what is termed the 'Adipoinsular Axis'[20]. Insulin has an adipogenic effect on adipocytes, increasing energy storage, which is an unwanted process if nutrients and energy storage are already in excess. Therefore, leptin exerts an inhibitory effect on pancreatic β-cells and reduces insulin secretion which, in turn, prevents further energy storage within adipose tissue. The same process is also thought to work in reverse; as adipose stores decrease so too does leptin production, which then permits an increase in insulin production, and thus, increases the deposition of adipose stores[20].
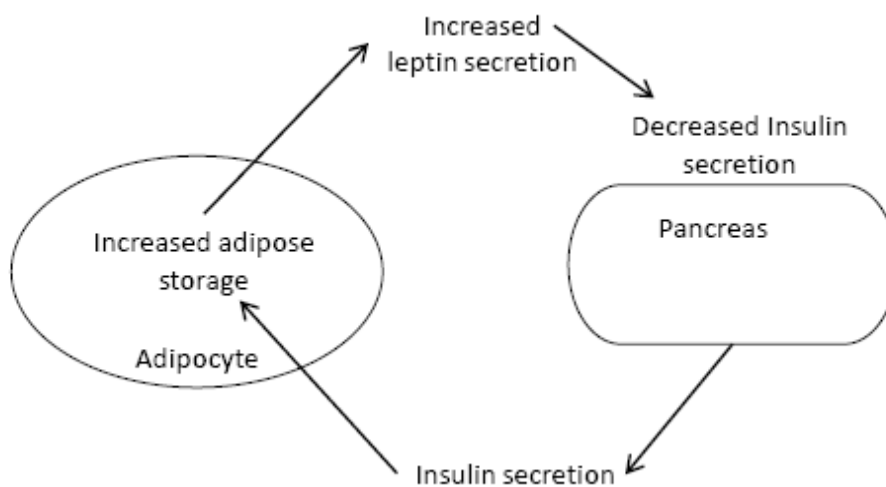
Figure 1-2- Schematic diagram of the Adipoinsular axis

Therefore, it is not hard to see how, if leptin levels were elevated, insulin secretion from the pancreas would be inhibited leading to disturbed glucose homeostasis leading to the development of hyperglycaemia.

Another adipokine, known to be altered in obesity, and which is also important for glucose homeostasis and insulin sensitivity is adiponectin.

1.1.5. Decreased Adiponectin Levels:

Adiponectin is a 30-kDa protein, which was thought to be produced, and secreted, exclusively by adipocytes[21]. Recent data now suggests that other cell types may contribute to adiponectin production; however the specific cell types involved is currently unknown. Apidonectin exerts its actions through two main receptors; AdipoR1, and AdipoR2[22]. AdipoR1 and AdipoR2

have been shown to increase AMP kinase (AMPK) and PPARα activity, respectively. This activation leads to increased insulin sensitivity through fatty acid oxidation and increased glucose uptake. AdipoR1 has been shown to be present in the hypothalamus and increased AMPK activity is linked to increased food intake[22].

It would therefore appear that leptin and adiponectin work as antagonists of one another. When adipose stores are high, leptin is secreted decreasing insulin sensitivity, and food intake. And when adipose stores are low, adiponectin is secreted increasing insulin sensitivity, and food intake. One could therefore assume that the two hormones work in synchrony to maintain an appropriate level of food intake and energy storage[22]. This assumption is further supported by the fact that circulating plasma concentrations of adiponectin are inversely related to circulating plasma concentrations of leptin[19]. Therefore, as circulating leptin levels rise with increased adiposity, adiponectin levels fall[19]. Decreased adiponectin levels then lead to diminished insulin sensitisation, and coupled with the effects of leptin, insulin resistance and hyperglycaemia ensue. For this reason, both hyperleptinaemia and decreased adiponectin, are positively associated with the development of insulin resistance and type 2 diabetes mellitus[12].

1.1.6. Chronic-Low Grade Inflammation:

An important and recent development is the finding that obesity is characterised by chronic, low-grade inflammation[23]. The term 'low-grade' inflammation is used to distinguish this type of inflammation, found in obesity, from the classical inflammation found in response to injury. Rarely are the classical symptoms, such as redness, swelling, pain, and fever, associated with obesity-induced inflammation. Although some of the same mediators and pathways are used, it must be made clear that the acute inflammation seen in response to injury or infection is very different to the long-term inflammation witnessed in obesity[7]. Obese individuals have been reported as having increased circulating levels of the pro-inflammatory factors TNF-α, IL-6, IL-8, and IL-18, and increased circulating levels of the acute-phase proteins C-reactive protein (CRP) and haptoglobin[23].

1.1.6.1. Acute Phase Proteins:

Other acute phase proteins, mainly pro-coagulant proteins, known to be positively correlated with obesity are tissue factor, PAI-1, and factor VII[16]. Synthesis of PAI-1 has been shown to originate from within WAT, however the source of the other factors is less clear. PAI-1 is a crucial factor in the maintenance of vascular homeostasis, inhibiting the action of plasminogen, the precursor of plasmin, thus inhibiting the degradation of fibrin. Together, with the other pro-coagulant proteins, raised PAI-1 levels permit fibrin accumulation, resulting in atherothrombotic disease. This clearly demonstrates how one of the morbidities associated with obesity can be directly linked to alterations in adipokine production, secondary to the increased fat mass[14].

However, the biggest problem associated with obesity, is the raised levels of pro-inflammatory proteins as they have widespread effects on multiple physiological processes.

1.1.6.2. Pro-inflammatory Markers:

Circulating TNF-α levels are raised in obesity, and although TNF-α is expressed in, and secreted by, adipocytes, the vast majority of TNF-α is secreted by cells of the stromal vascular and matrix fractions, including macrophages[16]. The extent to which adipocytes contribute to the elevated circulating levels of TNF- α is still under debate. It should be noted, that in lean individuals WAT has not been identified as a major source of TNF-α.

TNF-α is said to act as an influential local regulator, stimulating many processes including apoptosis[24]. It also acts as a regulatory factor in the production of other adipokines, mainly IL-6, which is also secreted by adipocytes. In obesity, IL-6 circulating levels are also elevated, which has been attributed to WAT secretion secondary to the finding that IL-6 gene expression is increased within WAT of obese individuals. The functional role of IL-6 in obesity is unknown, however IL-6 receptors have been found within the hypothalamus, indicating that it may exert direct central actions[24]. Therefore, in addition to leptin, IL-6 may contribute to energy regulation by conveying information from adipocytes to the hypothalamus[14].

TNF-α has also been shown to stimulate IL-8 release and, once again, increased gene expression has been found in WAT suggesting it is the source for raised IL-8 levels in obesity[14]. IL-18 has also been found to be raised in obesity, and levels have been shown to decline with weight loss, leading to speculation that it also originates from WAT; however this has yet to be proven[14].

A key question, which remains to be fully answered, is what is the source of the raised pro-inflammatory cytokines? Current research speculates that there are three possibilities:

1. The inflammatory markers originate from a source other than adipose tissue, primarily the liver and the cells of the immune system[14];

2. The inflammatory markers originate from an alternative source but are stimulated to do so by factors secreted by the WAT e.g. hepatic production of CRP is known to be increased in response to IL-6 secretion from the expanded WAT[14];

3. The inflammatory markers originate from within the adipocytes themselves and the increased plasma levels are 'spill over' from the overall increased adipose mass[14].

The most prevalent hypothesis is that the excess WAT initially produces excess cytokines which subsequently leads to the stimulation of cytokine production by immune cells which have been recruited to the WAT[7]. In the context of obesity, WAT is known to contain macrophages, and macrophage precursor cells; however what is not known is why the macrophages are initially recruited[7].

Several theories propose that in the obese individual, adipocytes begin to secrete low levels of TNF-α which, in turn, stimulates both pre-adipocytes, and endothelial cells, to produce monocyte chemoattractant protein-1 (MCP-1), leading to the recruitment of macrophages[7]. Research in mice, has suggested that the initial cytokine secretion by the adipocytes may be as a consequence of hypoxia[14]. Research has shown that as obesity progresses and fat mass increases, the supporting vasculature is overwhelmed with the increased metabolic demands leading to oxidative injury of the vasculature and hypoxia within the cells. The hypoxic adipoctyes then respond by secreting inflammatory markers, such as TNF-α and IL-1β, in an attempt to stimulate angiogenesis. This, in turn, then

activates transcription factors, such as hypoxic-inducible factor-1 (HIF-1) within the adipocytes, and stromal vascular cells[14]. HIF-1 then targets the genes encoding for vascular endothelial growth factor (VEGF), plasminogen activator inhibitor-1 (PAI-1) and leptin. This then leads to altered angiogenesis and an increase in blood supply[14].

In addition to the altered angiogenesis, hypoxia also induces necrotic death of large numbers of adipocytes[18]. It should be noted that necrosis-like death is the primary method of cell death in WAT, opposed to apoptotic cell death, and unlike apoptosis, necrotic cells die in an uncontrolled manner, requiring macrophages to engulf the necrotic debris. Therefore, inflammatory mediators, such as MCP-1, are released by the necrotic cells[18]. MCP-1 secretion coupled with TNF-α and IL-1β secretion, leads to large numbers of macrophages being recruited into adipose tissue, which also adhere to the endothelial cells[23]. It is presumed that macrophage recruitment, in this case, would be for phagocytosis of the necrotic adipocytes[23].

However, there are also some alternative theories about macrophage recruitment into WAT. Other research, in mice and humans, has shown that there is increased macrophage gene expression directly proportional to body weight, suggesting that macrophage content of adipose tissue is correlated to adiposity and BMI[16]. Accumulation of macrophages within the WAT increases as adipocyte volume increases, and along with BMI, can be used as a predictor for macrophage numbers within WAT[16]. In this instance, the role of the macrophages may be to act as a paracrine messenger between enlarged adipocytes whose function has become altered as a result of obesity[16]. Other research has suggested that the macrophages infiltrate the adipose tissue first and that the ensuing inflammatory response is a direct result of the invading macrophages[23]. However, their initial stimulus for invasion, and/or their function within the WAT, has not specified, and therefore, these remain rather inconclusive theories.

Nonetheless, regardless of their function, or the initial recruitment stimuli, these recruited macrophages, along with the adipocytes, are integral to the production of the pro-inflammatory cytokines associated with obesity[23]. The initial macrophage recruitment culminates in a vicious cycle of increased cytokine production leading to increased macrophage recruitment[23]. Adipocyte dysfunction is disturbed further with exacerbated alterations in gene expression, mainly related to metabolic and inflammatory pathways, leading to an overall worsening inflammatory and metabolic status of the obese individual[23].
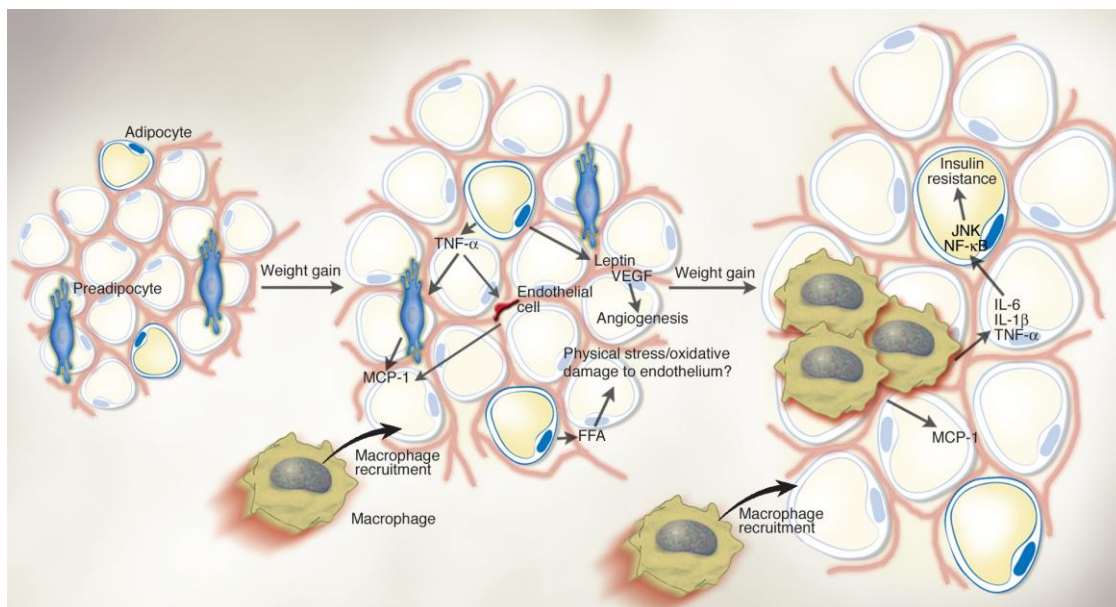
Figure 1-3 - Diagram to illustrate perpetuating cycle of macrophage recruitment and increased production of pro-inflammatory cytokines.[23]

Metabolic dysfunction is mainly instigated by the profound inhibitory effects the pro-inflammatory cytokines, especially TNF-α, have on insulin sensitivity and action[25].

Initiation of the insulin signalling pathway is induced by insulin binding to an insulin receptor (IR), resulting in receptor auto-phosphorylation and, subsequent phosphorylation of IR substrates (IRS)[25]. IRSs, in turn, associate and activate down-stream effectors which ultimately have the ability to regulate, and induce, end-point glucose transporters[25].

TNF-α has been shown to promote serine phosphorylation of IRS-1, impairing its association with the IR, inhibiting insulin signalling, and insulin-regulated glucose uptake[25]. Ultimately this mediates an insulin resistant state. Research has proven that when exogenous TNF-α is administered to obese humans, the development of insulin resistance is promoted[25]. There is also evidence demonstrating that obese mice with non-functioning TNF-α have improved insulin sensitivity and better glucose homeostasis[26]. These findings support the theory that TNF-α, and the activation of inflammatory pathways, are critical for the regulation of insulin action in the obese individual. Whether TNF-α is solely responsible for the effect on insulin action, or it is the inflammatory pathways, remains to be discovered[7]. TNF-α and IL-6 have also been implicated in the development of hypertriglyceridaemia and raised serum fatty acid concentration which is thought to originate by stimulating lipolysis and increased hepatic triglyceride secretion[27].

Cumulatively, all these disturbances in adipokines, including: leptin, adiponectin, TNF-α, and interleukins; leaves the obese individual exposed to perturbations in glucose and triglyceride homeostasis, and alterations in blood coagulation. Thus, as a direct result of increased WAT obese individuals are predisposed to the development of hypertriglyceridaemia, insulin resistance, and atherothrombotic disease[7].

The physiological changes documented by no means explain all of the associated morbidities with obesity but they do go part way to explaining the origins of the clustering of metabolic diseases surrounding obesity. This 'clustering' of diseases has subsequently been termed, 'Metabolic Syndrome' and is said to encompass: obesity, insulin resistance, glucose intolerance, hypertriglyceridaemia, low high-density lipoprotein (HDL) cholesterol, hypertension and accelerated atherosclerosis[28]. Individuals with metabolic syndrome

are at increased risk of type 2 diabetes mellitus and cardiovascular disease[7].

What is intriguing is that over the past few decades, it has been noticed that these metabolic pathologies are beginning to emerge in children. In 2000, the first cases of type 2 diabetes mellitus were reported in overweight girls aged nine to sixteen[29]. There are now approximately 1,400 children diagnosed with type 2 diabetes mellitus in the UK[29].

One factor believed to be involved in the increasing incidence of metabolic pathologies in children is maternal obesity[30]. Epidemiological data has indicated that the intrauterine period is one of vulnerability, and if present, a suboptimal intrauterine environment, such as the chronic low-grade inflammation associated with obesity, has the potential to jeopardise the long-term health of the foetus[30]. Barker et al coined the phrase, 'foetal origins of health', to describe the molecular and physiological changes that can occur in foetal tissue secondary to an early in-utero event, or ongoing environment such as obesity, leading to altered growth[31].

1.1.7. Metabolic Syndrome and its Link with Maternal Obesity:

Longitudinal studies have proven that maternal obesity is a risk factor for the development of metabolic syndrome in offspring[32]. Neonates whose birth weights are greater than, or equal to, the 90th centile are said to have the greatest risk of developing metabolic syndrome in later life[33]. One study reported that infants of obese mothers were two and a half times more likely to be obese at 2-4 years of age when compared to infants of lean mothers[34]. Whether this trend could be attributed to the postnatal environment was not commented upon and therefore could be an contributing factor to this finding.

Several studies have shown that there is a positive linear relationship between maternal BMI and neonatal birth weight[35].

Catalano et al stated that, over the past thirty years there has been a mean increase in birth weight by 116g in term singleton pregnancies, and that maternal weight at delivery correlated most strongly with this increase[36]. This is in conjunction with other studies conducted in North America and Europe, which also reported a correlation between maternal obesity and increased birth weight[37,38].

Studies have shown that much of the variability in birth weight of neonates can be accounted for by the volume of adipose tissue present[39]. Foetal lean mass has been shown to remain relatively constant, even between small-for-gestational-age (SGA) and large-for-gestational-age (LGA) foetuses, and changes in a consistent manner over time[40]. This is unlike foetal fat mass which vary significantly between neonates[30]. Neonates of obese mothers when compared to neonates of lean mothers have been shown to have significantly increased fat mass and percentage body fat[30]. Studies have also demonstrated that neonates of obese mothers have a raised ponderal index[41]. Ponderal index is a neonatal anthropometric measure of obesity, which is the equivalent to BMI in adults, and is calculated by birth weight (kg)/length^3 (m^3)[42]. Thus neonates of obese mothers are not only more likely to be heavier, but they are also to be more obese.

There is even some evidence to suggest that metabolic compromise is apparent before birth[41]. In a cohort study of fifty three lean and sixty eight obese women, Catalano et al measured the umbilical cord concentrations of insulin, glucose, leptin, and IL-6. They reported that the neonates of the obese women had higher fasting cord levels of insulin, glucose, and leptin, and concluded that the neonates were more insulin resistant than their lean counterparts. The neonates were also found to have increased adiposity which positively correlated to the level of insulin resistance. Insulin resistance was also reported to be positively correlated to maternal pre-gravid BMI. This study, therefore illustrates a link between

maternal pre-gravid BMI and the development of foetal metabolic compromise in utero, and the importance maternal pre-gravid BMI has on foetal development[41].

Maternal weight gain during pregnancy has also been highlighted as an important factor associated with neonatal birth weight. Rossner and Ohlin studied the relationship between birth weight, initial maternal weight, and total maternal weight gained during pregnancy of 2,295 women of varying BMI in Stockholm[43]. Total maternal weight gain was shown to be the strongest predictor of birth weight, followed by initial maternal weight. This positive correlation was only demonstrated up until a BMI of 24kg/m^2, which after this point the strength of association decreased[43].

This somewhat confuses the literature as this finding would indicate that maternal obesity is not positively associated with a rise in birth weight. However, this may be explained by the fact that maternal obesity is not only associated with macrosomic neonates but also SGA neonates[44]. Maternal obesity has been implicated in the development of both macrosomia and SGA neonates and, interestingly, both groups are at increased risk of developing metabolic syndrome[45]. To date, the literature does not yet have any definitive evidence specifying the patho-physiology linking the development of these two neonatal outcomes to maternal pre-gravid obesity and also to the subsequent development of metabolic syndrome[46].

Before proceeding, it is important the some definitions are clarified. Macrosomia is classified as a birth weight of greater than 4000 grams, irrespective of gestational age[47]. Whereas, LGA is typically defined as a birth weight greater than or equal to the 90% centile on a birth weight centile chart[48]. The terms appear to be used interchangeably and are both associated with multiple obstetric and neonatal problems. Therefore, it was decided that the term macrosomia will be used throughout this thesis.

For the purposes of this thesis, the literature review will explore the impact of maternal obesity and its associated chronic low-grade inflammation on placental function and foetal development, focusing primarily on the mechanisms leading to macrosomic term neonates, and possibilities linking this to the development of metabolic syndrome.

1.1.8. Placental Factors Implicated in Foetal Growth and Alterations Related to Maternal Obesity:

Pregnancy is a natural state of inflammation and altered metabolism which is critical for the development of the foetus. When this is combined with pre-gravid obesity and its associated metabolic dysfunction, an even more disturbed metabolic state is created. Obese pregnant women have been shown to have dyslipidaemia, hyperinsulinaemia, hyperleptinaemia, cytokine up-regulation and impaired endothelial function[49]. There has been a wealth of studies exploring the effect of these metabolic disturbances on pregnancy, and foetal outcome, however; the precise full effect is still yet to be elucidated.

Maternal nutrition and metabolism, utero-placental blood flow, trans-placental substrate concentration gradient, placental size, and its solute transfer capabilities, *all* affect placental function and subsequent foetal growth[50]. However, as the interface between mother and foetus, providing all foetal support, the placenta is one of the most important factors governing foetal growth[51].

1.1.8.1. Placental Structure:

The placenta is a circular discoid organ which is approximately twenty two centimetres in diameter and weighs roughly 500grams. Morphology of placentae varies greatly and is dependent on a number of factors such as delivery mode[52].

The placenta is mainly derived from trophoblastic tissue which differentiates from external cells from the morula as it becomes a

blastocyst[52]. Once the blastocyst has attached to the endometrium the polar trophoblast differentiates and its mononuclear cells fuse to form the syncytiotrophoblast[53].
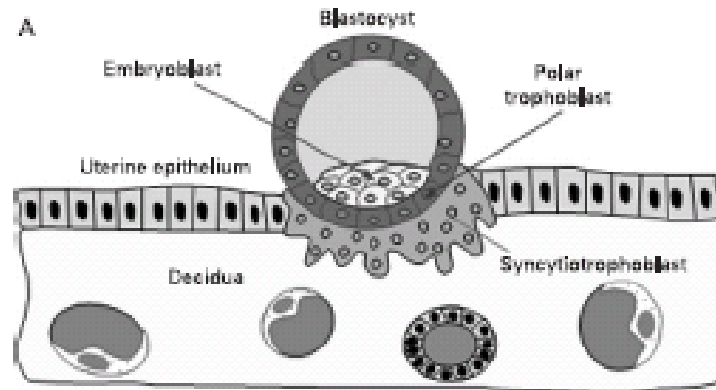
Figure 1.4 – Figure to show formation of the placenta from the blastocyst[53].

The syncytiotrophoblast then rapidly proliferates invading the uterine epithelium cells and at around day twelve the embryo is completely implanted[53]. The remaining mononuclear trophoblastic cells are now referred to as the cytotrophoblast, and form a second inner circle inside the syncytiotrophoblast. The cells of the cytotrophoblast act as stem cells and ultimately fuse with the syncytiotrophoblast. At around nine days, lacunae form within the syncytiotrophoblast establishing foetal-placental circulation. Maternal and foetal circulations are separated by the syncytiotrophoblast and the foetal capillary endothelium[52]. The syncytiotrophoblast which remains in between the lacunae are referred to as trabeculae and cumulatively these two sections go on to form the villous trees and intervillous spaces of the placenta[53]. The syncytiotrophoblast has two aspects: the microvillous plasma membrane (MVM) (maternal aspect) and the basal plasma membrane (BM) (foetal aspect)[54]. And it is through these two membranes that nutrient and waste transport from mother to foetus, and vice versa, is thought to be regulated[55].

1.1.8.2. Altered Insulin Sensitivity and Glycaemic Control:

The ability to regulate nutrient balance during pregnancy is critical to maternal and foetal health. In a normal pregnancy the placenta secretes a multitude of hormones including; oestrogen, progesterone, cortisol, human placental lactogen (HPL), prolactin and growth hormone[56]. These hormones are believed to create an insulin resistant state, which has profound effects on maternal energy metabolism. In fact, insulin sensitivity is thought to decrease by around sixty percent[57]. This insulin resistant state is necessary because, in utero, the foetus primarily relies on glucose as its main energy substrate[58]. As gestation progresses, foetal demand increases, and so too does placental hormone secretion, decreasing maternal insulin insensitivity further[59]. Studies have shown there is also a thirty percent increase in hepatic production of glucose and elevations of blood glucose levels are more frequent and pronounced during pregnancy[53,60]. Obviously these adjustments in glucose levels and insulin sensitivity are not completely unopposed otherwise this would result in the development of gestational diabetes mellitus (GDM). Therefore, there is also an increase in endogenous secretion of maternal insulin[61].

This situation however appears to be altered in the context of obesity. Non-diabetic, obese pregnant women have been demonstrated to have hyperinsulinaemia and they have also been shown to be less sensitive, or more resistant, to insulin than overweight, or lean, mothers[41]. As maternal BMI increases insulin sensitivity has been shown to diminish[41]. This may be attributed to the fact that non-pregnant obese women are initially more insulin resistant than non-pregnant lean women as a result of hormone and cytokine derangements associated with obesity[54]. The difference in insulin sensitivity is most pronounced during early pregnancy, with a less prominent difference in late pregnancy[54].

The decreased insulin sensitivity of obese mothers predisposes them to episodes of mild hyperglycaemia. If the episodes were more pronounced and long term, GDM would be diagnosed, however not all obese mothers are diagnosed with GDM. There are studies to suggest that these brief excursions away from euglycaemia may be sufficient to induce foetal overgrowth. Ericsson et al induced transient states of hyperglycaemia in pregnant rats in early pregnancy, and observed that as few as three episodes of transient hyperglycaemia in early pregnancy, but not late pregnancy, were sufficient to cause foetal overgrowth[62]. As Catalano demonstrated that insulin insensitivity is most prominent in early pregnancy in obese mothers, this seems a plausible mechanism for foetal overgrowth.

Several hypotheses have been suggested with regard to the mechanism by which maternal hyperglycaemia may lead to foetal overgrowth. The first hypothesis was suggested in 1954 by Pederson[63]. He observed that many women who suffered from GDM during their pregnancies produced macrosomic foetuses. He proposed that there must be a link between the maternal hyperglycaemia and the development of foetal macrosomia[63]. It was known that placental glucose transfer was mediated by facilitated diffusion, and that this was greatly dependent upon maternal glucose levels. For that reason, Pederson hypothesised that maternal hyperglycaemia could promote increased placental glucose transfer, resulting in foetal hyperglycaemia and subsequently, foetal hyperinsulinaemia[63]. This seems a reasonable theory for mediating foetal overgrowth, as insulin is known to have profound effects on foetal growth patterns, stimulating the growth of all foetal tissues except for the brain[64]. Therefore, foetal macrosomia many originate by this mechanism.

However, foetal macrosomia has also been observed in diabetic mothers with strict glycaemic control. If Pederson's theory were absolute then these women, should deliver appropriate-for-gestational-age (AGA) infants[65]. This would suggest that

hyperinsulinaemia, secondary to maternal hyperglycaemia, is not the only mechanism leading to increased foetal growth and fat accretion in foetuses of obese/diabetic mothers.

1.1.8.3. Leptin:

Another hormone which is known to be elevated in obese pregnancy is leptin, which is not surprising given that leptin is raised in the non-pregnant obese individual. In fact, leptin levels in obese mothers are said to double that of the circulating levels in lean mothers[41].

In non-obese pregnancy, leptin is known to peak in the second trimester, plateau in the third, and then fall below pre-gravid concentration around birth[66]. Although the rise in leptin could be attributed to the increase in adipose tissue gained during pregnancy, changes in maternal BMI do not correlate with the gestational increase in leptin concentration[67]. The placenta has been shown to mainly secrete leptin into maternal circulation with a smaller, but significant, amount being released into the foetal circulation. Secretion in foetal circulation is evidenced by higher concentrations of leptin in the umbilical vein than the umbilical arteries[67]. There is also a marked postnatal decrease in leptin levels in both mother, and foetus, and for that reason the placenta has been ascribed as the principal source for raised leptin levels experienced during pregnancy[68]. Within the placenta, leptin mRNA and protein is known to be co-localised to the syncytiotrophoblast, and cytotrophoblast and appears to be identical to leptin produced by adipose tissue; with the only exception being that placental leptin has an upstream enhancer, suggesting that its regulation is different[69].

Concentrations of leptin within umbilical cord blood have been positively correlated with foetal birth weight, length, head circumference, and ponderal index, suggesting a regulatory role of

leptin in foetal growth[70]. SGA neonates have been associated with low concentrations of circulating and placental leptin, and macrosomic neonates of diabetic mothers have been associated with raised concentrations of circulating and placental leptin[71]. One hypothesis how leptin may regulate foetal growth is through stimulation of amino acid nutrient transporters. Jansson et al cultured primary villous fragments from placentae obtained from term, uncomplicated deliveries and stimulated them with leptin and insulin. Leptin, in a concentration dependent fashion, was shown to increase the uptake of an amino acid transporter called System A[72].

System A is widely expressed on the MVM of the syncytiotrophoblast and mediates sodium-dependent uptake of neutral amino acids, and is implicated in foetal growth[73]. There are three isoforms of the sodium-coupled neutral amino acid transporter (SNAT); 1, 2, and 4. These are encoded for by the genes *Slc38a1*, *Slc38a2*, and *Slc38a4*. SNAT1 and SNAT2 operate via similar means, whereas SNAT4, has a lower affinity for neutral amino acids, and mediates the uptake of cationic amino acids[74].

As leptin has been proven to stimulate increased transport by system A, this poses a possible mechanism for leptin regulation of foetal growth. Pregnancies complicated by diabetes, where macrosomic foetuses are common, have been shown to have increased system A activity.[61] Therefore, it would not seem foolish to speculate that the raised levels of leptin, present in obesity, might lead to increased system A activity and mediate increased foetal growth in that manner. However, studies have shown that reality is, in fact, to the contrary.

Farley et al conducted similar studies to Jansson et al, but instead compared placental system A activity in placentae from seven lean and seven obese women[75]. Placental leptin protein content and leptin

receptor expression were also measured. Placentae of obese women, with circulating hyperleptinaemia and appropriate-for-gestational-age infants, were shown to have decreased SNAT 4 activity and decreased syncytiotrophoblastic expression of the leptin receptor and SNAT 4. In contrast, leptin was found to significantly stimulate system A in the placentae of lean women which is in agreement with Jansson et al[72]. Farley et al concluded that due to the increased leptin levels in the obese women down-regulation of the leptin receptor was precipitated, resulting in leptin resistance[76]. This research would imply that raised leptin levels, in association with obesity, are not involved in the up-regulation of system A and subsequent foetal overgrowth[44]. However, this finding may be attributed to the fact that the obese women had delivered AGA neonates, opposed to macrosomic neonates. It would therefore be useful to conduct this study again and examine the effect leptin has on system A activity in the placentae of obese women who deliver macrosomic infants. Having said that, this study highlights an important finding; that placentae from obese women appear to be leptin resistant.

Placental leptin has also been implicated in the regulation of placental growth during pregnancy[77]. Umbilical cord concentrations of leptin have been shown to correlate with placental size suggesting that leptin may act in a paracrine manner stimulating placental growth, possibly by angiogenesis; however the precise mechanism is unknown[78]. Studies have demonstrated that there is a positive correlation between uncomplicated, full term, singleton placental weight and increased maternal BMI[79]. Therefore, increased placental size, secondary to hyperleptinaemia in the obese mother, could potentially result in increased nutrient transfer and foetal growth. How this is actually mediated, remains to be established.

As the intracellular signalling mechanisms are so poorly understood, even in lean pregnancy, it is hard to determine whether elevated circulating leptin levels have a regulatory role in increased foetal growth. The raised levels associated with obesity could simply represent an overall increase in placental and adipose mass. However, it is possible that leptin is implicated in the regulation of cellular tissue growth, opposed to overall foetal growth. In the majority of studies examining the regulatory role of leptin, birth weight is used as the primary outcome measure, whereas animal studies examining specific cells, such as pancreatic islet cells, have shown that leptin can induce cell proliferation[80]. Muhlhausler et al conducted studies in sheep and demonstrated that prenatal exposure to increased maternal nutrition and hyperleptinaemia during late gestation resulted in increased fat deposition in postnatal life[81]. Increased leptin mRNA in perirenal and subcutaneous adipose tissue were also reported[80]. Thus they have demonstrated how maternal over-nutrition alters postnatal adipose stores[81]. More importantly, further studies in sheep and non-human primates by Muhlhausler et al demonstrated that the nutritional environment to which a foetus is exposed in utero and perinatally has long term consequences on the function of appetite regulation within the brain affecting its feeding behaviour and energy balance throughout its lifetime[82]. These findings illustrate how an infant may be predisposed to obesity in later life simply by in-utero exposure to over-nutrition. These findings remain to be proven in humans but implicate hyperleptinaemia as a potential regulator in determining the development of metabolic syndrome in later life.

This, however, still does not explain how maternal obesity leads to foetal overgrowth. Due to the altered levels of cytokines known to be associated with obesity, research has therefore explored this avenue to see if this sheds any light on the situation.

1.1.8.4. Inflammatory cytokines:

Non-obese pregnancy is associated by raised circulating maternal TNF-α levels, which initially decrease during early pregnancy, but then increase in late pregnancy[55]. Like leptin, maternal circulating TNF-α levels drop rapidly after delivery and therefore, the rise in TNF-α has been ascribed to the placenta[83].

Placental resident macrophages, also known as Hofbauer cells, syncytiotrophoblastic cells, and cytotrophoblastic cells have all been shown to produce and secrete the following pro-inflammatory factors; IL-1β, IL-6 and TNF-α[15]. The individual contribution of each cell line to the cytokine network is difficult to determine due to overlapping cell-cell communications. Virtually all cell types identified in utero-placental tissues have been shown to participate in cytokine networks[26].

The role of pro-inflammatory cytokines in pregnancy is, however, a little unclear. Research has implicated some placental cytokines in the establishment of pregnancy; IL-1β is known to be implicated in implantation.[84] One prevalent hypothesis is that these placental cytokines contribute towards the alterations in maternal metabolism, which is illustrated in Figure 1:4.

Figure 1-5 - Schematic overview of the production of placental cytokines, and the physiological processes they affect[26].
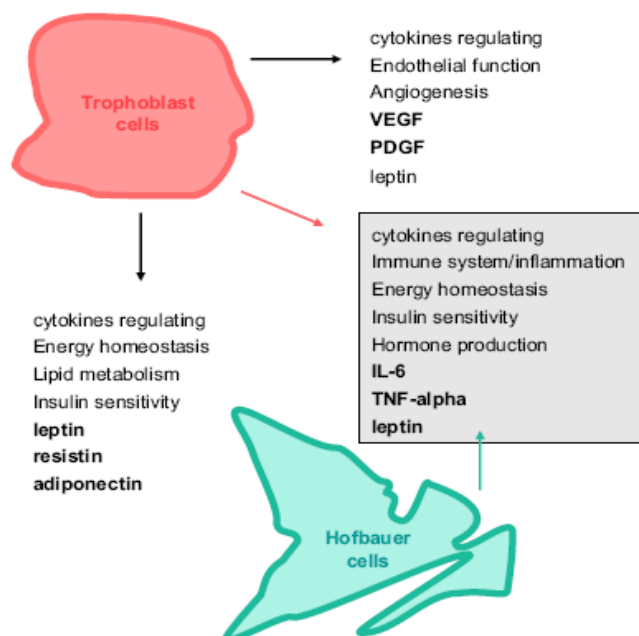
Once villous circulation is established, TNF-α, and IL-6 are secreted into the maternal circulation, with a minority entering the foetal circulations[79]. These cytokines could then potentiate alterations in maternal metabolism through the mechanisms detailed earlier in relation to obesity-induced metabolic changes. However, further research is needed to expand upon these hypotheses in the context of pregnancy and to detail the precise functional role(s) of placental cytokines, and their extra-placental target(s).

Changes in circulating maternal TNF-α levels have been shown to mirror insulin sensitivity changes and, therefore, suggests that TNF-α may be a crucial mediator in the development of insulin resistance in pregnancy[56]. Several older studies positively correlated changes in insulin sensitivity to placental secretion of human placental lactogen (HPL), progesterone and oestrogen[56]. However, although these hormones are increased during pregnancy, they have now been shown to have very little predictive power for insulin sensitivity[85]. More recent studies have shown that, in women with normal glucose tolerance (NGT), and women with GDM, TNF-α is a greater predictor of insulin resistance[55]. Women with GDM were found to produce more TNF-α than women with NGT, however whether TNF-α is the sole contributor leading to increased insulin resistance in the development of GDM is unknown[86].

Having said all that, it is therefore intriguing that studies have reported no differences in the level of circulating TNF-α between obese and lean pregnancies[87]. This is surprising given that in the non-pregnant state, obese women are known to have elevated circulating levels of TNF-α[87]. This is also surprising given that circulating TNF-α is a strong predictor of insulin insensitivity, and therefore, one would expect levels to be raised in the more insulin resistant, obese pregnant women[87]. The finding that maternal circulating TNF-α levels are similar in lean and obese women suggests two things; firstly, there must be other factors implicated

in mediating insulin resistance in the obese mother, and; secondly, that maternal circulating TNF-α is not implicated in the development foetal overgrowth in the context of obesity.

However, in a study investigating the placentae of obese neonates TNF-α has been implicated in foetal overgrowth. Varastehpour et al studied the placentae of eight obese and seven lean neonates following elective caesarean delivery[88]. Obesity in a neonate was defined as having greater than 16% body fat. Placentae of obese neonates were shown to contain increased TNF-α, and leptin, mRNA expression and protein content[88]. They found that the increased TNF-α, and leptin, induced increased gene expression of the secretory phospholipase A2 (sPLA2) groups IIA, and V[88]. The PLA2 family is a group of lipolytic enzymes that catalyses the hydrolysis of membrane phospholipids, releasing arachidonic acid (AA), docosahexaenoic acid (DHA), and other polyunsaturated fatty acids (PUFA)[88]. It was suggested that the increased gene expression of sPLA2 led to increased generation of PUFAs which can serve as substrates for adipogenesis. PUFAs can also amplify local inflammation within the placenta which can cause alterations in the phospholipid bi-layer, increasing membrane fluidity, and ultimately materno-foetal nutrient transfer[88]. Varastehpour et al demonstrated that accumulation of omega-3 fatty acids within the placentae was strongly associated with high neonatal adiposity. Therefore, the authors proposed that up-regulation of sPLA2 by TNF-α and leptin was a mechanism for increased fat accretion and the development of obesity in-utero[88].

This is in contrast to Farley et al's findings that leptin gene expression is decreased in the placentae of obese women[75]. However, these two studies differ as Varastehpour et al examined the placentae of obese neonates, and Farley et al examined the placentae of obese women with AGA neonates. It would therefore appear that in neonates which are obese, TNF-α and leptin are increased within the placentae and are implicated in foetal growth, but AGA neonates with

obese mothers have decreased leptin gene expression within their placentae which is not implicated in growth. It remains to be established what the protein content and gene expression of leptin and TNF-α is in the placentae of obese mothers who deliver macrosomic infants.

Maternal obesity during pregnancy has, however, been associated with increased circulating levels of IL-6, in comparison to lean pregnant women, which have been positively correlated to foetal adiposity at birth. In experiments conducted on rats, exogenous IL-6 was administered during pregnancy and the offspring were found to have increased fat accretion, and reduced insulin sensitivity, implicating IL-6 as a regulatory factor of foetal growth[89].

Jones et al have demonstrated, in cultured human primary trophoblast cells, that physiological concentrations of IL-6 and TNF-α had a pronounced stimulatory effect on the activity of system A[74]. Doses of IL-6, similar to the physiological levels seen in obesity, were shown to mediate the activation of the signal transducer and activator of transcription 3 (STAT3), leading to increased gene and protein expression of the system A isoform SNAT2[74]. TNF-α was shown to mediate increased protein expression of SNAT1 and SNAT2, however, without altering STAT3 signalling or SNAT1 gene expression. STAT3 has not been previously identified as a cellular mediator in the regulation of system A nutrient transport. Unlike leptin, neither TNF-α, nor IL-6, had any effect on the expression of SNAT4. The authors also reported, in unpublished data, that SNAT2 appears to be increased, whereas SNAT4 remains unchanged, in the placentae of obese women who deliver macrosomic infants[74]. However, as TNF-α is not raised in obese pregnancy it is unlikely that this mechanism is applicable to the development of foetal overgrowth. What this research does show is that IL-6 leads to increased SNAT 2 activity, and as increased levels of IL-6 are associated with obese pregnancy and have been correlated to increased foetal adiposity, this

identifies a molecular link how the inflammatory status of the obese mother may potentiate foetal overgrowth[74].

Although maternal obesity is not associated with increased maternal circulating TNF-α levels, placentae of obese women have been demonstrated to contain macrophages with increased gene expression for TNF-α, IL-6 and IL-1[90].

1.1.8.5. Increased Macrophage Numbers Are Found in the Placentae of Obese Women:

As mentioned previously, obesity is associated with excess of accumulation of macrophages within WAT, and it appears that macrophages also accumulate within the placenta of obese women. Challier et al, functionally and phenotypically characterised the placentae of twenty obese and fifteen lean women[90]. Placentae of obese women were reported as containing twice as many macrophages as placentae of lean women, and that these macrophages were activated with increased gene expression for pro-inflammatory cytokines. Some macrophages (CD14+) also demonstrated increased gene expression encoding for MCP-1[90].

Studies in support of this finding have been conducted in obese baboons, which also reported increased numbers of intra-villous macrophages present within the placenta, however they did not speculate as to their origin[91]. Challier et al speculated that the activated monocytes present within the obese mother's circulation contributed to the accumulation of macrophages present in the placenta, and that the macrophages were recruited by increased placental expression of MCP-1[90]. The reason for the initial increase of MCP-1 gene expression was unknown. Elevated levels of IL-6 and CRP were also demonstrated in the obese women, with, again, no difference in TNF-α concentration. In addition, when umbilical blood was assessed, there was no reported increase in gene expression for pro-inflammatory cytokines in mononuclear cells, implying these

foetal macrophages were not activated. From these findings it was assumed that the pro-inflammatory status of the obese mother was confined to the mother and placenta, and not directly conveyed to the foetus[90].

There was no definite reason found for the increased numbers of macrophages within the placentae of obese women, but it could be speculated that their recruitment occurs via the same mechanism as macrophage recruitment occurs in adipose tissue in obesity. Their presence, however, raises the possibility that the activated macrophages, found in the placentae of obese women, may be involved in the regulation of placental nutrient transporters, such as sPLA2 and system A, resulting in altered foetal growth and development. Future research into this topic to elucidate the initial recruitment stimulus and true functional role of macrophages in the placentae of obese women is needed.

Research has also been conducted into how alterations in cytokines, leptin and insulin may affect overall placental structure and how this may affect foetal growth. Radaelli et al profiled the expression of genes altered in the placenta in response to the diabetic insult[92]. A total of 110 genes were shown to be modified in response to GDM, with a third of the genes regulating inflammatory responses and endothelial reorganisation. The authors' hypothesised that the inflammatory environment present in GDM had induced the recruitment of TNF-α, IL-1, and leptin receptors to the MVM of the syncytiotrophoblast, which were then activated by the inflammatory mediators, present within the maternal circulation[92]. This then led to the activation of multiple genes and transcription factors, mainly involved in the regulation of placental structure. For example, TNF-α, IL-1, and leptin receptor activation were shown to induce genes encoding for fibronectin and matrix metalloproteinases (MMPs)[92]. MMPs have been shown to degrade extracellular matrix (ECM) proteins resulting in alterations in the cellular network and

release of growth factors in the placenta, culminating in increased angiogenesis and disturbed placental structure[92].
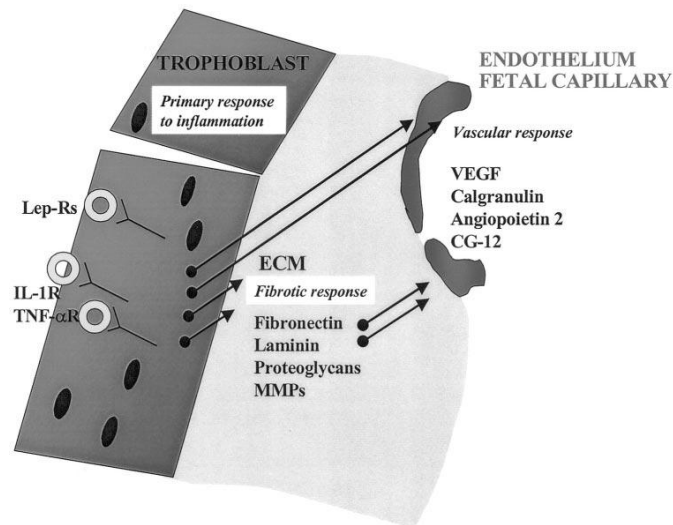
Figure 1-6 - Diagram illustrating the molecular interactions occurring in the syncytiotrophoblast in response to the inflammatory environment characteristic of GDM[92].

This diagram demonstrates how the inflammatory status of the diabetic mother may lead to dysregulation of placental structure. Potentially this dysregulation could alter the function of the placenta and the nutrient supply to the foetus, and ultimately foetal growth. How, or even if, this actually occurs is, however, unknown.

A recent study, more applicable to the situation of obesity, examined the effect of mild hyperglycaemia on placental structure and function. Pietro et al reported that placentae from women with mild hyperglycaemia were shown to contain more terminal villi than placentae from women with NGT, GDM, or clinical diabetes mellitus[93]. This was deemed as a response to hyperglycaemic induced hypoxia. Hyperglycaemia is known to cause increased glycosylation of haemogloblin, causing alterations in its structure, and impairing oxygen transport. Therefore, by increasing the number of terminal villi, a larger surface area for materno-foetal gas exchange is provided, overcoming the induced hypoxia[93]. It was then speculated that this would also lead to increased surface area for nutrient

exchange, possibly providing a link between maternal hyperglycaemia and foetal macrosomia[93].

As obesity is also associated with transient episodes of hyperglycaemia, and elevated levels of pro-inflammatory cytokines, it could be postulated that similar mechanisms may also apply to placentae of obese women, leading to altered placental structure and foetal growth. However, although thorough searches have been conducted, there appear to be no studies documenting placental changes in obese women who deliver live, AGA, or macrosomic, neonates. Therefore, without knowing what structural changes are associated with obesity it is impossible to speculate what may be occurring within the placenta or how this may influence foetal growth.

1.1.9. Summary:

In summary, obesity is associated with multiple metabolic changes and when this is combined with the inflammatory status of pregnancy, further metabolic alterations are instigated. Obese mothers are known to produce SGA neonates and macrosomic neonates, both of which are at increased risk of developing metabolic syndrome in later life. The mechanisms for the development of either one of these outcomes is unclear, or why they are predisposed to metabolic syndrome.

With regard to the development of macrosomia, several factors have been implicated:

- Hyperinsulinaemia and decreased insulin sensitivity leading to transient episodes of hyperglycaemia and increased placental glucose transport,
- Hyperleptinaemia which has been shown to affect placental size and the development of appetite regulation within the foetus,
- Increased numbers of activated macrophages within their placentae whose function is unknown,
- Elevated levels of circulating IL-6 which have the potential to increase amino acid transport via system A.

See Figure 1.7 overleaf for schematic diagram of possible theories implicating maternal obesity in the development of foetal macrosomia.
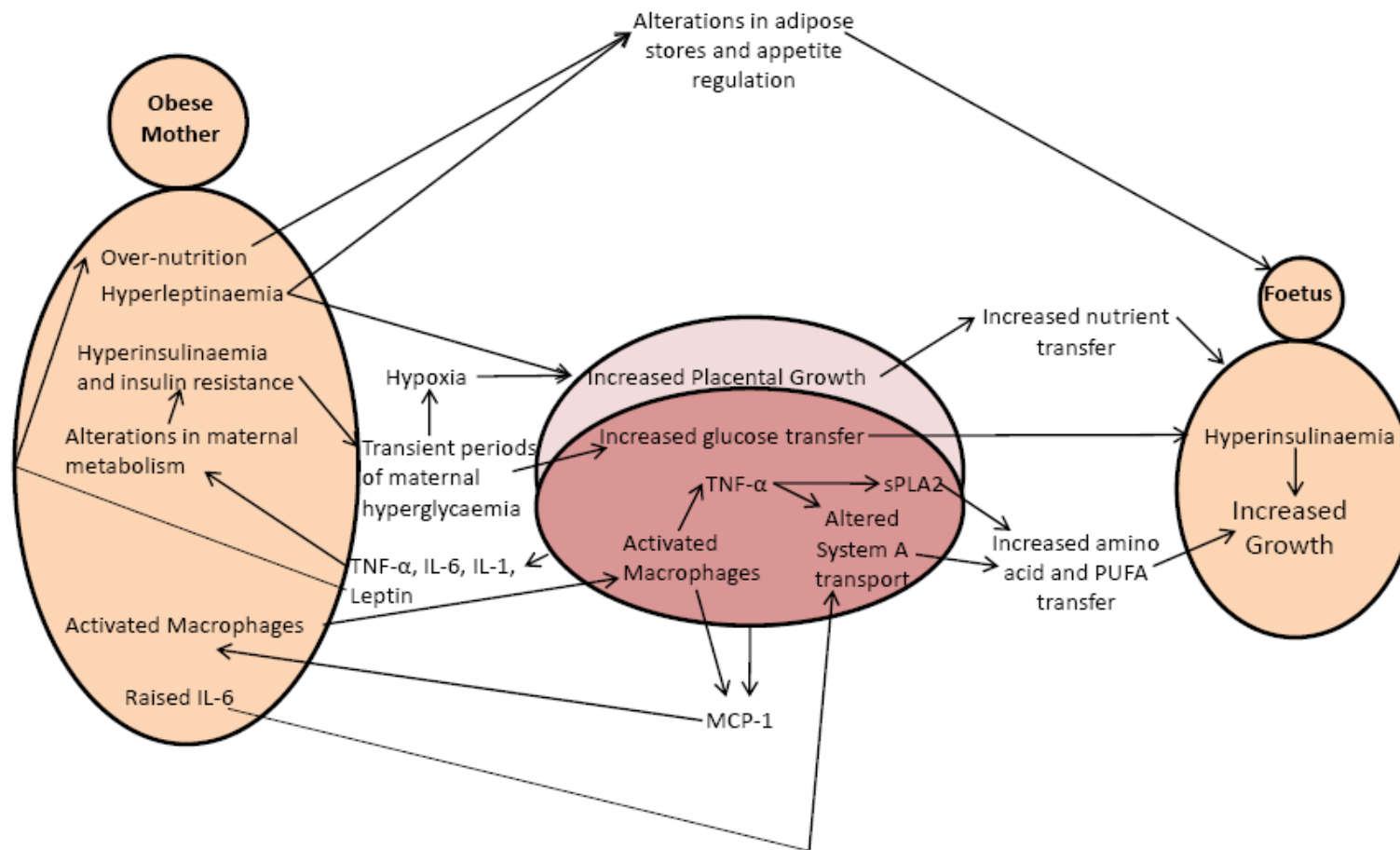
Figure 1.7: Schematic diagram summarising the possible theories implicating maternal obesity in the development of foetal macrosomia.

However, much of this is hypothetical and based on animal models, with no definitive answers coming from human studies. Very few of the regulatory factors and the intra- and extracellular mechanisms in foetal overgrowth are known. With so much that is unknown about the cellular interactions relating to obesity alone, it is nigh on impossible to unravel the multitude of interactions occurring in obese pregnancy, and the potential implications these may have to mother and foetus.

Obesity, however, *is* rising in adults, and in 2009 approximately 33% of pregnant women were overweight and 23% were obese in the UK alone[94]. The WHO speculates that by 2015 in the UK, 73% of women aged thirty or over will be overweight.[95] This will mean that more and more foetuses will be exposed to a detrimental in utero environment, putting them at increased risk of becoming obese themselves. It is therefore pertinent that these interactions are unveiled in an attempt to break the perpetuating cycle of obesity-inducing obesity in subsequent generations. If no efforts are made to break this cycle then we are simply condemning future generations to a short-lived life of obesity.

This study, by no means, aims to address all of the mechanisms related to the development of foetal macrosomia in relation to obesity. What it does seek to explore are the following factors:

- The relationship of maternal variables, especially BMI, and neonatal birth weight and adiposity. It is hypothesised that, in agreement with previous studies, that an increase in birth weight will be seen in neonates of obese mothers which will correlate to maternal pre-gravid BMI.
- The morphology of placentae of obese women. It has yet to be discovered whether the placentae of obese women, who do not suffer from GDM, have any associated placental morphological

changes on histological examination. The null hypothesis is that there will be no morphological changes detected on light microscopy in the obese participants' placentae when compared to lean participants' placentae. Placental morphology will then be examined in relation to foetal adiposity and any correlations noted.

- Determine whether glucose, leptin, TNF-α, or insulin have any regulatory effect on the production and/or secretion of placental cytokines from placentae of lean and obese women which are cultured in vitro. It is hoped that the results will help elucidate the placental regulation of cytokine production. The null hypothesis is that lean and obese placentae produce the same quantities of pro-inflammatory cytokines.

In accordance with this, the aims of the study as illustrated in Figure 1.7 are:

1. To describe the association between maternal demographics and neonatal anthropometry,
2. To compare the patterns of placental morphology between lean and obese pregnant women and its association with neonatal anthropometry,
3. To compare basal, and stimulated, production, and secretion, of pro-inflammatory cytokines between lean and obese pregnant women.
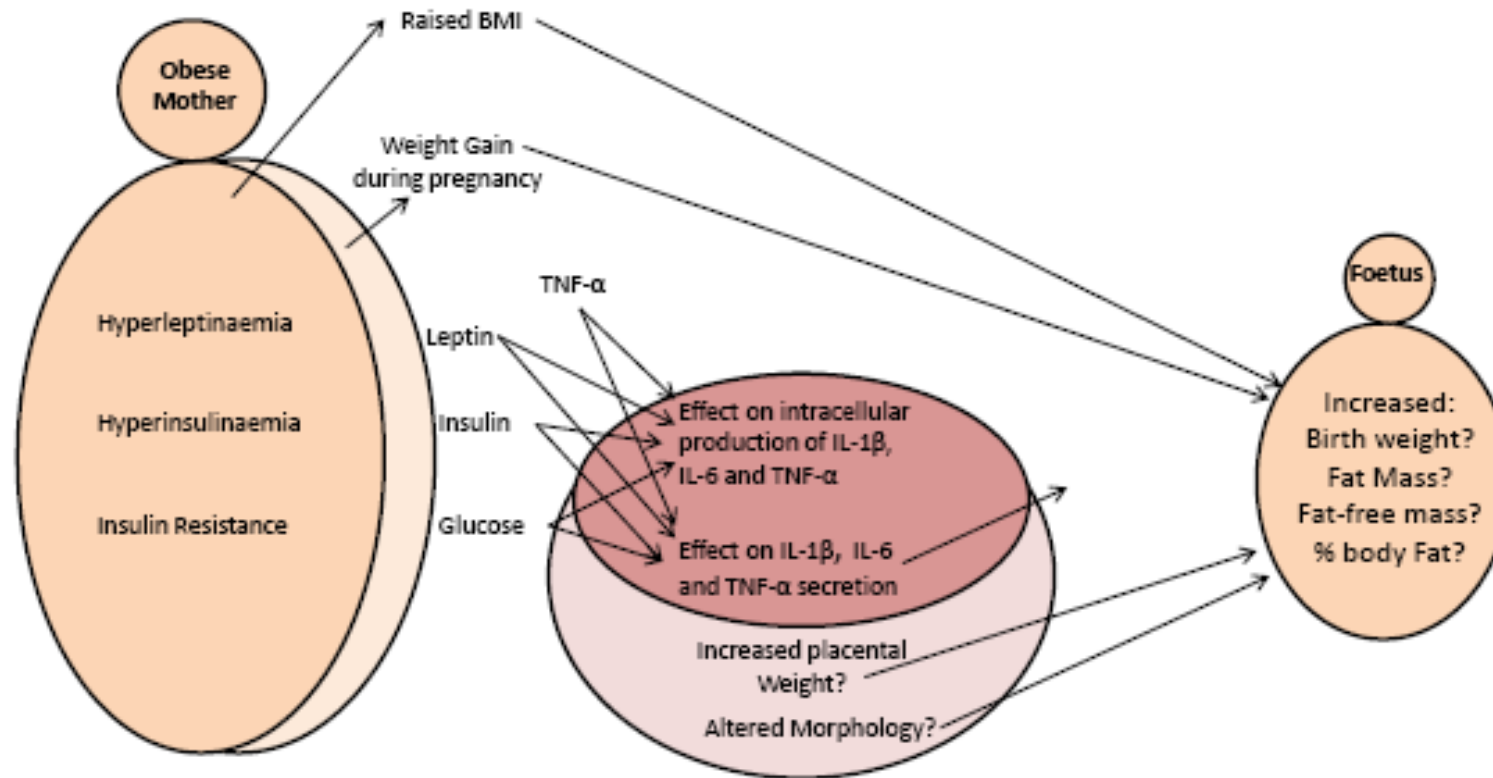
Figure 1-8 - Diagram summarising the aims of the study.

Chapter 2: Materials and Methods:

2.1. The 'Fit for Birth' Study:

Participants were recruited as part of the 'Fit for Birth' (FFB) study; a two year prospective study, based at the Liverpool Women's hospital, investigating obese, pregnant women. The 'Fit for birth' study has two overall aims:

> 1. *'To determine the scale of the problem of obesity in pregnancy, in the Liverpool PCT area.'*
> 2. *'To generate information that can be used to develop services and plan further research.'*

By gathering this information the study hopes to improve the care, and obstetric outcomes, of obese women in Liverpool during pregnancy. It also hopes to reduce the long-term risks of poor health in obese women, and their families.

Recruitment of participants was conducted over a one year period, from June 2009 to June 2010, and was based on the body mass index (BMI) of women during their first booking appointment with their midwife. Dependent on their booking BMI, participants were eligible for one of three subgroups:

Booking BMI	Section of study participant is eligible for	What participation involves
Less than 30	Not eligible for any section of the study	No participation required, patient just attends normal antenatal appointments.
30-34.9	Level one - 'Fit for birth'	Attends regular midwife appointments and is weighed at each appointment.
Greater than 35	Level two - 'Fit for Birth Plus'	Is invited to the Liverpool Women's Hospital to attend specialised clinics (see below for further information)

Table 2.1 - Table to show the eligibility of women from the Liverpool area with dependent upon BMI to the FFB study.

Level one of the study, simply entitled 'Fit for Birth', only required participants to be weighed at their routine antenatal appointments by their community or hospital midwife. Maternal and obstetric data was then extracted from their medical notes. This level aims to calculate descriptive statistics of NHS attendance rates, and summarise weight gain during pregnancy, of obese pregnant women in Liverpool.

Level two of the study, 'Fit for Birth Plus', entails a much more detailed analysis of the participant. Each participant was invited to attend three specialist clinics at twelve, twenty-eight and thirty-six weeks gestation. During each appointment information was gathered about the participant's diet, physical activity, health-related quality of life, and sleep. As part of this level the participant was also invited to consent for histological examination of their placenta and neonatal anthropometric measurements of their baby. This level aims to generate detailed longitudinal, descriptive statistics to aid service delivery and, analyse the impact of maternal variables on obstetric and neonatal outcome. (See Appendix I for patient information sheet).

Consent for the FFB study was performed by the woman's midwife or by a member of the FFB team who was based at the booking centre. The candidate did not participate in the selection or consenting process.

2.1.1. Placental Histological Examination Recruitment:

This section of the 'Fit for Birth' study was conducted by the candidate over a ten month period, from September 2009 to June 2010, with the kind help of the midwives on the delivery suite and midwifery-led unit at the Liverpool Women's hospital.

2.1.1.1. Eligible participants:

For placental histological examination, the following inclusion criteria were applied to the participants:

- Participants must have qualified and consented to the 'Fit for Birth Plus' level;
- The gestational age at time of delivery must be greater than 36 weeks;

Exclusion criteria were:

- Participants under the age of eighteen (due to legal reasons deemed by the ethics comittee).

Participants were entitled to withdraw their consent for placental histology and neonatal anthropometric measurement, at any time, without withdrawing their consent from other aspects of the 'Fit for Birth Plus' level of the study.

In order to ensure participants' placentae were sent for histological examination the candidate designed a form to highlight their participation within the study to their delivering midwife (Appendix II). In order that these forms were not overlooked, they were placed at the front of the patient's hand-held maternity notes. Initially, these forms were distributed during the participant's third visit to the 'Fit for Birth' clinic as the appropriate gestation for inclusion would have been reached. However, due to

unforeseen reasons, many participants who had consented for 'Fit for Birth Plus' at their booking appointment, were not given follow-up appointments at the 'Fit for Birth' clinic. Out of a possible one hundred and thirty eight participants consented to the 'Fit for Birth Plus' level of the study during the ten month period, only seventy nine were given appointments to the clinic. During investigation into this problem it evolved that there had been communication problems regarding whose responsibility it was to book participants' appointments; the community midwife or the study administrator. During this time period irretrievable data was lost as many participants' gestational ages were too advanced to attend the clinics. In an attempt to improve recruitment rates for the placental histology aspect of the study, the candidate sought ethical approval to contact the participants by post. This was granted in late November and from then onwards, reminders were distributed to all participants by post (Appendix III).

Recruitment of placentae for histological examination relied heavily on the co-operation of the midwives who attended to the participants during their delivery. Therefore, much effort was made to advertise the study to all the midwives on delivery suite and midwifery-lead unit to ensure that the study was well publicised. This was achieved by regularly attending staff handovers and distributing posters detailing information about the study (Appendix IV).

Participant admission to the hospital was monitored on a daily basis. This was done by checking the various maternity wards' admission boards and the hospital computer system; 'Meditech'. Depending upon whether the participant was antenatal or postnatal when their admission was detected, one of the following four options was followed:

- Antenatal - If a participant had been admitted and was yet to deliver, consent for placenta histology was verified, and if confirmed, a form was placed within their notes, if there was not one already there. Their midwife was then informed of their participation and notified of the following procedure; immediately after delivery the placenta was to be placed in a 'Fit for Birth' histology bucket and placed in the fridge, to prevent decomposition, with a corresponding, completed 'Fit for Birth' histology form (Appendix IV). The individualised 'Fit for Birth' histology bucket and form were designed to highlight the placentae as part of the study to the Alder Hey pathologists who were to conduct the histological examination.

- Postnatal and placenta **had been sent** for histological examination – If a participant had already delivered prior to their admission being detected and their midwife had already sent their placenta for histological examination then, irrespective of whether the participant had attended a 'Fit for birth' clinic, neonatal anthropometric measurements were performed.

- Postnatal and placenta **had not been sent** for histological examination but participant **has attended** at least one 'Fit for Birth' clinic – If this was the case then neonatal anthropometric measurements were still performed as these could be correlated to other maternal information gathered during the FFB clinic appointment(s).

- Postnatal and placenta **had not been sent** for histological examination and participant **has never attended** a 'Fit for Birth' clinic – although neonatal anthropometric measurements could be conducted there would be no maternal or placental information to correlate it to and, therefore, it was decided that it was futile to measure the neonates of these participants. Every effort was

made to avoid this situation through rigorous monitoring of the wards and advertisement to the midwives to prevent loss of vital data.

2.1.2. Neonatal Anthropometric Measurements:

One variable of particular interest was neonatal body composition, especially the percentage of total body fat, which can be estimated in several different ways. The measurement of total body water is one method; however this requires the use of a mass spectroscopy facility, which due to funding restrictions the candidate did not have access to. The second method is to estimate total body electrical conductivity (TOBEC), which requires access to specialist equipment, which again, the candidate did not have access to.[96] The third method is to calculate the fat mass of the neonate using a statistical model. The first group of researchers to propose a statistical model for estimating neonatal fat mass was Dauncey et al in 1977. It was based on the following assumptions:

- The head is a sphere devoid of fat;
- The trunk is a cylinder covered by a layer of subcutaneous fat whose circumference is equal to the chest circumference, and whose length is equal to the crown-rump length minus the diameter of the head;
- The upper limbs are cylinders covered by subcutaneous fat whose circumference equals the circumference of the upper arm, and whose length equals the length of the upper and lower arm;
- The lower limbs are cylinders covered by subcutaneous fat whose circumference equals the mean of the mid-thigh and calf circumferences, and whose length equals the length of the upper and lower leg.

In order to calculate the overall volume of the subcutaneous fat, first the depth of the subcutaneous fat covering each of the cylinders needs to be known. This can be estimated by measuring the

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overlying skin fold of each 'cylinder' using callipers. The volume of each cylinder is then calculated by:

- length x circumference x depth of subcutaneous fat

Total body fat can then be estimated by summing all the volumes of fat covering the cylinders and multiplying by 0.9, the density of fat.[97]

This model remained unchanged until 1995 when Catalano et al redefined this equation. Fat mass was estimated on one hundred and ninety four neonates using TOBEC and the anthropometric statistical model. From their research, Catalano et al derived that the following statistical model had better correlation with estimated neonatal fat mass than the previously proposed statistical model by Dauncey et al:

'(0.39055*birthweight(kg))+(0.0453*Flank skinfold thickness(mm))-(0.03237*length(cm))+0.54657'
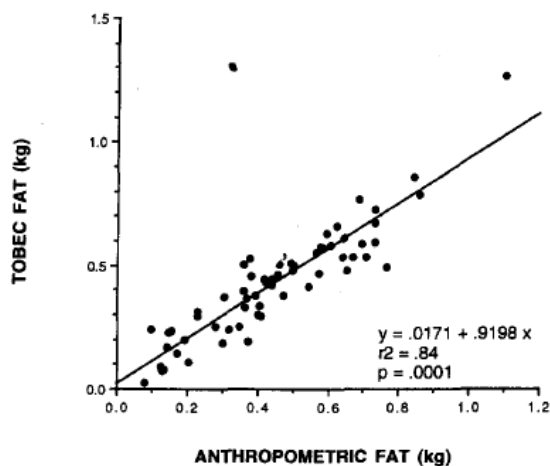
Fig. 1. Correlation between our derived anthropometric model to estimate neonatal fat mass and total body electric conductivity (TOBEC)–estimated neonatal fat mass. $R^2 = 0.84$, $p = 0.0001$.
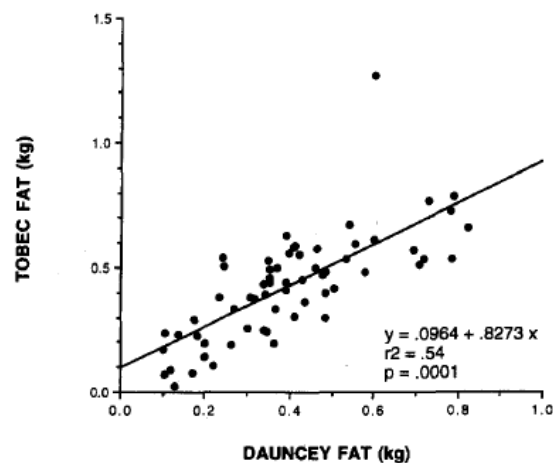
Fig. 2. Correlation between Dauncey anthropometric model to estimate neonatal fat mass and total body electric conductivity (TOBEC)–estimated neonatal fat mass. $R^2 = 0.54$, $p = 0.0001$.

Figure 2-1 - Graphs taken from Catalano et al's paper entitled 'Anthropometric estimation of Neonatal body composition'. The graphs illustrate the correlation of

TOBEC estimated neonatal fat mass with: a) Neonatal fat mass calculated from the model devised by Catalano et al, and b) Neonatal fat mass calculated by the model devised by Dauncey et al[96].

As can be seen from the graphs Catalano et al's statistical model had an R^2 = 0.84 when compared to TOBEC fat mass estimation, whereas Dauncey et al's only had an R^2 = 0.54[10]. Based on these results, it was decided that the statistical model proposed by Catalano et al would be used in the study to estimate neonatal fat mass, as TOBEC could not be performed due to financial constraints.

Therefore, in view of calculating neonatal fat mass and free-fat mass, the following anthropometric measurements were performed:

- Length – the length of the neonate was measured to the nearest millimetre using a one metre, portable roll mat. The neonate was laid supine with their head and feet exposed. The participant, or the participant's partner, was asked to hold the neonate's head against the head board while the candidate straightened the neonate's legs whilst moving the adjustable foot board towards the feet. The reading was taken once the legs were straight and the feet were pressed flat against the foot board. On three occasions, the roll mat had to be placed inside the neonate's cot as there was no other available space for the roll mat to be placed on. The reading was, therefore, much more difficult to perform and due to the manner in which the roll mat had to be placed gave a greater than actual reading.

- Flank skin fold thickness – this was measured to the nearest 0.2 of a millimetre in the mid-axillary line just above the superior iliac crest using Harpenden callipers. The skin-fold was held using the thumb and index finger, ensuring no underlying tissue was included, and the callipers applied for approximately two seconds until the needle steadied (see Appendix VI for reference of method). The measurement was performed twice, however if the second measurement was greater or less than ten percent of the first measurement then the measurement was performed for a third time. An average of the two, or three, measurements was then taken. All measurements were performed on the right hand side of the neonate.

- Head circumference – the occipital frontal circumference was measured in centimetres using a disposable, paper tape measure. This measurement was performed once.

These measurements were only performed by the candidate once it had been verified that a participant had either;

- Had their placenta sent for histological examination irrespective of attending a 'Fit for Birth' clinic, or;

- Had not had their placenta sent for histological examination but had attended a 'Fit for Birth' clinic.

Before visiting the participant on the ward, the candidate first ensured that the participant had been deemed healthy to receive visitors by their midwife. Once confirmed, the participant's consent for neonatal anthropometric measurements was checked. Five participants chose to withdraw their consent at this point, as they deemed it detrimental to the care of their baby. If, however, consent was reconfirmed then the anthropometric measurements listed above were performed.

				Reasons for not obtaining placenta for histological examination		Reasons for not obtaining neonatal anthropometric measurements	
Number of placentae sent for histological examination	74	49	Participants had attended at least one 'Fit for birth' clinic		N/A	25	Neonatal anthropometric measurements performed
						2	Participant was repeatedly unavailable and was discharged prior to measurements being obtained
						1	Premature baby – placenta should not have been sent – neonate too critically ill to be handled by candidate
						1	Intra-uterine death therefore neonate not measured
						1	Homebirth
						12	Candidate was not contacted by the attending midwife
						7	The candidate was unavailable to perform the neonatal anthropometric measurements
		25	Participants had never attended a 'Fit for birth' clinic		N/A	13	Neonatal anthropometric measurements performed
						1	Participant withdrew their consent
						1	Participants were repeatedly unavailable
						4	The candidate was unavailable to perform the neonatal anthropometric measurements
						6	The candidate was not contacted by the attending midwife
Number of placentae not sent for histological examination	62	28	Participants had attended at least one 'Fit for Birth' Clinic	3	No reminder in notes as did not attend third 'Fit for Birth' clinic appointment, prior to ethical approval for contact via post.	3	No form in notes therefore the candidate could not be contacted by midwife and no neonatal anthropometric measurements were performed
				4	Participants had not placed the form in their hand-held notes – checked by candidate	5	Neonatal Anthropometric measurements performed
				4	Participants withdrew their consent	4	Participants withdrew their consent
				3	Participants had preterm deliveries	3	Premature babies – neonates too critical to be handled by the candidate
				12	Participants' midwives did not send their placenta for histological examination or, the participant had not placed the form in their notes (notes unavailable for candidate to check)	11	The candidate was not contacted by the attending midwife
				1	Participant moved away	1	Participant moved away
				1	Emancipated	1	emancipated
		34	Participants had never attended a 'Fit for Birth' clinic	5	Participants had preterm deliveries	5	Premature babies – neonates too critical to be handled by the candidate
				26	Participants' midwives did not send their placenta for histological examination or, the participant had not placed the form in their notes (notes unavailable for candidate to check)	26	No neonatal anthropometric measurements were performed as no other maternal or placental histological data to correlate it to
				3	Delivered at alternative hospital	3	Delivered at alternative hospital

Table 2.2 - Table to summaries numbers of placentae obtained and neonatal anthropometric measurements performed.

2.1.3. Control Participants:

The 'Fit for Birth' study postulated that, over the two year period, approximately one hundred and fifty placentae would be obtained from participants with a BMI of thirty five or greater. In order to give an 80% power to detect a 2.5-fold increase in the rate of maternal vascular underperfusion among obese participants, fifty control participants with a BMI of nineteen to twenty five would need to be recruited.

During the candidate's clinical placement, eighteen control participants with a BMI between nineteen and twenty five were recruited. The following method was used to identify and consent control participants:

1. The theatre list for elective caesarean sections for the following day was obtained.
2. All patients on the theatre list were cross-referenced with their BMI.
3. Patients with a BMI between nineteen and twenty five were noted and located on the ward.
4. The candidate introduced themselves, the 'Fit for Birth' and the reason for approaching the patient.
5. Patients were given a 'Patient Information Sheet' and given time to read it (Appendix VII).
6. The candidate then answered any questions the patient had with regard to participation within the study.
7. The candidate then asked if the patient would like to participate in the study. It was made clear that the patient was eligible to decline the offer and it would affect their treatment during their hospital stay.
8. If the patient agreed, three consent forms were filled in and signed by the patient (Appendix VIII). The candidate

then dated and countersigned these and placed a Liverpool Women's Hospital patient identification label on them.

9. One copy of the consent form was then placed in: the participant's brown hospital medical records; the participant's hand-held maternity notes; and finally one copy was kept and given to the study administrator.

10. A control placental histology reminder form was then placed at the front of the participant's hand-held maternity notes to identify their participation within the study to the theatre midwife.

11. The candidate then informed the theatre administrator that the participant had consented and that their placenta required sending for histological examination. (Prior to consenting any controls, the candidate checked there were sufficient placental histology buckets available).

12. Following delivery the placenta was collected from theatre in the placental bucket and with the corresponding, completed placental histology form the placenta was sent for histological analysis.

13. The next day, neonatal anthropometric measurements were performed on the neonate.

Only seventeen of the eighteen control participants consented to neonatal anthropometric measurements. One participant withdrew their consent as they did not want their baby 'poked and prodded'.

2.1.4. Data collection:

The following information was gathered from Meditech following a participant delivery:

Maternal variable	Neonatal variable
Age	Gestational age at birth (days)
Weight (kg)	Birth weight (g)
Height (cm)	Outcome of birth
Body mass index (kg/m^2)	Apgar score at one minute
Smoking status	Apgar score at five minutes
Ethnicity	Level of resuscitation received at delivery
Gravity	Foetal problems during labour
Parity	Sex
Onset of labour	
Mode of delivery	
Presentation of foetus	
Maternal problems during labour	

Table 2.3- Table to show information gathered from Meditech.

Along with the neonatal anthropometric data, these variables were entered into a Microsoft Excel database.

The following variables were then calculated and entered into the main Excel database:
- Neonatal Fat Mass (kg):

    Using the statistical model by Catalano et al

- Neonatal free fat mass (kg):

    = Birth weight(kg) – fat mass(kg)

- Neonatal percent body fat (%):

$$= \text{Fat mass(kg)/birth-weight(kg)}*100$$

- <u>Birth weight centile</u>:

    This was calculated by downloading a 'bulk birth weight centile calculator' from www.gestation.net. The following variables were necessary for the calculation and re-coded into:

    - Birth weight (kg)
    - Maternal height (cm)
    - Maternal weight (kg)
    - Parity
    - Ethnicity -   1 = European
                      2 = Indian
                      3 = Pakistani
                      4 = Bangladeshi
                      5 = African Carribean
                      6 = African
                      7 = Middle Eastern
                      8 = Far Eastern Asian
                      9 = South East Asian
                      10 = Unclassified
    - Gestational age (days)
    - Neonatal sex - 1 = male
                      2 = female

From this information the 'bulk birth-weight centile calculator' then calculated the individual birth-weight centiles for all of the neonates.[98] (Appendix IX).

Placental histology reports were received back approximately three weeks after the placenta had initially been sent for examination. The reports were then photocopied and read by the candidate. If any gross abnormality was detected in the report, the candidate reported

these to an appropriate consultant. The following variables were then inputted into the main Excel database:

- Placental untrimmed weight (grams),
- Placental trimmed weight (grams),
- Centile of placental trimmed weight (placental weight with the membranes and umbilical cord removed).

A consultant neonatologist, blinded to the BMI status of the participant, was then asked to classify the reports into the following four categories:

0 = Normal
1 = Maternal Vascular Underperfusion
2 = Chorangiosis
3 = Other Abnormality

A normal placenta was defined as having no obvious abnormalities on light microscopy as reported by a pathologist who was also blinded as to the participants' BMI cohort. The category of maternal vascular underperfusion (MVUP) was chosen as it is an important cause of foetal growth restriction, and therefore it was chosen to determine if there was any correlation between MVUP and SGA neonates. MVUP is diagnosed by the presence of the following abnormalities in a placenta (see Figure 2.3 below). Chorangiosis is defined by an increased number of capillaries in terminal villi and is believed to be a placental response to long term chronic hypoxia and its presence is a pathological feature. As obese mothers are known to suffer from transient periods of hyperglycaemia, which leads to poor oxygen transportation, chorangiosis was chosen to determine if placentae of obese participants were more hypoxic in comparison to placentae from lean participants. The category of 'other' included any other reported placental morphological

abnormalities such as foetal thrombotic vasculopathy and chorioamnionitis[99]. Once the reports had been categorised, their categories were then entered onto the database.

Diagnostic terminology	Definition
Intervillous space	
Increased syncytial knots	Aggregates of syncytiotrophoblast nuclei along stem villi or at one or more poles of distal villi
Mild–moderate	Excessive for gestational age in ≤ 30% of parenchyma
Severe	Excessive for gestational age in > 30% of parenchyma
Villous agglutination	Clusters of adherent distal villi (> 2, < 20) agglutinated by fibrin(oid) and/or bridging syncytial knots accompanied by stromal fibrosis, cellular degeneration, or karyorrhexis
Increased intervillous fibrin	Abnormal amounts of intervillous fibrin(oid) either coat proximal stem villi (Langhan stria) or are eccentrically adherent to (or within, following reepithelialization) distal villi
Distal villous hypoplasia	Modal diameter of distal villi is decreased. Number of distal villi decreased relative to the number of stem villi. Stem villi either have muscularized vessels or dense fibrotic cores (> 30% of parenchyma affect ed).
Arterial wall and implantation site	
Acute atherosis, decidual arteries	Red–blue glassy degeneration (fibrinoid necrosis) of arterial smooth muscle *plus* subendothelial or medial foam cells (macrophages) in muscularized maternal arteries of basal plate, marginal zone, and/or membranous decidua
Mural hypertrophy, membrane arterioles	Thickening (mean wall diameter > 30% of mean circumference) of maternal arterioles in the decidua parietalis due to any combination of medial or subendothelial hyperplasia, hypertrophy, and interstitial matrix deposition
Muscularization, basal plate arteries	Persistence of smooth muscle cells in the wall of a large spiral artery in the basal plate (includes by definition basal plate acute atherosis—see text)
Increased placental site giant cells, decidua basalis	Numerous trophoblastic giant cells (three or more nuclei) in the *deep* basal plate (near plane of separation from uterus) of the basal plate surrounded by loose decidual tissue without accompanying intermediate trophoblast or fibrinoid
Immature intermediate trophoblast, decidua basalis	Tightly cohesive groups of 10–20 (or more) eosinophilic and/or vacuolated immature intermediate trophoblast arranged in sheets or clusters in the *superficial* basal plate (near anchoring villi). Adjacent fibrin(oid) is often excessive and may show cystic degeneration and lamination
Other	
Thin umbilical cord	No completely sampled representative umbilical cord segment has a maximum cross-sectional diameter of > 8.0

Figure 2-2 - Figure taken from a paper by Redline RW et al. It details the placental reaction patterns, nomenclature, and definitions used the diagnosis of MVUP[100].

2.2. Placental Analysis:

2.2.1. Summary of Placental Explant Incubation:

By isolating placental tissue in the laboratory, information can be obtained that would otherwise be very difficult to elucidate in the complex in-vivo situation. One short-term model for studying the placenta in-vitro is placental explants incubation. Placental villous tissue is extracted from the placenta and incubated in a supportive medium whilst different variables are examined. The viability of this model has been proven for up to four hours, after this time period intracellular vacuoles are seen to form within the cells. This model provides a simple technique to ascertain the assortment of characteristics, such as nutrient transport, or sensitivity to stimuli, possessed by the human placenta[101]. It also presents a straightforward method for comparing normal placentae to placentae from pathophysiological conditions, such as, pre-eclampsia, or gestational diabetes[102]. Based on this, it was therefore proposed that placental explant culturing would be a feasible means of investigating cytokine production and secretion within the placentae of obese, and lean, subjects.

Five placentae from both obese and lean participants were incubated for three hours in Tyrode's medium. In order to minimise variability, all placentae were obtained from elective caesarean sections, as previous research has shown differences in the cytokine secretion of vaginally, and caesarean, delivered placentae[84]. Following a caesarean section, placentae were found to produce more IL-1β when stimulated with a range of TNF-α doses, whereas placentae obtained from a vaginal delivery were found to produce more IL-6[84]. Based on this research, and ease of predicting delivery time, the decision was made to use placentae delivered by caesarean section.

2.2.1.1. Methodology for Villous Tissue Preparation and Explant Incubation:

Due to the nature of this procedure and the necessity for speed, this section of the method was carried out by the candidate, with the help of the lab technician, Lisa Heathcote, and the research assistant Jo Drury.

Placentae were transferred from the delivery theatre to the university class II laboratories in sealed placental buckets in conjunction with the University and Liverpool Women's NHS Foundation Trust Safety guidelines[103].

Prior to the arrival of each placenta all equipment and solutions needed for the experiment were prepared in the laboratory (see appendix X for all specific protocols relating to laboratory work).

Once a placenta had arrived from theatre the maternal and foetal aspects were photographed. This was done in order to ensure that there was an accurate record of the placenta that the pathologist could refer to if the dissection had majorly interfered with the overall placental structure.

Three $1cm^3$ full thickness samples were then cut from the placenta. The samples were taken randomly from three different cotyledons of the placenta, to ensure a representative sample was taken, avoiding areas directly below the cord and from the periphery. The samples were then washed three times in primary buffering solution (PBS) to remove as much maternal blood as possible.

The chorionic and decidual edges were removed and each sample was dissected into approximately $0.25 \ cm^3$ fragments. Six fragments, from a mixture of the three initial samples, were then placed into

scintillation vials containing 4ml Tyrodes' solution. Vials were run in duplicate as one set was intended for enzyme-linked immunosorbent assays (ELISA) and the other for immunohistochemistal analysis.

Each scintillation vial was then subjected to one of the following stimulants: leptin, insulin, glucose, and TNF-α (see table overleaf).

Vial Number	Stimulant concentration used during incubation period
1	Control Tyrodes (5 mM glucose)
2	15 mM glucose Tyrodes
3	25 mM glucose Tyrodes
4	Control Tyrodes+100 ng/ml leptin
5	Control Tyrodes+1 ng/ml TNF-α
6	Control Tyrodes+10 ng/ml TNF-α
7	Control Tyrodes+30 ng/ml TNF-α
8	Control Tyrodes+0.1 nM Insulin
9	Control Tyrodes+1 nM Insulin
10	Control Tyrodes+10 nM Insulin
11	Control Tyrodes+100 nM Insulin

Table 2.4 – table to show concentrations of stimulants used during incubation.

The fragments were then incubated in a shaking waterbath at for three hours at 37°C. During their incubation the fragments were exposed to the atmosphere to allow normal gaseous exchange.

It should be noted here that during validation, two sets of vials for TNF-α were trialled, one set containing 4ml Tyrode's solution and the second set containing 4ml Tyrode's solution + 1 µl protease inhibitor cocktail (PI). Initially, it was thought that the TNF-α would degrade too rapidly and alter the overall cytokine

concentration. However, after several trials and subsequent analysis, the additional protease inhibitor was deemed unnecessary.

After three hours the fragments from the first set of vials were removed from the stimulants and placed in 2ml of H_2O. A PI was added at this point to prevent protein degradation of the fragments. The fragments were then incubated again for ≥18 hours at 4°C without exposure to the atmosphere. The supernatant that the fragments were initially incubated in was frozen at -80°C in aliquots to be used later for ELISA analysis (these aliquots were labelled as 'extracellular' and were to be used to assess the concentration of cytokine the placental cells excreted in response to the different stimuli). The fragments from the second set of vials were removed and placed in 4% neutral buffering formalin (NBF) and left overnight to fix. The supernatant from these fragments was discarded.

Following the second incubation period the fragments were removed and placed in 10ml of 0.3M NaOH, and incubated for $\geq$ 6 hours at 37°C. This was to lyse the fragments in order for protein analysis to be conducted later. The supernatant from the fragments was then aliquotted and frozen at -80°C and was also intended for ELISA analysis (these aliquots were labelled as 'intracellular' and were to be used to assess the concentration of cytokine which remained within the placental cells following incubation). Following the third incubation period the vials were removed and stored at 4°C until protein analysis had been performed.

2.2.2. Summary of Methodology for Processing of Fixed Samples and Wax Embedding

Due to time constraints on the candidate, the wax embedding was conducted by the candidate, Lisa Heathcote, and Jo Drury.

The fragments which had been fixed in 4% NBF for 24 hours were removed and processed in the Shandon Citadel Processor. In summary the following process is followed:

- 4% formalin in neutral buffer   45 minutes
- 60% Ethanol   1 hour
- 70% Ethanol   1 hour
- 90% Ethanol   1 hour
- 100% Ethanol   1 hour
- 100% Ethanol   1 ½ hours
- 100% Ethanol   2 hours
- Xylene 1   1 hour
- Xylene 2   1 ½ hours
- Xylene 3   2 hours
- Wax 1   2 ½ hours
- Wax 2   3 ½ hours

Once finished, the blocks were then removed and further embedded in wax and stored at room temperature. These blocks are to undergo immunohistochemical staining and analysis at a later date by another MPhil candidate and were never intended to be included in this candidate's thesis.

2.2.3. Summary of ELISA process:

In order to assess the concentrations of the cytokines within the supernatant ELISAs were performed for the following cytokines:
- Interleukin-6
- Interleukin-1β
- Tissue Necrosis Factor – α (TNF-α)

ELISA is a method for detecting an antibody or antigen within a sample. There are several variants of methodology, with varying sensitivities. 'Sandwich-ELISA' was chosen as the technique to be used in this instance as provides the most sensitive method.

In summary, 'Sandwich' ELISA involves the following:

a. A known quantity of a specific, primary monoclonal antibody is applied to a plastic microtiter plate.
b. Once the primary antibody has bound, it is removed, and then any non-specific binding sites are blocked using a blocking buffer. This ensures that the cytokines within the samples only bind to this primary antibody and not any other non-specific sites.
c. The samples, containing the cytokines under investigation, are then incubated in the 'coated' plate.
d. After a designated period of time, any unbound sample is removed and a polyclonal biotinylated antibody is added. This secondary antibody is specific to the cytokine which has bound to the primary antibody[104].
e. Again after a designated period of time, any unbound secondary antibody is removed. A biotin-labelled detection antibody e.g. Streptavidin horseradish peroxidise (HRP), is then added. Streptavidin HRP consists of streptavidin which is covalently conjugated to the horseradish peroxidise enzyme (HRP). The

streptavidin binds to the biotin of the secondary antibody and the HRP enzyme converts a specific substrate[105].

f. The appropriate substrate, in this instance a ready-to-use solution of 3,3',5,5' - tetramethylbenzidine (TMB), is applied. In the presence of HRP, the TMB and peroxide contained in the substrate solution react to produce a blue by-product. The intensity of the blue colour is proportional to the amount of HRP activity, which in turn is related to the amount of cytokine bound to the primary antibody.

g. A 'stop' solution, sulphuric acid in this case, is then added and changes the colour to yellow, inhibiting any further enzyme activity[106].

h. The optical density of each well on the microtitre plate is then read in a spectrophotometer and compared to a standard curve. The concentration of cytokine within each well is then generated.
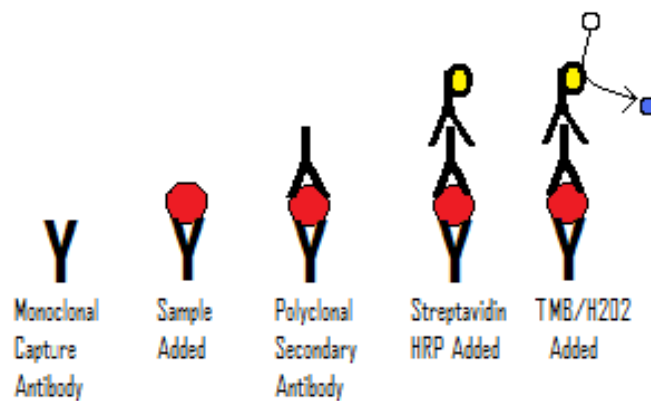
Figure 2-3 - Schematic diagram of the process used in 'sandwich' ELISA.

2.2.3.1. Methodology of ELISA analysis:

ELISAs were performed for the above mentioned cytokines. This method has been written as if three plates were running simultaneously, one for each of the cytokines assayed.

NB.   - The diluent used throughout the run for IL-6 and IL-1β was PBS/1% BSA

   - The diluent used throughout the run for TNF-α was PBS/ 0.1% BSA

Initially, the diluent for TNF-α contained 0.05% Tween 20, however this was omitted after several unsuccessful ELISA runs using this diluent.

A plan detailing where the samples to be assayed were to be placed on the microtiter plate was prepared prior to the ELISA run. Samples were run in duplicate and an average of the two concentrations was taken.

Antigen coating the plates:
- The primary antibodies for each cytokine were prepared:
  - For each cytokine:
     55µl of capture antibody was added to 9945µl of appropriate diluent.
  - (Final concentrations: IL-6 = 200ng/ml, IL-1β = 100ng/ml and TNF-α = 4µg/ml).
- Each plate was then coated with 100 µl per well of primary antibody and covered with plate sealer.
- The plates were then incubated at room temperature overnight.

The plates were then washed using wash buffer. The wells were filled forcefully and once the entire plate was full, the buffer was

decanted into the sink and the blotted vigorously. This was repeated 3 times in total. The wells were then filled with 300 µl of blocking buffer and incubated for 1 hour at 37ºC in a Dynatech Varishaker Incubator. The samples to be assayed were then removed from the freezer and allowed to defrost in ice.

A separate standard curve for each cytokine to be assayed was prepared:

- IL-1β:

    The highest concentration of IL-1β on the standard curve was 500pg/ml. All other standard curve concentrations were prepared by serial dilution using PBS/1% BSA.

Code for plate plan	Final concentration of IL-1β(pg/ml):
S1	500.00
S2	250.00
S3	125.00
S4	62.50
S5	31.25
S6	15.63
S7	7.81
S8	3.91

Table 2.5 - Table to show standard curve for IL-1B.

- IL-6:

    The highest concentration of IL-6 on the standard curve was 600pg/ml. Again all other standard curve concentrations were prepared by serial dilution from this first concentration.

Code for plate plan	Final Concentration of IL-6 (pg/ml):
S1	600
S2	300
S3	150
S4	75.0
S5	37.5
S6	18.75
S7	9.38
S8	4.69

▪ TNF-α:

The highest concentration of TNF-α on the standard curve was 1000pg/ml.Once again, all other standard curve concentrations were prepared by serial dilution.

Code for plate plan	Final Concentration of TNF-α (pg/ml):
S1	1000
S2	500
S3	250
S4	125
S5	62.5
S6	31.25
S7	15.6

Table 2.7 – Table curve for TNF-α.   to show standard

NB.   Blank   diluent   was used as S0 for all cytokines.

1:2 dilutions of the extracellular supernatants were prepared (150µl sample + 150µl diluents). This was done because when samples were run undiluted the generated cytokine concentration was greater than the highest concentration on the standard curve i.e. '>max'

1:4 dilutions of the intracellular supernatant were prepared (60µl sample + 180µl diluents). The reasoning for this was the same as for dilution of extracellular supernatant.

NB. All the samples were vortexed thoroughly prior to use to ensure a representative sample was used for the ELISA.

After one hour, the plates were removed from the incubator and washed three times with wash buffer. 100 µl of each standards,

sample or diluent was pipetted into the plate and incubated for a further hour at 37ºC.

The secondary antibodies were then prepared by performing a 1:180 dilution (55µl antibody + 9945µl diluents) giving the final concentrations of each cytokine were as: IL-1β = 100ng/ml, IL-6 = 200ng/ml, and TNF-α = 300ng/ml.

After one hour, the plates were removed from the incubator and washed three times with wash buffer. 100 µl of the secondary antibody was then added to each well and then incubated for a further hour at 37ºC.

A 1:200 dilution of the detection agent, Streptavidin HRP, was then prepared (50µl Streptavidin HRP + 9950µl diluents). After the hour, the plates were again removed from the incubator and washed three times with wash buffer. 100 µl of Streptavidin/HRP was added to each well and incubated for a further 30 minutes at room temperature. After 30 minutes, the plates were removed from the incubator and washed three times with wash buffer.

100µl of TMB/H_2O_2 was added to each well. The solution was inspected prior to use and was discarded if it already appeared blue. The plates were then incubated, in the dark, at room temperature, for 6-7 minutes, or until there was an adequate colour gradient along the standard curve. 50 µl of 1M H_2SO_4 was then added to each well to inhibit any further enzyme activity.

The plate was then placed on the plate reader, and the optical density of the wells was then read between the intensities of 450nm and 540nm. A graph, plotting cytokine concentration against net absorbance, was then generated, giving a standard curve. A line of best fit was then applied and the concentration of the cytokine

being assayed within each sample was automatically generated using the standard curve.

If any results were '>max' i.e. the concentration of cytokine within that well exceeded the highest concentration on the standard curve, then, if possible, this result was repeated on another run. If any results were '<min' i.e. the concentration of cytokine within that well fell below the lowest concentration on the standard curve, then, if possible, this result was repeated, undiluted, on another run.

2.2.4. Summary of Protein Concentration anaylsis for the Placental fragments:

In order to quantify the results generated by the ELISAs, the protein concentration of each placental villous tissue fragment needed to be determined. If this concentration remained unknown then any recorded differences in cytokine concentration could be attributed to variations in the volume of villous tissue of the samples as opposed to any true physiological differences induced by the stimulants.

To determine the protein concentration of the samples, Coomassie Plus (Bradford) Assays were performed on all samples. This procedure was chosen as it is a simple and cheap procedure for the determination of protein concentrations of multiple samples. The Bradford reagent contains coomassie G-250 dye, which when bound to protein, changes colour from reddy/brown to blue[107]. This results in a spectral shift of absorbance which can then, like in ELISAs, be read by a spectrophotometer.

2.2.4.1. Methodology for the Bradford Protein Assay Run:

The fragments which had been placed in 4ml of 0.3M NaOH and left to lyse were used for this section of the methodology. Samples were run in duplicate and an average of the two concentrations was taken. As there are only eleven samples per placenta, two placentae were assayed simultaneously. A standard curve was prepared as follows:

Code used on plate plan	Final Protein Concentration (µg/ml)
S0	0
S1	12.5
S2	62.5
S3	125.0
S4	187.5
S5	250.0

Table 2.8 – table to show standard curve used for protein assays.

The standards were pipetted onto the plate (20µl standard+180µl PBS) and then the samples were added to the plate (10µl sample+190µl PBS). A smaller volume of sample was added, otherwise if 20µl was added the protein concentration generated was greater than the highest concentration on the standard curve i.e. '>max'. 50µl of Bradford's dye reagent (at room temp) was then added to each well. The microtiter plate was then placed on the plate reader and mixed for 30seconds. The plate was read at an absorbance of 595nm. A graph, plotting protein concentration against net absorbance was then generated, giving a standard curve. A line of best fit was then applied and the concentration of protein within the samples was generated automatically using the standard curve.
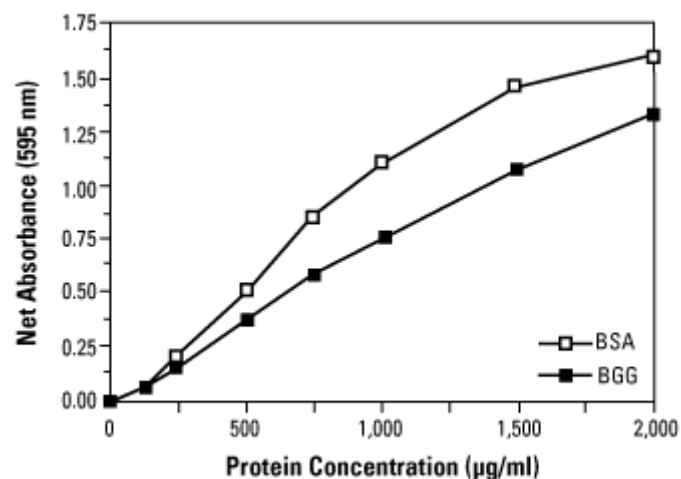
Figure 2-4 - A Typical standard curve for BSA and bovine gamma globulin (BGG) in the Pierce Coomassie Plus Protein Assay.[104]

Once the protein content of the villous tissue had been determined, the corresponding cytokine concentrations of the samples were divided giving a final cytokine concentration in pg/mg of protein.

Chapter 3: Results

3.1. Maternal, Neonatal, and Placental Histological Results:

Data which fell into the following categories was excluded from statistical analysis:

- Deliveries before 258 days gestation,
- Deliveries after 288 days gestation,
- Neonatal data from women diagnosed with GDM.

3.1.1. Maternal Demographics:

Means and standard deviations (s.d.) were calculated for all variables, and then independent samples t-tests were performed to compare means and s.d's between lean and obese participants. Differences in means were considered significant if the p value was less than 0.05. For all calculations, unless stated, equal variances were assumed.

	Lean, n = 17 (mean+/-S.D)		Obese, n = 85 (mean+/-S.D)		Mean Difference	95% Confidence Interval		P-value
	Mean	S.D	Mean	S.D		Lower	Upper	
Age (years)	30.82	4.84	29.35	5.95	-1.47	-4.52	1.58	0.341
Height (cm)	159.08	5.77	164.06	6.89	**4.99**	**1.44**	**8.53**	**0.006**
Weight (kg)	59.93	9.76	105.07	11.88	**45.14**	**39.04**	**51.23**	**0.000**
BookingBMI (kg/m2)	23.58	2.93	39.00	3.54	**15.42**	**13.60**	**17.24**	**0.000**
Weight Gain (kg)	-	-	*n = 18* 7.23	4.82	-	-	-	-

Table 3.1 – Table to show means and standard deviations of maternal demographics of lean and obese participants.

As per study design, obese participants had a higher booking weight, giving them an average booking BMI of 39.00. Obese mothers were also significantly taller than lean participants. Data on weight gained during pregnancy was only available for eighteen of the obese participants, and no data was available for lean participants. Obese participants had an average weight gain of 7.23 +/- 4.82kg.

Due to the skewed nature of the variables; gravidity, parity and ethnicity; medians and interquartile ranges (IQR) were calculated opposed to means and s.d's, then a Mann-Whitney U test was performed to compare the medians and IQRs between lean and obese participants.

	Lean, n=17		Obese, n=85		P-Value
	Median	Interquartile Range	Median	Interquartile Range	
Gravidity	3	1.5-3	2	2-4	0.698
Parity	1	0.5-1.5	1	1-2	0.437
Ethnicity	1	1-1	1	1-1	0.708

Table 3.2- Table to show medians and IQRs of the gravidity, parity and ethnicity of lean and obese participants.

There were no significant differences in the gravid, or parity, between the lean and obese participants. For ease, ethnicity was coded as specified in the method; the number one denotes for 'White British'. 100% of participants in the lean cohort were white British and, 93% of participants in the obese cohort were also white British. Therefore, there were no significant differences between the ethnicities of the two cohorts with the majority of participants, in both cohorts, being white British.

3.1.2. Neonatal Demographics:

For continuous neonatal data, independent sample t-tests were performed to assess a difference between infants of lean and obese participants.

	Lean, n = 17			Obese, n = 85			Mean Difference	95% Confidence Interval		P-value
	Mean	S.D	Range	Mean	S.D	Range		Lower	Upper	
Gestational age (days) *(equal variances not assumed)*	272.24	3.63	265-282	275.41	7.54	259-287	3.18	0.76	5.59	**0.011**
Birth weight (g)	3180.71	516.25	2160-4050	3637.98	518.54	2480-5210	457.27	184.13	730.41	**0.001**
Birth weight Centile	42.54	32.84	0.9-97.3	50.66	31.87	0.1-100	8.12	-8.76	25.01	0.342

Table 3.3 - Table to show means and standard deviations of neonatal demographics of infants born to lean and obese participants.

Infants born to obese participants had a higher birth weight than infants born to lean participants. Infants of obese participants were, on average 457.27g heavier than infants of lean participants. There was also a significant difference in gestational age of approximately 3days. There was no significant difference in the average birth weight centile between the two cohorts.

In order to examine birth weight centiles more accurately, the centiles were subdivided into the following groups: <2nd, ≤9th, 9th-91st, >91st and >98th. Also to examine if more neonates of obese participants were classified as macrosomic compared to neonates of lean participants, birth weight was also divided into two groups; birth weight <4000g and birth weight > 4000g. In order for this data to be analysed the data was first classified into separate sub-groups and then independent T-tests were performed within each sub-group.

Variable	Group	Lean, n=17			Obese, n=85			Mean Diff.	95% CI		P-value
		No. in each sub-group	Mean	S.D	No. in each sub-group	Mean	S.D		Lower	Upper	
Birth weight Centile	0-2nd centile	1	0.90	-	2	0.85	1.06	-0.05	-16.56	16.46	0.976
	2nd-9.00th centile	3	5.50	2.36	8	5.09	2.51	-0.41	-4.21	3.39	0.811
	9.01th-90.99st centile	11	46.80	23.63	65	50.83	25.95	4.03	-12.63	20.63	0.631
	91st-97.99th centile	2	95.50	2.55	7	94.40	1.96	-1.10	-4.99	2.79	0.525
	98th-100th centile	0	-	-	3	99.67	0.58	-	-	-	-
Birth weight	<4000g	16	3126.38	362.92	64	3416.2	486.4	289.8	73.69	505.80	**0.009**
	>4000g	1	4050.00	-	21	4314.1	264.4	277.4	264.1	-328.1	0.363

Table 3.4 - Table to show distribution of birth weight centiles and neonatal macrosomia in subdivided groups of lean and obese participants.

There was no significant difference between the lean and obese cohort for any of birth weight centile groups. There was a significance difference between the lean and the obese cohort for the number of neonates with a birth weight of <4000g (p=0.009). This is most probably attributable to the small numbers in the lean cohort.

In order to examine whether there was a difference of neonatal sex between the two cohorts a Chi-squared test was performed.

Variable	Category	Lean, n=17	Obese, n=85	Chi-squared (χ^2)	P-value
Neonatal Sex	Male	10	48	0.032	0.858
	Female	7	37		

Table 3.5 – Table to show p-value for number of male and female neonates within each cohort.

There was also no difference in the distribution of neonatal sex in lean and obese participants.

The determinants of birth weight were then explored. Birth weight and birth weight centile were plotted on a set of graphs against maternal BMI for lean and obese participants. Graphs were plotted as scatter plots, displaying means, 95% confidence intervals, and the R^2 value (square of the correlation coefficient).

Figure 3.1 – Graph to show relationship between maternal booking BMI and birth weight in obese participants.
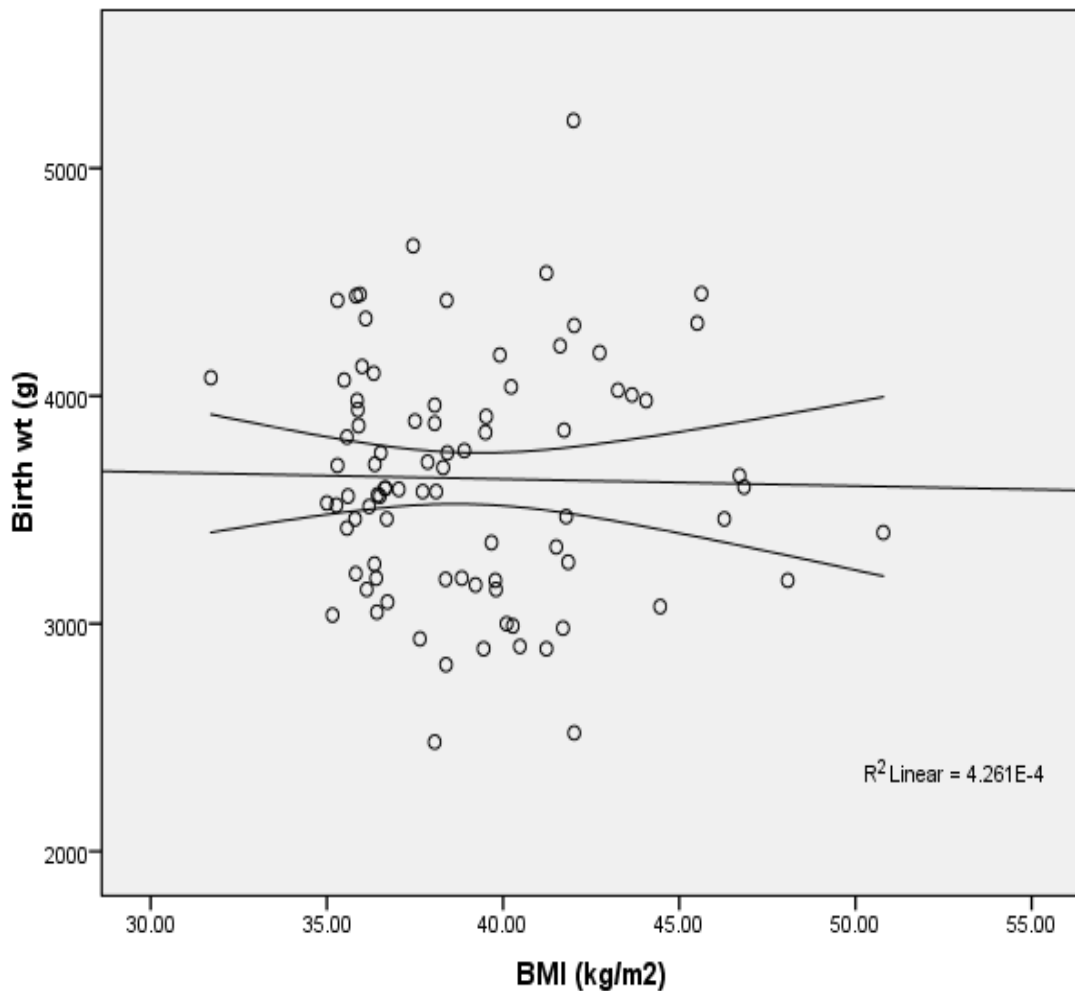
In obese participants, maternal booking BMI explained 0.042% of the variance in birth weight and therefore demonstrates that maternal BMI is not significantly correlated to birth weight.

Figure 3.2 – Graph to show relationship between maternal booking BMI and birth weight in lean participants.
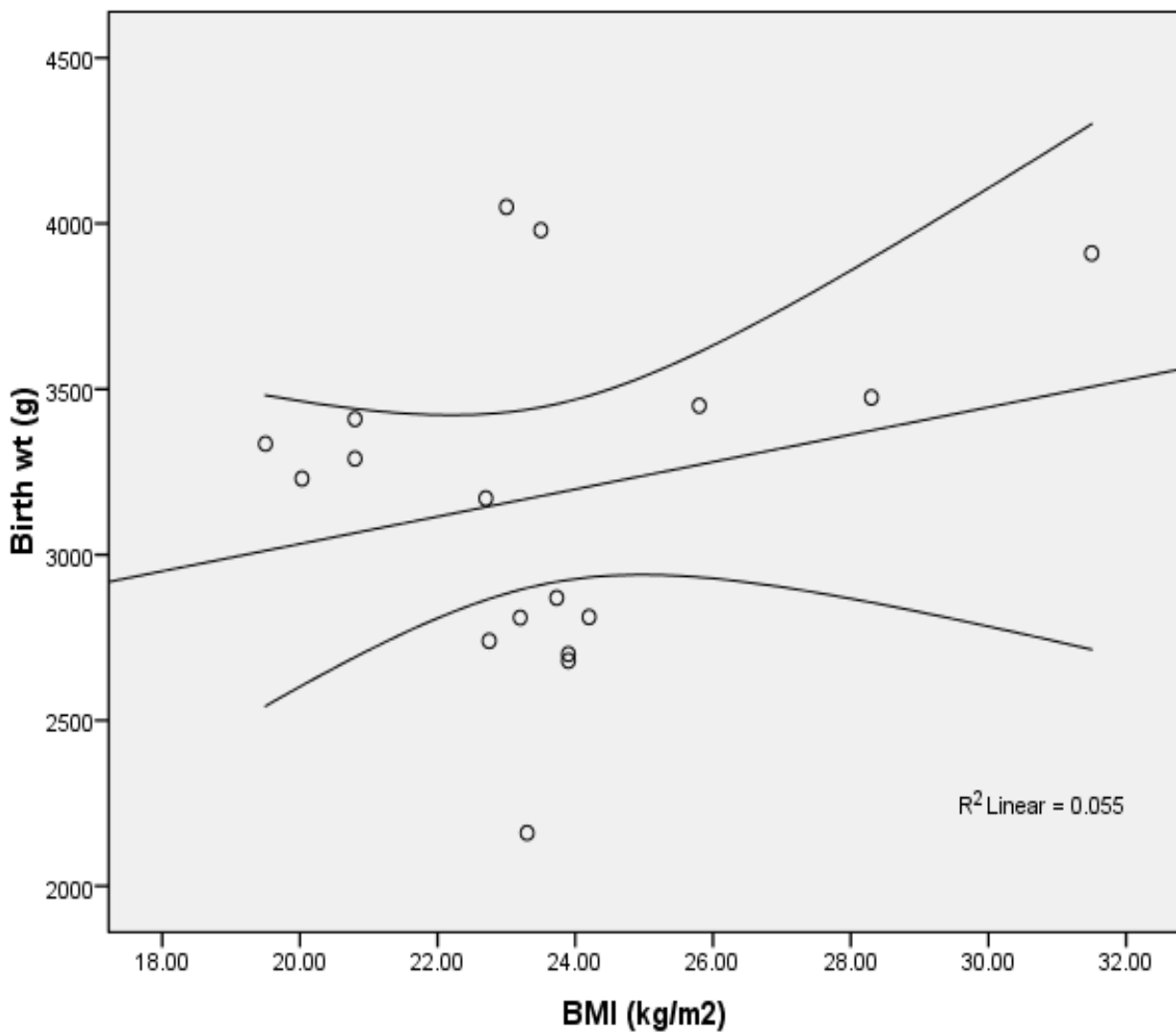
In lean participants, maternal booking BMI explained 5.5% of the variance in birth weight and therefore also shows that maternal BMI

is not significantly correlated to birth weight. However, this graph does show there is slightly more (5.458%) correlation between the lean cohort and neonatal birthweight compared to the obese cohort.

3.1.2.1. Neonatal Anthropometrics Measurements:

Means and standard deviations were calculated for infants born to lean and obese participants. Independent sample t-tests were then performed to assess whether there was a significant difference between the two cohorts.

	Lean, n = 15		Obese, n = 33		Mean Difference	95% Confidence Interval		P-value
	Mean	S.D	Mean	S.D		Lower	Upper	
Length (cm)	50.45	2.42	52.17	3.21	1.72	-0.15	3.60	0.071
HC (cm)	34.34	1.31	35.39	1.06	1.05	0.36	1.75	**0.004**
Flank SFT (mm)	4.04	0.91	4.68	0.71	0.64	0.160	1.13	**0.010**
Fat Mass (kg)	0.30	0.15	0.48	0.17	0.19	0.09	0.29	**0.001**
Free Fat Mass (kg)	2.78	0.32	3.14	0.37	0.36	0.14	0.59	**0.002**
% Body Fat	9.25	3.76	13.11	3.34	3.86	1.69	6.04	**0.001**

Table 3.6 - Table to show means and standard deviations of neonatal anthropometric measurements of infants born to lean and obese participants

In order to determine if there was a difference in the number of neonates which were classified as obese (defined by a % body fat > 16%) a Pearson's Chi squared test was performed.

Variable	Category	Lean, n=15	Obese, n=33	Chi-squared (χ^2)	P-value
% Body Fat	<16%	93.3%(14)	81.81%(27)	1.098	0.295

	>16%	6.66%(1)	18.18%(6)		

Table 3.7- Table to show p-value for number of obese neonates in lean and obese participants.

All anthropometric measurements, except length, were increased in neonates born to obese participants compared to neonates born to lean participants. On average, neonates of obese participants had a head circumference which was 1.05cm bigger, and a flank skin fold which was 0.64 mm thicker, than neonates of lean participants. Overall, fat mass was increased by 190g and fat free mass was increased by 360g in neonates of obese participants, which culminated in an increased percentage by fat 3.86%. There was no difference in the number of neonates classified as obese in either cohort.

The determinants of % body fat were then explored and plotted on a graph against maternal BMI for lean and obese participants. Graphs were plotted as scatter plots, displaying means, 95% confidence intervals, and the R^2 value (square of the correlation coefficient).

Figure 3.3 – Graph to show relationship between maternal booking BMI and neonatal % body fat in obese participants.

In obese participants, maternal booking BMI explained 0.2% of the variance in neonatal % body fat. Therefore this graph demonstrates that maternal BMI is not strongly correlated to neonatal % body fat in the obese cohort
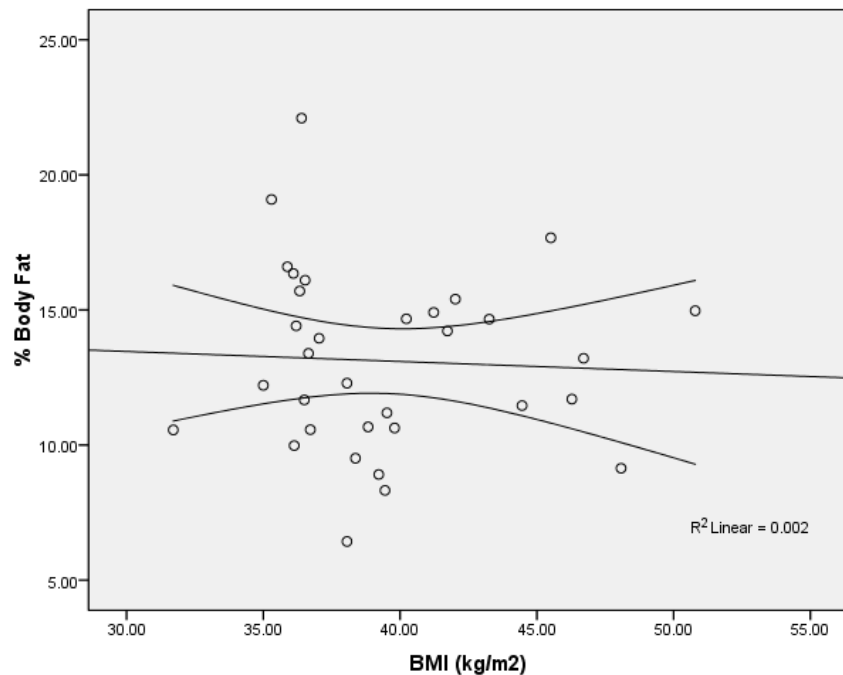
Figure 3.4 – Graph to show relationship between maternal booking BMI and Noenatal % body fat in lean participants.
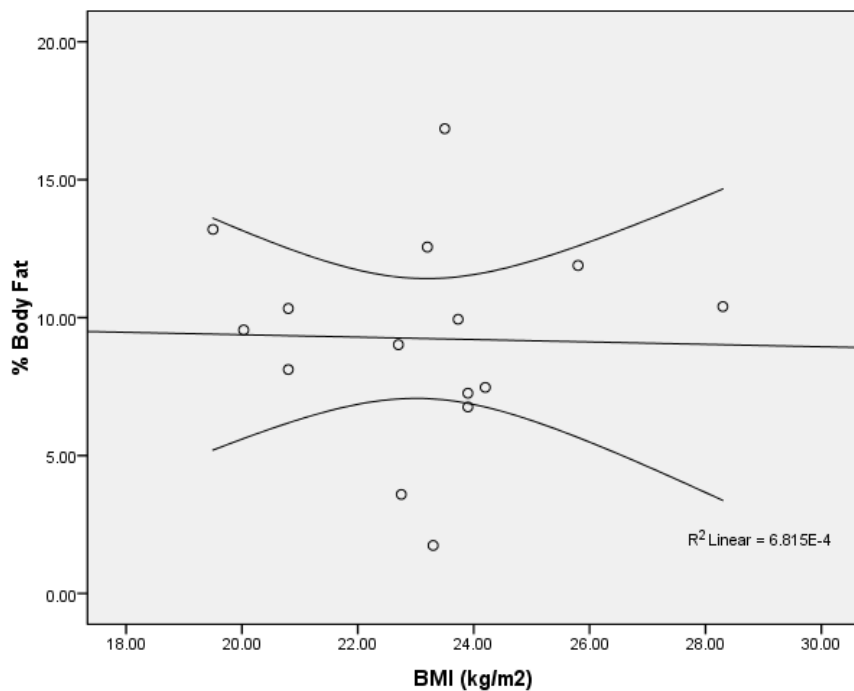
In lean participants, maternal booking BMI explained 0.068% of the variance in neonatal % body fat. This graph therefore demonstrates a similar finding to figure 3.3, illustrating that maternal BMI in the lean cohort is also not strongly correlated to neonatal % body fat.

The relationship between neonatal % body fat and birth weight centile was then explored to determine what proportion of the variation in birth weight centile was attributable to variation in body fat.

Figure 3.5 – Graph to show the relationship between % body fat and Birth weight centile in obese participants.
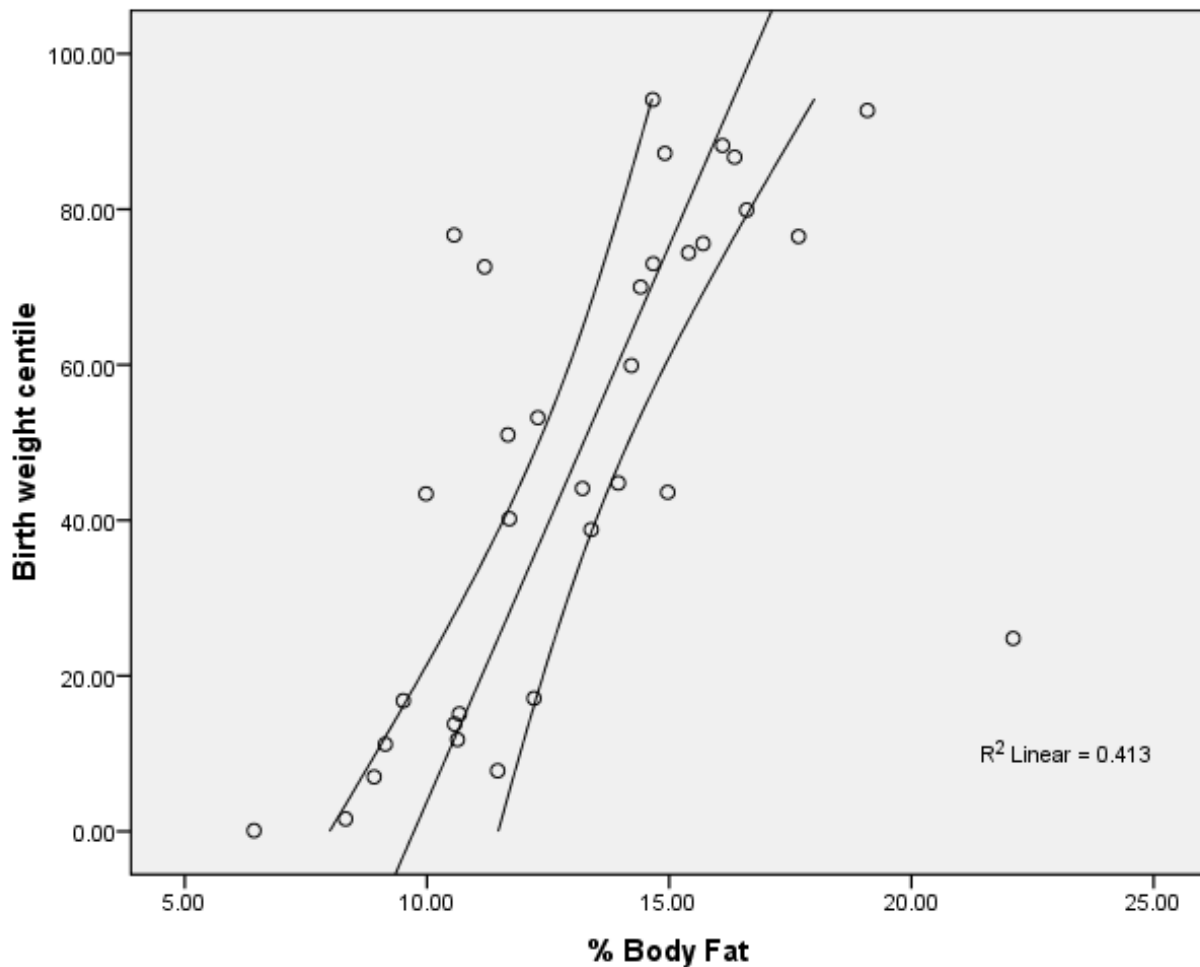
In obese participants, % body fat explained 41.3% of the variation in birth weight centile. This graph therefore demonstrates that 41.3% of the variation in birth weight centiles in the neonates from the obese cohort was attributable to changes in % body fat.

Figure 3.6 – Graph to show the relationship between % body fat and Birth weight centile in lean participants.
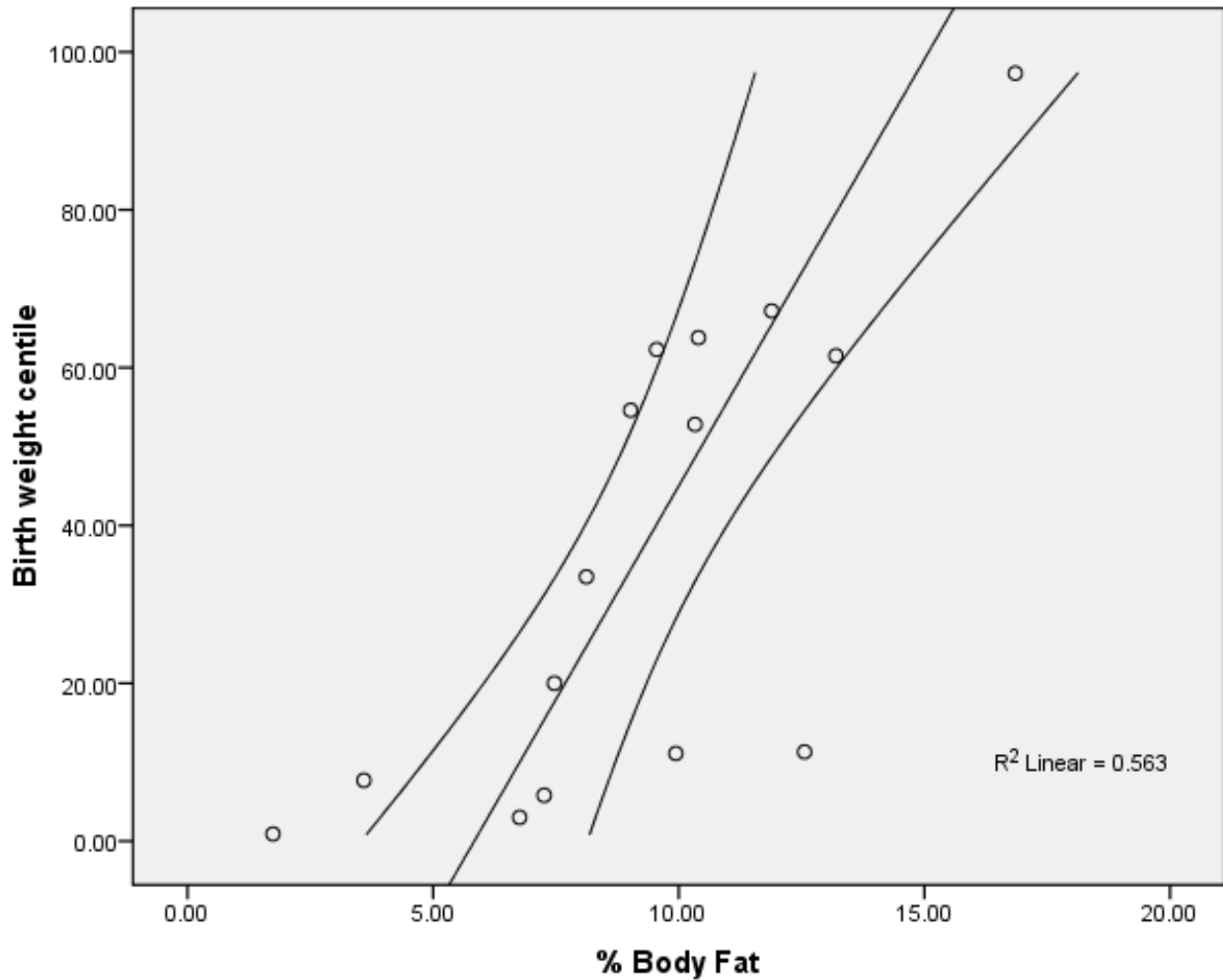
In lean participants, % body fat explained 56.3% of the variation in birth weight centile. This graph therefore demonstrates that a greater proportion of the variation in birth weight centiles in the neonates in the lean cohort was attributable to % body fat compared to neonates in the obese cohort.

3.1.3. Placental Demographics:

Four histological reports for lean participants and seven reports for obese participants were still outstanding; therefore these were excluded from analysis.

	Lean, n= 14		Obese, n = 50		Mean Difference	95% Confidence Interval		P-value
	Mean	S.D	Mean	S.D		Lower	Upper	
Placental weight (g)	568.15	79.99	670.51	124.44	102.36	27.49	177.23	**0.008**
Placental trimmed weight (g) *Equal Variances not assumed*	474.50	59.44	546.20	119.18	71.70	25.02	118.38	**0.003**

Obese participants had an increased untrimmed and trimmed placental weight in comparison to lean participants' placentae. Maternal booking BMI was then plotted against placental trimmed weight for lean and obese participants to determine if there was any correlation between the two variables.

Figure 3.7 – Graph to show the relationship between maternal booking BMI and placental trimmed weight in obese participants.

In obese participants, maternal booking BMI explained 1% of the variation in placental trimmed weight. Therefore
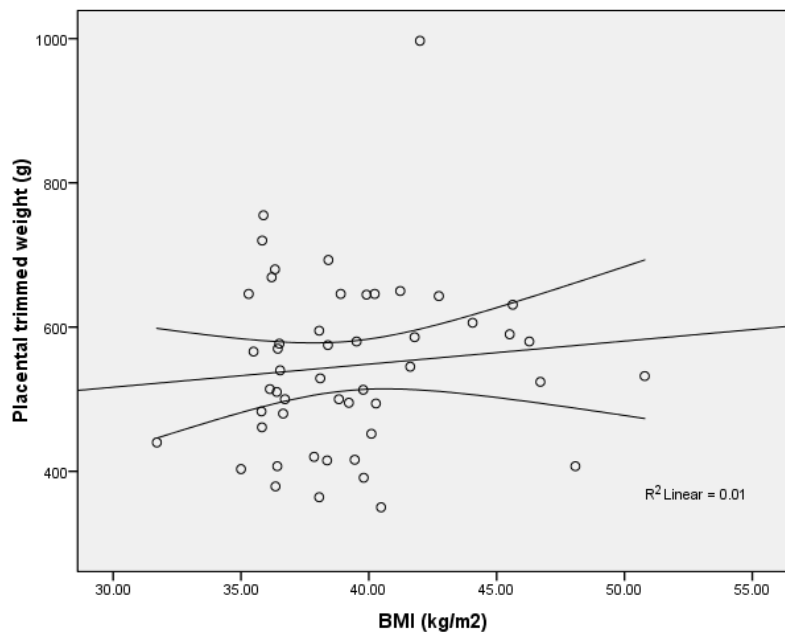
this demonstrates that there is no significant correlation between maternal BMI and placental trimmed weight in the obese cohort.

Figure 3.8 – Graph to show the relationship between maternal booking BMI and placental trimmed weight in lean participants.
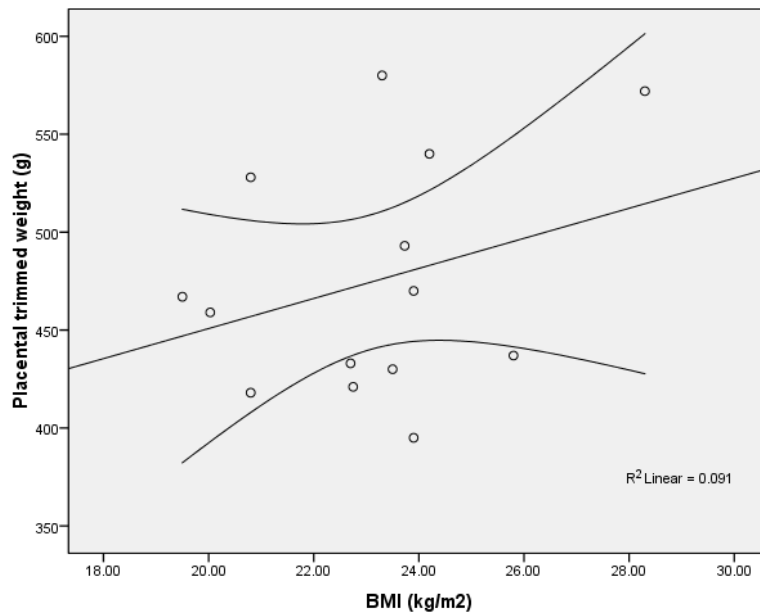
In lean participants, maternal BMI explained 9.1% of the variation in placental trimmed weight. This graph therefore demonstrates that in the lean cohort maternal BMI correlated more strongly (9.1% vs 1%) with placental trimmed weight when compared to the obese cohort.

Histological classification was then explored next. One report for an obese placenta was still waiting to be classified; therefore it was excluded from analysis.

		Lean, n =14		Obese, n =49		Chi-squared Value (χ^2)	P Value
		Actual Number	% of total	Actual Number	% of total		
Histological classification	**Normal**	3	21.43	11	22.45		
	Chorangiosis	10	71.43	18	36.73	6.828	0.078
	MVUP	1	7.14	12	24.49		

	Other	0	0.00	8	16.33		

Table 3.5 - Categorisation of patho-histological reports in lean and obese participants.

A significant difference between histological reports for lean and obese placentae was not found when birth weight was not categorised. There were similar percentages of reported normal placentae in lean and obese participants. However, 71.43% of lean placentae had reported chorangiosis compared to 36.73% of obese placentae, and 7.14% of lean placentae vs 24.49% of obese placentae had reported maternal vascular underperfusion. No lean placentae were reported as 'other', compared to 16.33% of obese placentae which were reported as 'other'.

In order to determine whether any of the histological categories related to the development of birth weight, neonates were subdivided into groups dependent upon their birth weight centile in both cohorts. A Pearson's Chi squared test was then performed for neonates of lean and obese participants.

		Histological Category								Chi-squared Value (χ^2)	P-value
		Normal		MVUP		Chorangiosis		Other			
		No.	%	No.	%	No.	%	No.	%		
Birth weight Centile	0-2nd centile	0	0%	1	2%	1	2%	0	0%	8.072	0.779
	2nd-9th centile	2	4%	1	2%	0	0%	0	0%		
	9.01th-90.99st centile	7	14%	8	16%	14	24%	7	14%		
	91st-97.99th centile	2	4%	1	2%	2	4%	1	2%		
	98th-100th centile	0	0	1	2%	1	2%	0	0%		

Table 3.6 - Table to show association between histological categories and birth weight centiles in obese participants.

		Histological Category								Chi-squared Value (χ^2)	P-value
		Normal		MVUP		Chorangiosis		Other			
		No.	%	No	%	No.	%	No.	%		
Birth weight Centile	0-2nd centile	0	0	0	0	1	7%	0	0	3.111	0.795
	2nd-9th centile	0	0	0	0	3	21%	0	0		
	9.01th-90.99st centile	3	21%	1	7%	5	36%	0	0		
	91st-97.99th centile	0	0	0	0	1	7%	0	0		
	98th-100th centile	0	0	0	0	0	0	0	0		

Table 3.7 - Table to show association between histological categories and birth weight centiles in lean participants.

Placental histological category had no significant effect on birth weight centiles in either neonates of obese, or lean, participants.

3.1.4. Univariate analysis

Associations between maternal and neonatal variables were explored next. Depending on the type of data (continuous or categorical) different statistical tests were performed.

Bivariate Pearson correlations were performed for the following continuous variables; maternal BMI, maternal weight gain and gestational age; and; birth weight, head circumference, length, flank skin fold thickness, % body fat and placental trimmed weight.

		Birth Weight (g)	Neonatal Head Circumference (cm)	Neonatal Length (cm)	Neonatal Flank Skinfold Thickness (mm)	Neonatal % Body Fat	Placental trimmed weight (g)
		n=85	n=33	n=33	n=33	n=33	n=50
Maternal BMI	P-value	0.851	0.782	1.000	0.517	0.790	0.481
	Pearson's Correlation Coefficient	-0.021	-0.05	0.000	0.117	-0.48	0.102
Parity	P-value	0.795	0.445	0.823	0.480	0.605	0.419
	Pearson's Correlation Coefficient	0.029	-0.138	0.04	-0.127	0.093	0.117
Gestational Age (days)	P-value	**0.025**	0.063	0.105	0.465	0.633	0.639
	Pearson's Correlation Coefficient	**0.243**	0.327	0.287	0.132	0.086	-0.068
Maternal Weight Gain (kg)	P-value	*n=20* 0.228	*n=20* 0.809	*n=20* 0.789	*n=20* 0.500	*n=20* 0.227	*n=18* 0.421
	Pearson's Correlation Coefficient	-0.282	-0.058	-0.064	-0.160	-0.283	-0.202

Table 3.8 - Table to show p-value when bivariate analysis was performed between the listed variables in the obese cohort.

In obese participants, the only variables which significantly correlated to one another were gestational age and birth weight.

		Birth Weight (g)	Neonatal Head Circumference (cm)	Neonatal Length (cm)	Neonatal Flank Skinfold Thickness (mm)	Neonatal % Body Fat	Placental trimmed weight (g)
		Lean n=17	Lean n=15	Lean n=15	Lean n=15	Lean n=15	Lean n=14
Maternal BMI	P-value	0.366	0.082	0.870	0.932	0.926	0.296
	Pearson's Correlation	0.234	-0.463	-0.046	-0.024	-0.026	0.301
Parity	P-value	0.471	0.700	0.666	0.248	0.810	0.346
	Pearson's Correlation	0.188	0.109	0.122	0.318	0.068	-0.272
Gestational Age (days)	P-value	**0.04**	**0.012**	**0.01**	0.725	0.192	0.759
	Pearson's Correlation	**0.502**	**0.630**	**0.643**	0.099	0.356	-0.09

Table 3.9 - Table to show p-value when bivariate analysis was performed between the listed variables in the lean cohort.

In lean participants, gestational age correlated to birth weight, head circumference, and length.

A Mann-Whitney U test was performed between the following continuous variables; birth weight, head circumference, length, flank skin fold thickness, % body fat and placental trimmed weight; and the following categorical variable; neonatal sex.

	Birth Weight (g)		Neonatal Head Circumference (cm)		Neonatal Length (cm)		Neonatal Flank Skinfold Thickness (mm)		Neonatal % Body Fat		Placental trimmed weight (g)	
	Obese n=85	Lean n=17	Obese n=33	Lean n=15	Obese n=33	Lean n=15	Obese n=33	Lean n=15	Obese n=33	Lean n=15	Obese n=50	Lean n=14
Neonatal Sex	**0.008**	0.558	0.202	0.324	0.362	0.722	0.386	0.862	0.512	0.643	0.340	0.277

Table 3.10 - Table to show the p-values when Mann Whitney U tests were performed for the listed variables.

In obese participants, birth weight correlated to neonatal sex. There were no other significant correlations with neonatal sex in lean or obese participants.

A Kruskal Wallis H-test was performed between the following continuous variables; maternal BMI, parity, gestational age, and weight gain; and the categorical variable; placental histological classification (placental histological categories were the same as those listed in Table 3.6 - normal, chorangiosis, MVUP, and other).

	Placental Histological Classification	
	Obese n=49	Lean n=14
Maternal BMI	1.000	0.394
Parity	0.648	0.881
Gestational Age (days)	0.758	0.179
Maternal Weight Gain	n=18 0.351	

Table 3.11 - Table to show p-values when a Kruskal-Wallis test was performed for the listed variables.

None of the maternal variables correlated significantly to placental histological classification in lean or obese participants.

A Kruskal Wallis H-test was also performed between the following continuous variables; birth weight, head circumference, length, flank skin fold thickness, % body fat and placental trimmed weight; and the categorical variable; maternal smoking status (smoker, non-smoker, ex-smoker).

The smoking status of one obese participant was unknown and therefore was excluded from analysis.

	Birth Weight (g)		Neonatal Head Circumference (cm)		Neonatal Length (cm)		Neonatal Flank Skinfold Thickness (mm)		Neonatal % Body Fat		Placental trimmed weight (g)	
	Obese n=84	Lean n=17	Obese n=32	Lean n=15	Obese n=32	Lean n=15	Obese n=32	Lean n=15	Obese n=32	Lean n=15	Obese n=49	Lean n=14
Maternal Smoking Status	0.479	0.646	0.437	0.826	0.331	0.211	0.271	0.699	0.686	0.458	0.481	0.322

Table 3.12 - Table to show the p-values when a Kruskal-Wallis test was performed for the listed variables.

Maternal smoking status did not significantly correlate to any of the continuous variables in lean or obese participants.

Pearson's Chi-squared tests were performed for the following categorical variables; placental histological classification, maternal smoking status, and neonatal sex.

	Placental Histological Classification	
	Obese n=49	Lean n=14
Maternal Smoking Status	0.373	0.326
Neonatal Sex	0.668	0.420

Table 3.13 - Table to show p-values when a Pearson's Chi-squared test was performed between the following variables

Placental histological classification did not correlate to either maternal smoking status, or neonatal sex, in lean or obese participants.

3.1.5. Multivariate analysis:

Due to so many insignificant results when univariate analysis was performed, no multivariate analysis was performed.

3.2. Placental Tissue Fragment Incubation Results:

3.2.1. Demographic variables of Placentae used in Placental Tissue Fragment Incubation:

Means and standard deviations (s.d.) were calculated for the variables, and then independent samples t-tests were performed to compare means and s.d's between lean and obese participants. Differences in means were considered significant if the p value was less than 0.05. For all calculations, unless stated, equal variances were assumed.

	Obese n=5		Lean n=5		Mean Difference	95 % Confidence Interval		P-Value
	Mean	S.D	Mean	S.D		Lower	Upper	
Age (yrs)	30.20	4.15	33.40	3.91	3.20	-9.08	2.68	0.245
Height (cm)	161.40	9.66	159.80	5.96	1.60	-10.11	13.31	0.764
Weight(kg)	101.00	7.18	55.40	5.77	45.60	36.10	55.10	**0.001**
BMI	39.17	5.94	21.72	2.87	17.45	2.95	10.65	**0.001**
Gestational Age (days)	276.00	6.78	270.20	6.87	5.80	4.32	-4.16	0.216
Birth weight (g)	3664.00	315.48	333.90	524.17	325.00	-305.92	955.92	0.269
Birth Weight Centile	49.52	24.91	59.72	27.90	-10.20	-48.77	28.37	0.559
Length (cm)	54.54	2.46	51.18	2.10	3.36	0.28	6.69	0.049
Head Circumference (cm)	35.34	0.77	34.84	2.01	0.50	-1.72	2.72	0.618
Flank Skin Fold Thickness (mm)	4.08	0.92	5.00	0.83	-0.92	-2.19	0.35	0.135
Fat Mass (kg)	0.396	0.067	0.420	0.161	-0.02	-0.20	0.156	0.766
Fat Free Mass (kg)	3.27	0.27	2.92	0.38	0.35	-0.14	0.84	0.135

% body Fat	10.81	1.48	12.27	3.00	-1.46	-4.91	1.99	0.358
Placental Weight (g)	580.50	205.77	510.00	52.89	70.5	-296.73	437.73	0.584

The only significant difference between the two cohorts was maternal weight and BMI which was expected given the two separate cphorts. All participants in the two cohorts were white British, non-smokers. Both cohorts had an average parity of one, and a gravidity of two. Case summaries were performed for all cytokines measured and conditions used. General linear modelling was performed to examine the effect of; BMI, the stimulant, and BMI and the stimulant in combination. These statistics were performed to examine whether:

a) There was any difference under basal conditions between lean and obese placental cells ('BMI' column);

b) The stimulant alone had any effect on cytokine production ('stimulant' column) and finally;

c) There was a difference in the behaviour of the lean and obese placental cells in response to the stimulant ('BMI*Stimulant' column).

The terms intra- and extra-cellular have been used throughout; extra-cellular denotes to the quantity of cytokine secreted, and intra-cellular denotes to the residual amount of cytokine left within the cells.

Tables are subdivided according to which cytokine was measured.

3.2.2. Effect of Glucose, Leptin, TNF-α and Insulin on IL-1β Production by Lean and Obese Placental Tissue Fragments:

	IL-1β Extracellular			IL-1β Intracellular		
	BMI	Stimulant	BMI* Stimulant	BMI	Stimulant	BMI* Stimulant
5 mM glucose 15mM Glucose 25 mM glucose	0.371	**0.000**	0.482	0.548	**0.000**	0.963
Leptin 100ng/ml	0.087	0.718	0.522	0.487	0.997	0.815
TNF-α 1ng/ml TNF-α 10ng/ml	0.200	0.163	0.791	0.689	0.716	0.765

TNF-α 30ng/ml						
0.1 nM Insulin	0.007	0.986	0.668	0.508	0.785	0.741
1 nM Insulin						
10 nM Insulin						
100 nM Insulin						

Table 3.14 - Table to show P-values when General linear modelling was performed for IL-1B concentrations

Graphs, displaying standard error bars, were then made for any results with a p-value<0.05.

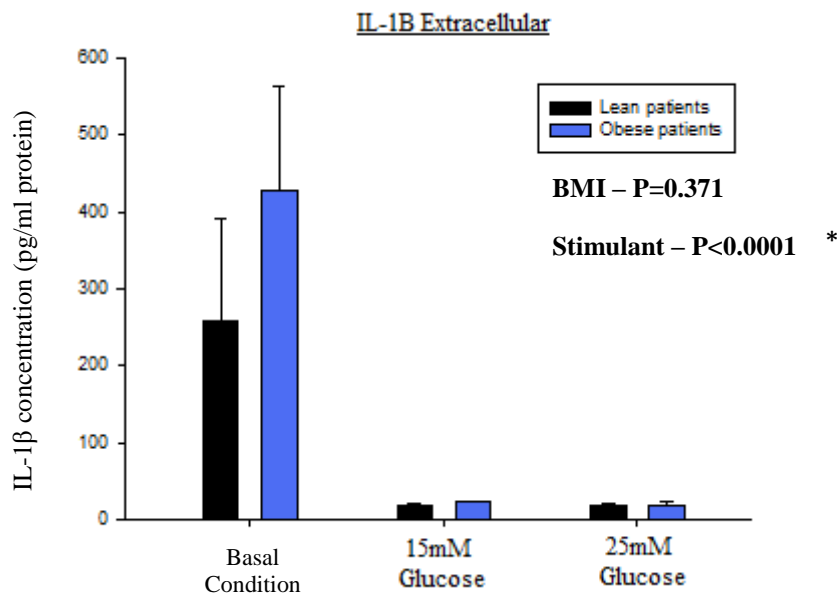## 3.2.3. Glucose stimulation:

Figure 3.9 – Histogram to show IL-1β secretion when stimulated with increasing concentrations of glucose.
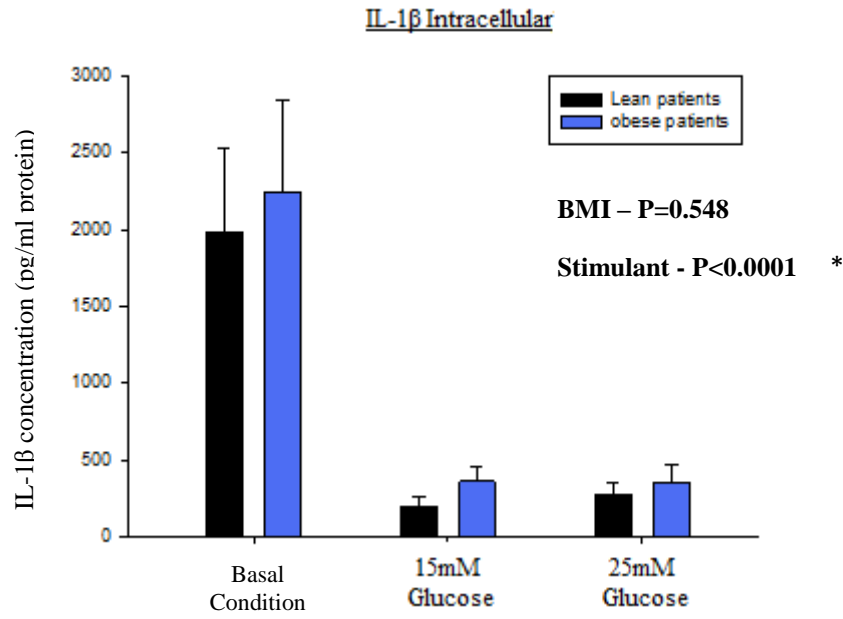
Figure 3.10 – Histogram to show intracellular IL-1β concentration when stimulated with increasing concentrations of glucose.
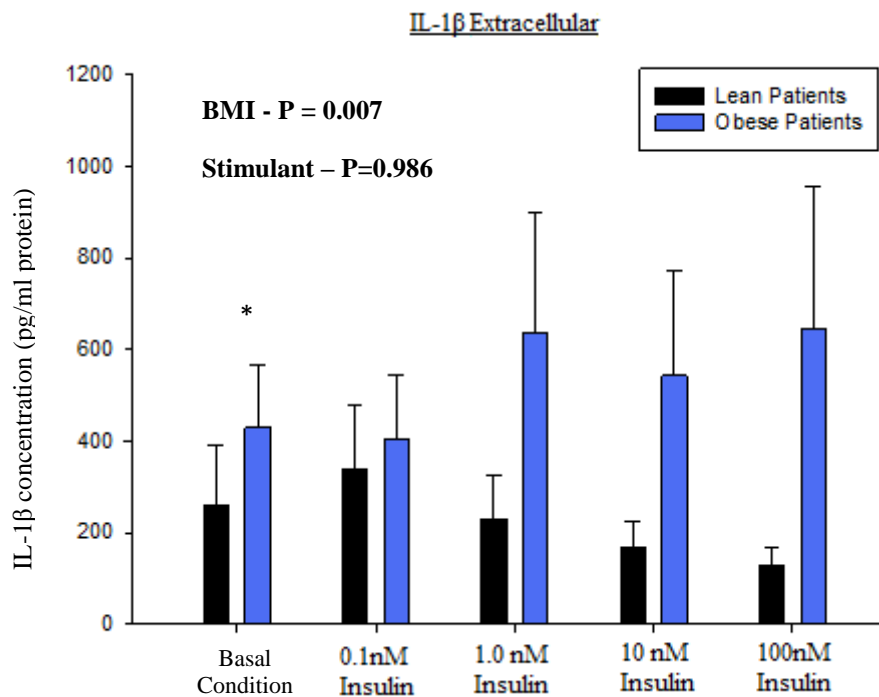
3.2.3.1. Insulin Stimulation:

Figure 3.11 - Histogram to show extracellular IL-1β concentration when stimulated with increasing concentrations of insulin.

Figures 3.9, and 3.10, show that lean and obese placental fragments behave the same way to increasing concentrations of glucose (p=0.482, p=0.963). Intracellular and extracellular concentrations of IL-1β were significantly reduced in response to increasing glucose concentrations in both cohorts (p=0.000). Figure 3.11 shows that under basal conditions obese placental fragments secreted (extracellular concentration) of IL-1β under basal conditions (p=0.007). There was no difference in the response of lean and obese placental fragments to insulin stimulation. Insulin, leptin and TNF-α did not significantly affect the intra- or extra-cellular concentration of IL-1β secreted from the placental cells.

3.2.4. Effect of Glucose, Leptin, TNF-α and Insulin on IL-6 Production by Lean and Obese Placental Tissue Fragments:

	IL-6 Extracellular			IL-6 Intracellular		
	BMI	Stimulant	BMI* stimulant	BMI	Stimulant	BMI* condition
5 mM glucose 15mM Glucose 25 mM glucose	0.000	0.000	0.545	0.000	0.000	0.502
Leptin 100ng/ml	0.011	0.607	0.683	0.002	0.710	0.868
TNF-α 1ng/ml TNF-α 10ng/ml TNF-α 30ng/ml	0.138	0.031	0.968	0.000	0.006	0.981
0.1 nM Insulin 1 nM Insulin 10 nM Insulin 100 nM Insulin	0.010	0.524	0.644	0.002	0.847	0.934

Table 3.15 - Table to show P-values when general linear modelling was performed for IL-6 concentrations

Under basal conditions the obese placental fragments were shown to secrete and contain a higher residual volume of IL-6 compared to the lean placental fragments (denoted by the 'BMI' column, p=0.000).

Increasing concentrations of glucose were shown to significantly reduce the amount of IL-6 secreted from the fragments and the residual concentration of IL-6 within the cells (denoted by the 'stimulant column' p=0.000). The response of the lean and obese placental fragments was the same to this stimulant (denoted by no significant p-value in the 'BMI*stimulant' column. If there was a difference in response of the two cohorts then this p-value would be significant).

TNF-α stimulation was found to have a significant effect on the intra- and extra-cellular concentrations of IL-6 in both lean and obese placental fragments (p=0.031 and p=0.006). There was no difference in the response from the two placental cohorts (p=0.968 and p=0.981).

Neither leptin nor insulin stimulation were found to have any effect on the intra- or extra-cellular concentrations of IL-6 in either cohort (p=0.607, p=0.710, p=0.524, p=0.847).
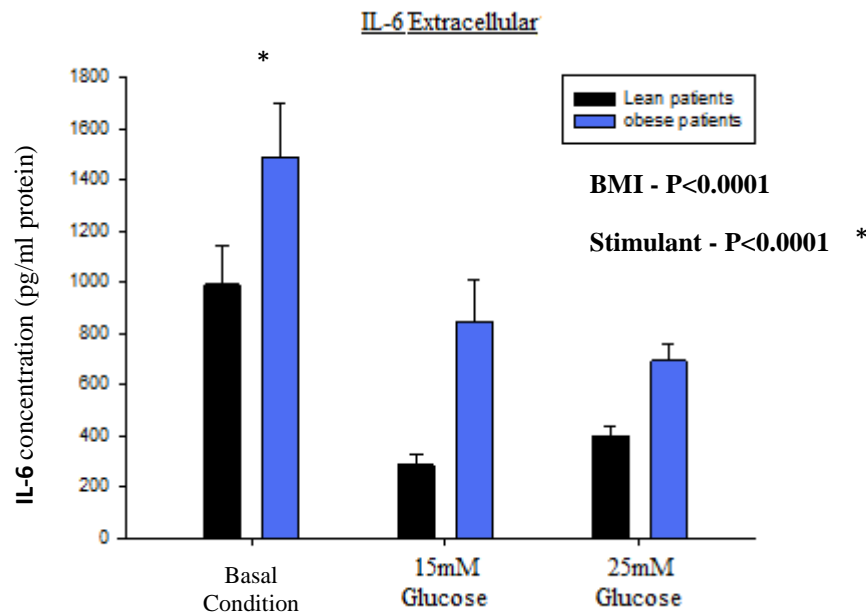
Glucose Stimulation:

Figure 3.12 - Histogram to show IL-6 secretion when stimulated with increasing concentrations of glucose.
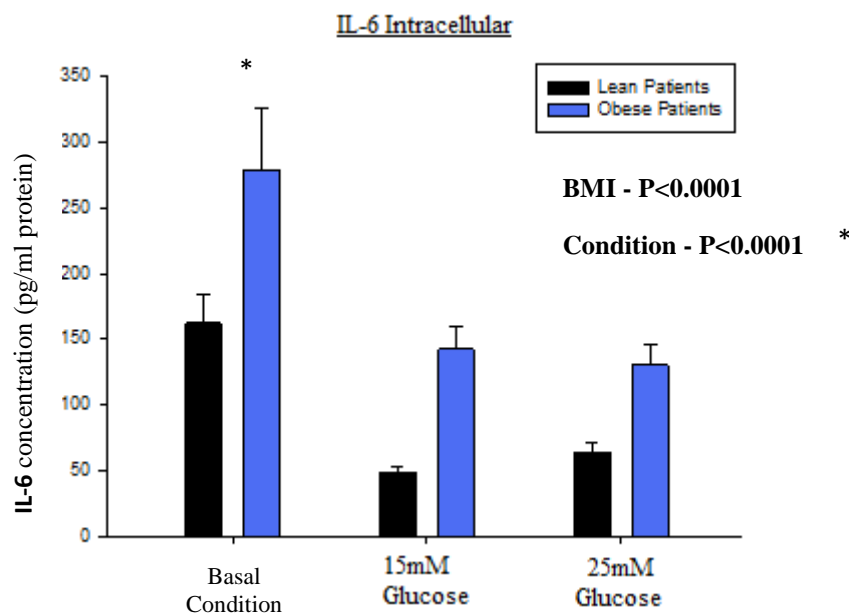

Figure 3.13 - Histogram to show IL-6 production when stimulated with increasing concentrations of glucose.
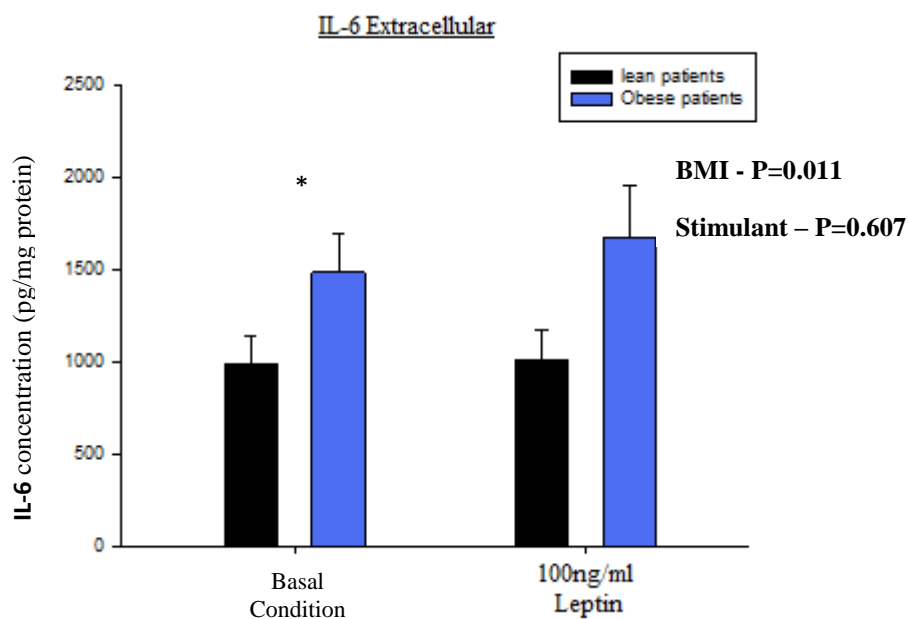
3.2.4.1. Leptin Stimulation:

IL-6 Extracellular

Legend: lean patients (black), Obese patients (blue)

BMI - P=0.011

Stimulant – P=0.607

Y-axis: IL-6 concentration (pg/mg protein), 0 to 2500

X-axis: Basal Condition, 100ng/ml Leptin

Figure 3.14 - Histogram to show IL-6 secretion when stimulated with 100ng/ml leptin.

IL-6 Intracellular

Legend: Lean Patients (black), Obese Patients (blue)

BMI – P=0.002

Stimulant– P=0.710

Y-axis: IL-6 concentration (pg/mg protein), 0 to 350

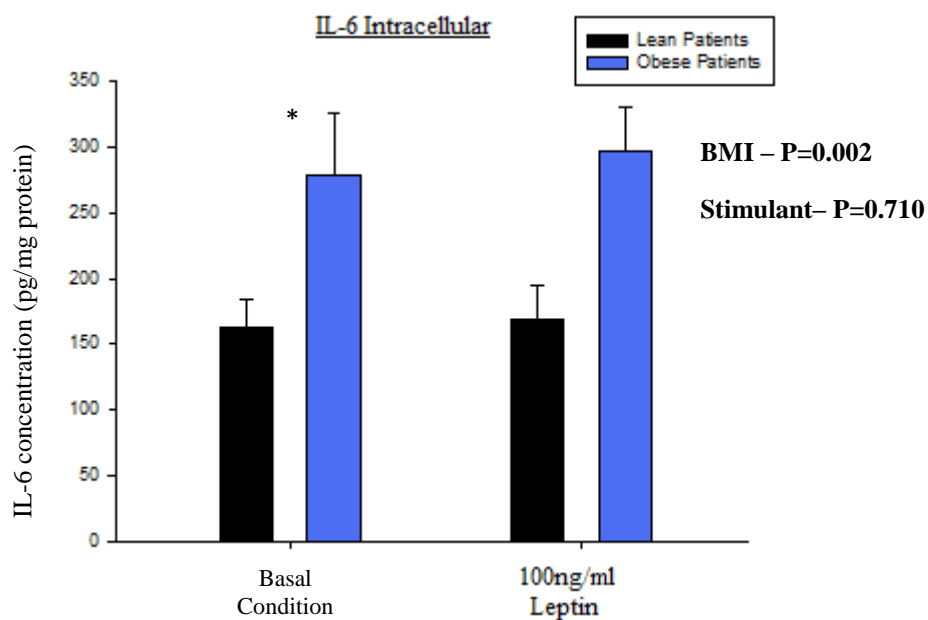*X-axis: Basal Condition, 100ng/ml Leptin*

Figure 3.15 - Histogram to show IL-6 production when stimulated with 100ng/ml leptin.
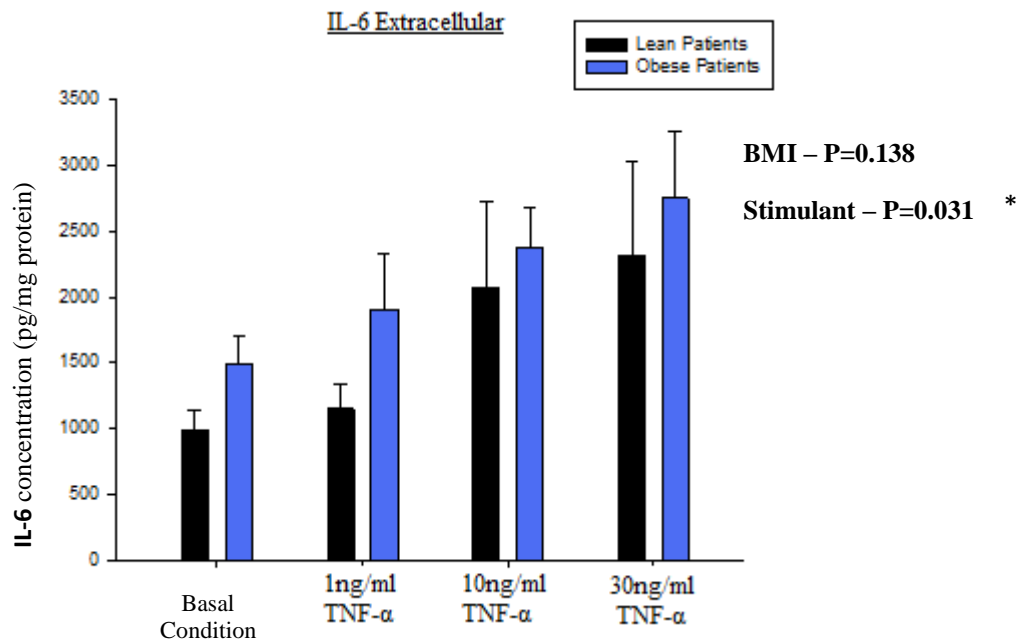
3.2.4.2. TNF-α Stimulation:

Figure 3.16 - Histogram to show IL-6 production when stimulated with increasing doses of TNF-α.
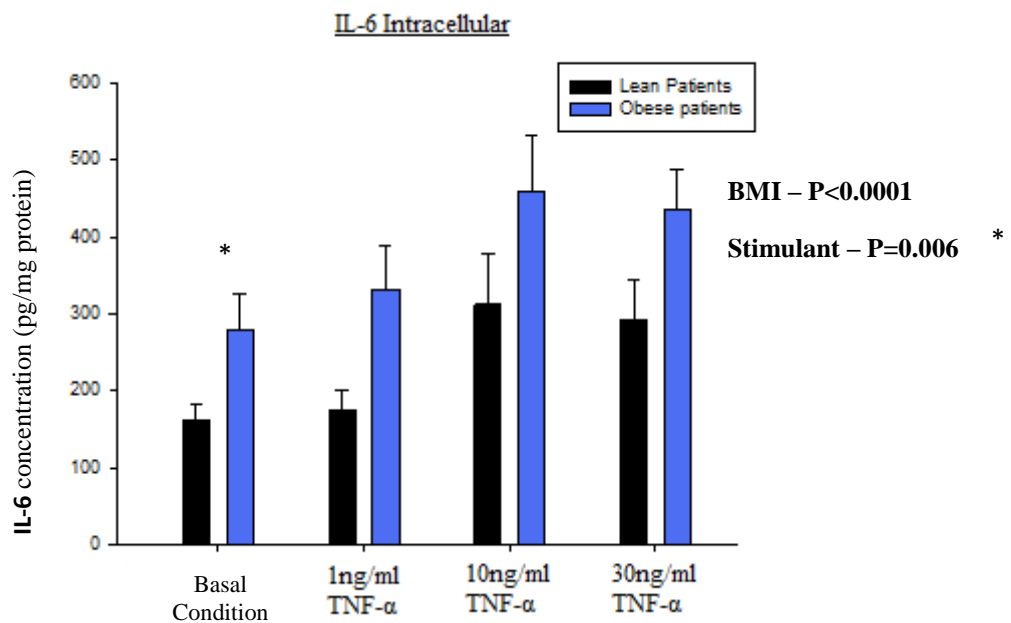

Figure 3.17 - Histogram to show IL-6 production when stimulated with increasing doses of TNF-α.
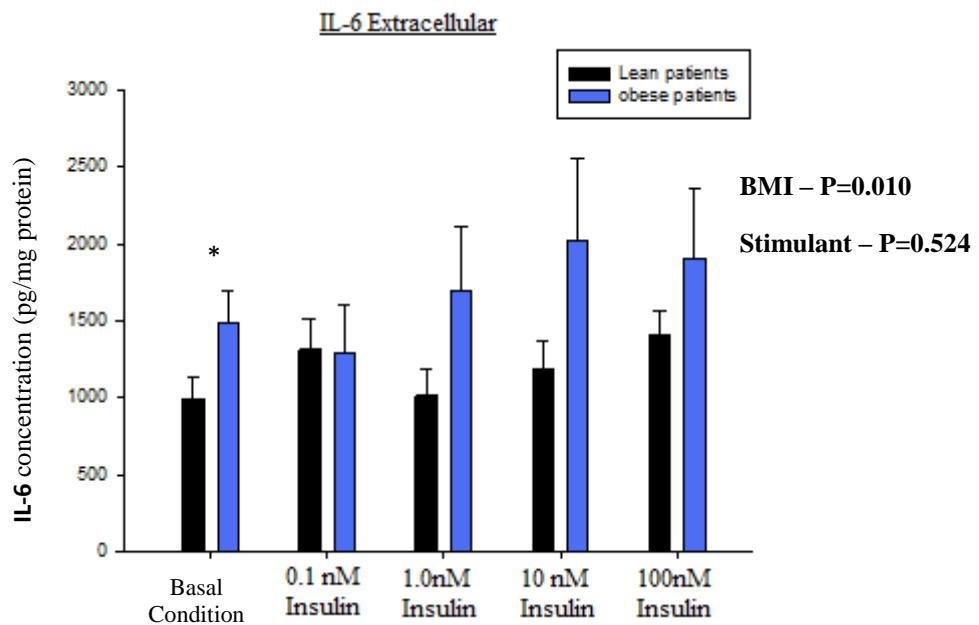
3.2.4.3. Insulin Stimulation:

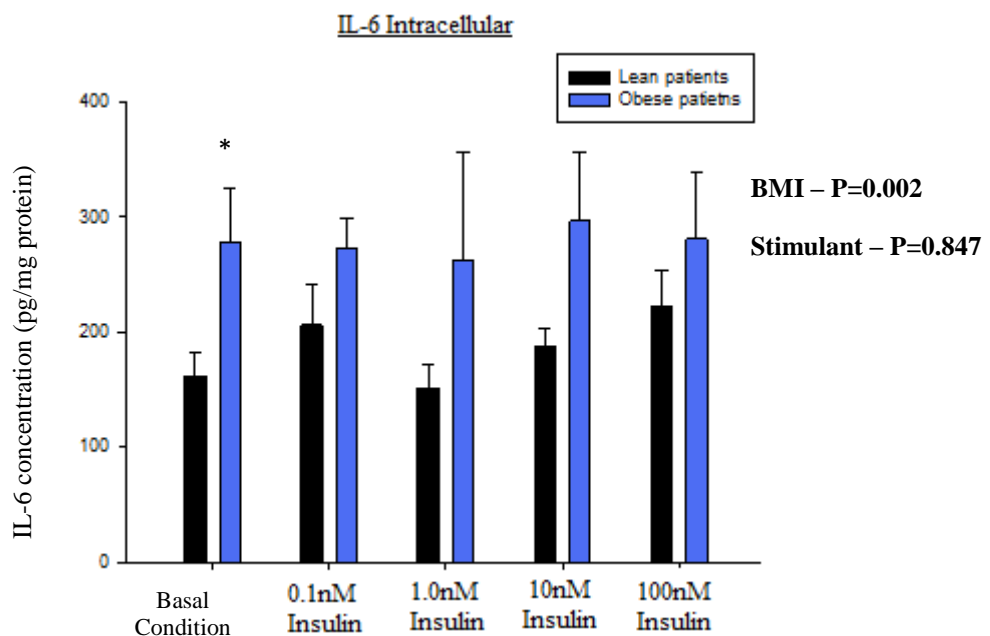**Figure 3.18 - Histogram to show IL-6 secretion when stimulated with increasing doses of insulin.**

Figure 3.19 - Histogram to show IL-6 production when stimulated with increasing doses of insulin.

3.2.5. Effect of Glucose, Leptin, and Insulin on TNF-α Production by Lean and Obese Placental Tissue Fragments:

	TNF-α Extracellular			TNF-α Intracellular		
	BMI	Stimulant	BMI* Stimulant	BMI	Stimulant	BMI* Stimulant
5 mM glucose / 15mM Glucose / 25 mM glucose	0.858	**0.000**	0.981	0.202	**0.000**	0.959
Leptin	0.562	0.577	0.465	0.295	0.802	0.919
TNF-α 1ng/ml / TNF-α 10ng/ml / TNF-α 30ng/ml	n/a	n/a	n/a	n/a	n/a	n/a
0.1 nM Insulin / 1 nM Insulin / 10 nM Insulin / 100 nM Insulin	0.455	0.739	0.372	0.573	0.219	0.970

Table 3.16 - Table to show P-values when General linear modelling was performed for TNF-a concentrations

In keeping with IL-1β and IL-6, increasing concentrations of glucose were shown to have an inhibitory effect on the intra- and extra-cellular concentration of TNF-α in both lean and obese placental tissue fragments (p=0.000). The response from both placental cohorts was the same (p=0.981, p=0.959). Leptin and insulin stimulation were found to have no effect on the intra- and extra-cellular concentrations of TNF-α in either cohort, and both cohorts were found to produce the same concentration of TNF-α under basal conditions.
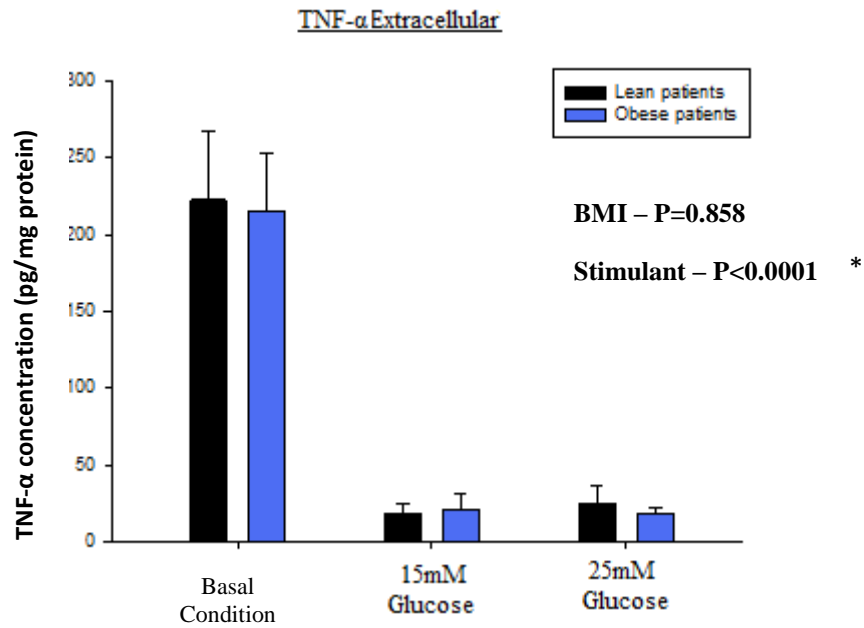
3.2.5.1. Glucose Stimulation:

Figure 3.20 - Histogram to show TNF-α secretion when stimulated with increasing doses of glucose.
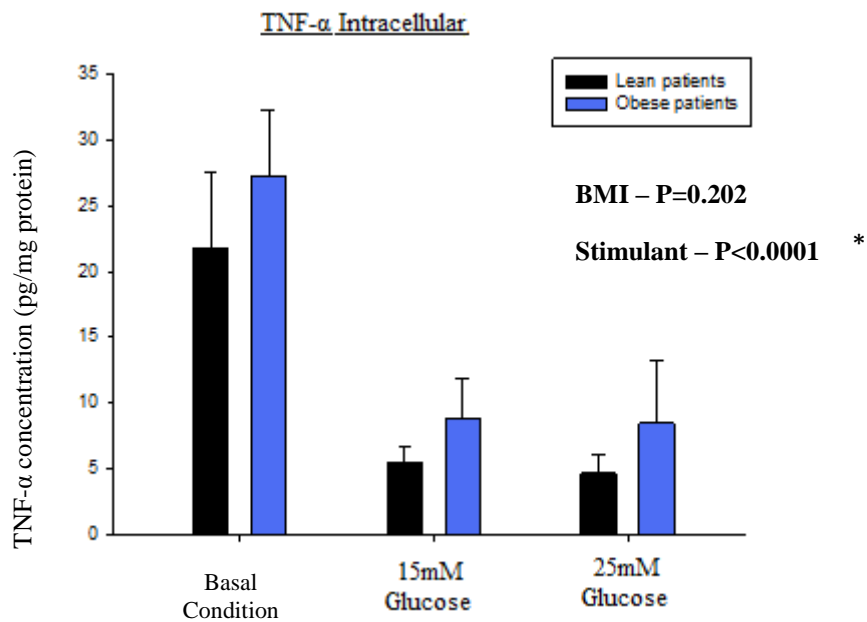

Figure 3.21 - Histogram to show TNF-α production when stimulated with increasing doses of glucose.

3.2.6. Dose response Calculations:

Stimulants which were shown to have an effect on the intra- and extra-cellular concentrations of cytokines were then assessed to determine if there was a dose response relationship. General linear modelling was performed and the effect of each concentration was assessed in contrast to the effect of the preceding concentration.

As glucose concentrations were found to affect all intra- and extra-cellular levels of the measured cytokines, this was analysed first.

	IL-1β E/C	IL-1 β I/C	IL-6 E/C	IL-6 I/C	TNF-α E/C	TNF-α I/C
15mM vs 5mM glucose	0.000	0.000	0.000	0.000	0.000	0.000
25mM vs 15mM glucose	0.026	0.007	0.003	0.004	0.000	0.012

Table 3.19 – Table to show p-values for dose response relationship of glucose on IL-1β, IL-6, and TNF-α production.

Increasing concentrations of glucose were found to have an inhibitory effect on the intra- and extra-cellular levels of all the cytokines measured.

TNF-α was also found to significantly affect intra- and extra-cellular levels of IL-6, and therefore this was also assessed for a dose response relationship.

	IL-6 E/C	IL-6 I/C
1 ng/ml TNF-α vs 0 ng/ml TNF-α	0.537	0.529
10 ng/ml TNF-α vs 1 ng/ml TNF-α	**0.043**	**0.002**
30 ng/ml TNF-α vs 10 ng/ml TNF-α	**0.028**	**0.068**

Table 3.20 – Table to show p-values for dose response relationship of TNF-α on IL-6 production.

There was no effect on intra- or extra-cellular levels of IL-6 when stimulated with a 1ng/ml TNF-α compared to 0ng/ml TNF-α (control). When the concentration of TNF-α was increased to 10ng/ml there was a significant effect on both intra- and extra-cellular levels of IL-6. However, when the concentration of TNF-α was increased to 30ng/ml, there was a significant effect on extra-cellular levels of IL-6, but no significant effect on intra-cellular levels of IL-6.

Chapter 4: Discussion:

This study has found that maternal obesity affects neonatal anthropometry by increasing head circumference, flank skin fold thickness, fat mass, free fat mass and percentage body fat. Histological examination revealed that although no statistically significant results were obtained, maternal vascular underperfusion appeared to be more common in the obese cohort. None of the placentae examined had evidence of inflammation. Incubation of placental tissue fragments revealed that the placentae of obese participants produced more IL-6 and IL-1β than lean participants, and that glucose had an inhibitory effect on the production of IL-6, IL-1β and TNF-α, which was not modified by obesity.

4.1. Maternal and Neonatal Demographics:

4.1.1. Maternal Demographics:

Obese participants, by definition, had an increased booking BMI compared to lean participants, with an average booking BMI of 39.00 (kg/m^2) compared to 23.58 (kg/m^2). Obese participants were also significantly taller than their lean counterparts. This should be taken into account as maternal height is an important determinant of birth weight, and therefore any observed difference in birth weight between the two cohorts could, in part, be attributed to difference in maternal height[108].

Average maternal weight gain in the obese participants was 7.23 +/- 4.82 kg. This is compatible with the Institute of Medicine's (IOM) 2009 guidelines for recommended weight gain during pregnancy, which specifies that the total weight gain for a woman with a BMI>35 should be between 5-9.09kg (11-20 pounds).[109] Maternal weight gain was not recorded in lean participants and therefore cannot be commented on.

4.1.2. Neonatal Demographics:

Overall, infants of obese participants had a higher birth weight than infants of lean participants. In addition, infants of obese participants also had a greater head circumference, flank skin fold thickness, fat mass, fat free mass, and percentage body fat. The rise in fat mass and fat free mass is in proportion to the overall raised birth weight. This study has shown that maternal pre-gravid obesity is associated with a rise in birth weight with, on average, infants of obese participants being 457.27g heavier than infants of lean participants.

One possible reason for this finding may be that there was a significant difference in the gestational ages between the two cohorts. The gestational age for neonates of lean participants was on average 272 days, and for neonates of obese participants the gestational age was 275 days, giving a p-value of 0.011. Gestational age was then shown to significantly correlate with birth weight in both cohorts (lean p-value=0.04, obese p-value=0.025). Gestational age was also shown to significantly correlate with neonatal head circumference (p=0.012) and length (p=0.01) in the lean cohort. This discrepancy in gestational age between the two cohorts could therefore be the reason for the difference in birth weight seen between the two cohorts. Further analysis needs to be performed when the study numbers have increased to see if there is still a significant difference in gestational ages and whether this may have any implications for the study's findings.

This finding is, however, in agreement with other studies which have also demonstrated increased birth weight of infants born to obese mothers[37]. Surkan et al demonstrated that neonates of mothers with a high pre-pregnancy BMI were two to three times more likely to be overweight[38]. When statistical analysis was performed for the candidate's study, to determine if more neonates of obese participants were; a) macrosomic, and b) obese; no statistical

significance was found. Whilst twenty-one neonates born to obese participants had a birth weight of greater than 4000g and six were classified as obese, compared to one macrosomic and one obese neonate born to a lean participant; this was not deemed statistically different. Thus, it can be assumed that in this study that birth weights of neonates of obese participants were increased but not sufficiently to deem them as macrosomic.

When further statistical analysis was performed, neonatal birth weight was not shown to significantly correlate to maternal booking BMI. Maternal booking BMI accounted for 5.5% of the variance in birth weight in the lean participants, whereas in obese participants, only 0.042% of the variance was accounted for by maternal booking BMI (Figures 3.1 and 3.2). This is therefore in opposition of Szostak et al's findings which stated that there is a general linear relationship between maternal BMI and neonatal birth weight[35]. From the graphs it would appear that there is, however, a greater association between neonatal birth weight and maternal BMI in the lean participants.

A possible reason for a lack of statistical correlation between maternal booking BMI and birth weight may due to the fact that all other birth weights, irrespective of whether the infant was macrosomic, were included. As stated previously, obese women are known to deliver macrosomic, and SGA, neonates and therefore, the presence of the two outcomes together may mask the correlation maternal booking BMI has to either outcome.[44] In order to determine more accurately whether maternal booking BMI correlates to the development of macrosomic, and SGA, neonates, neonatal birth weights should be subdivided and then analysis performed. This was unable to be performed due to insufficient numbers within each category, and therefore the lack of correlation between maternal booking BMI and birth weight, may be attributable to insufficient recruitment numbers. It does appear that macrosomia is more common in neonates

of obese participants but no statistical significance was found, which indicates that perhaps inadequate participant data was obtained to prove a statistical association. It will be interesting to see, as the study progresses over the next year, whether a statistical association is proven with increased participant numbers.

The findings of this study are also in opposition of Catalano et al who also found a correlation between maternal weight at delivery and neonatal birth weight[36]. However, this may be explained by the fact that Catalano et al correlated neonatal birth weight to maternal weight at delivery, whereas this study was attempting to find a correlation between maternal booking BMI and birth weight. Therefore, the timing of when the maternal weights were taken may account for the lack of correlation between birth weight and maternal BMI in this study. However, what this does highlight the possibly that maternal weight gain during pregnancy may be an important factor governing birth weight, as this study found no association between maternal booking BMI and birth weight but there has been a proven association between maternal weight at delivery and birth weight. Gestational weight gain of more than 25 lb has previously been associated with the development of macrosomic neonates in morbidly obese women[110]. Therefore, this could be a potential factor mediating increased foetal growth in the obese cohort of participants. However, it is hard to speculate as so few participants within this study have data on the amount of weight gained during their pregnancies. The few participants where maternal weight gain was known would suggest that the obese participants are not gaining excessive amounts of weight, and that this did not correlate to neonatal birth weight. However, as so little data was known this may not be a true representation of the whole cohort, and therefore weight gain could still be a feasible regulatory factor implicated in increased foetal growth.

It would be interesting to relate the detailed studies on nutritional intake of the obese participants, which is being gathered as part of the FFB study, to neonatal birth weight. As maternal nutritional state, and substrate availability, are such important factors governing neonatal birth weight one would expect correlations between the two. In the future it would be beneficial to gather detailed data on weight gain, nutritional intake (subdivided into food groups), and birth weight, in lean and obese participants, and determine if there are any positive, or negative, correlations present.

One positive finding of this study, which is in disagreement with previous studies, is that neonatal fat mass and fat free mass are increased in the context of obesity. Previous studies by Hull et al, and Sewell et al, have found that fat free mass, also known as lean body mass, does not tend to differ between neonates and it is mainly fat mass that alters[30,111]. In this study, neonates of obese participants were found, on average, to have an increased fat mass of 190g, and an increased fat free mass of 360g. As neonates of obese participants also had a raised percentage body fat, fat mass must have been increased more than fat free mass was. This is in agreement findings of Catalano et al who also found that neonates of obese mothers had a raised fat mass[41]. The increased fat mass in neonates of obese participants could be attributed to factors such as those mentioned in the introduction, for example, maternal insulin resistance promoting increased glucose transport and foetal hyperinsulinaemia, or raised inflammatory cytokines affecting system A activity. However, as no information is known on the levels of insulin, or circulating cytokines levels, within the obese participants it is difficult to speculate with any real conviction as to why the neonates of obese participants have a raised fat mass. The finding that fat free mass was raised in neonates of obese participants may reflect specific elements of nutrition among this

population, either due to the diet among pregnant women in Liverpool, or, due to influences on placental nutrient handling.

As no correlations between maternal and neonatal variables were found, it must, therefore, be considered that other factors, which were not measured in this study, are implicated in the development of the increased neonatal birth weight and anthropometric measurements in the neonates of obese participants. All that can be drawn from this study is neonatal percentage body fat did not correlate to maternal booking BMI, parity, gestational age, maternal weight gain, or maternal smoking status in either cohorts of participants.

4.2. Placental Demographics and Histopathology:

Placentae of obese participants were reported as having an increased trimmed, and untrimmed, weight when compared to lean participants. Placentae from obese participants were approximately 71.70g heavier, once trimmed, than placentae of lean participants. However, when univariate analysis was performed placental trimmed weight did not correlate significantly to maternal BMI, parity, gestational age, weight gain, neonatal sex, or smoking status.

This is in conflict with Swanson et al who reported a positive correlation between uncomplicated, full-term, singleton placental weight and maternal pre-gravid BMI. They reported that women with a higher pre-gravid BMI were more likely to produce heavier placentae and neonates[78]. This study has not found this correlation in this sample; however, it does seem feasible that an increased placental weight could lead to an increased surface area mediating increased placental nutrient transport and subsequent foetal growth. Stereology might be used to examine this possibility. However, in a study conducted by Farley et al which investigated the placental volumes of placentae from obese and lean baboons no differences was reported[75]. This finding, however, remains to be proven in human placentae.

Again, the lack of correlation found in this study may be attributable to insufficient participant numbers. Placental weights have been shown to be increased in the obese cohort and perhaps, given the relatively small numbers, they do not have sufficient statistical power to prove an association.

When placental histological reports were examined from the lean and obese participants, no significant difference was shown. Similar numbers of placentae were reported as normal, and as having chorangiosis, in both cohorts. The finding that similar numbers of

choriangiosis were reported in obese and lean placentae indicates that this finding is not exclusive to placentae of lean or obese participants, and that neither cohort are likely to be exposed to greater levels of hypoxia. Possible reasons for similarities in cohorts may be attributable to smoking, which was common in both cohorts, leading to decreased oxygen transport to the placenta and subsequently to the foetus.

Although it was not statistically significant, it does appear that more obese placentae were reported as having MVUP. Common features within placentae of obese participants, classified as having MVUP, were increased: syncytial knotting, intervillous fibrin, nucleated erythrocytes, thrombosis and infarcts. It is interesting that fibrin was increased within placentae of obese participants, as non-pregnant obese individuals have been demonstrated to have increased levels of PAI-1, a pro-coagulant factor known to lead to the accumulation of fibrin and atherothrombotic disease. This could be speculated as to the reason for increased fibrin deposition within the placentae of obese participants, however, further work to verify this are needed.

When statistical analysis was performed to assess an association between different categories of birth weight centile and placental histological categories, no significant difference was found for either cohort. This finding is, however, a little confusing. This study has been focused on finding a link between maternal obesity and the development of foetal overgrowth. MVUP is a placental finding associated with foetal intrauterine growth restriction (IUGR), not foetal macrosomia and, therefore, one would expect to see a significant difference of placental histological category in different birth weight categories. This would suggest that no specific placental morphology is associated with the development of different birth weights in either cohort of participants.

4.3. Incubation of Placental Tissue Fragments:

Firstly, a brief summary of the results for each cytokine measured is given, and then the effect of each stimulant condition is discussed in more detail.

4.3.1. Intra- and extra-cellular Cytokine Concentrations:

4.3.1.1. IL-1β:

Increasing concentrations of glucose were shown to reduce intra- and extra-cellular concentrations of IL-1β. Placental explants from lean and obese participants responded in the same manner to the glucose concentrations, and there was no difference in the intra- or extra-cellular concentrations of IL-1β in either cohort.

When the placental explants were stimulated with increasing concentrations of insulin, insulin itself had no effect on the extra-cellular levels of IL-1β, and the response of the two groups was the same. However, the placental explants from the obese participants were shown to secrete significantly more IL-1β than the lean participants' explants.

No other stimulants had a significant effect on the intra- or extra-cellular concentrations of IL-1β, nor was there any significant difference between the intra- and extra-cellular concentrations of IL-1β in the two cohorts.

4.3.1.2. IL-6:

Glucose stimulation elicited an inhibitory effect on the intra- and extra-cellular concentrations of IL-6 in placental explants from lean and obese participants. The explants from the obese participants were shown to produce significantly more IL-6, under

all glucose concentrations, than the explants from the lean participants.

Intra- and extra-cellular concentrations of IL-6, in both cohorts, were increased by TNF-α stimulation. There was a dose response effect seen when the explants were stimulated with 10ng/ml and 30ng/ml concentrations of TNF-α. Once again, the explants from the obese participants were shown to contain more intra-cellular IL-6 compared to the lean participants, however extra-cellular levels did not differ significantly between the two groups.

When stimulated with insulin and leptin, the presence of maternal obesity had no effect on IL-6, but placental explants from obese participants were shown to have higher intra- and extra-cellular concentrations of IL-6 compared to the lean participants.

4.3.1.3. TNF-α:

In keeping with the other two cytokines measured, intra- and extra-cellular concentrations of TNF-α were decreased by increasing concentrations of glucose. Both 15mM and 25mM glucose concentrations had an inhibitory effect on amount of TNF-α produced by placental explants from lean and obese participants.

No other stimulants were found to have a stimulatory, or inhibitory, effect on the production of TNF-α.

4.3.2. Effects of Stimulation of Cytokines Measured:

4.3.2.1. Glucose Stimulation:

Increasing glucose concentrations were shown to have an inhibitory dose response effect on the intra- and extra-cellular concentrations of IL-1β, IL-6, and TNF-α.

Coughlan et al, using similar conditions to those used in this study, demonstrated that placentae from lean women, and women diagnosed with GDM, differ in response to increasing glucose concentrations. Cultured placental explants from lean women were found to decrease TNF-α production in response to raising glucose concentrations, whereas placental explants from women diagnosed with GDM were shown to increase TNF-α production.[83] This is in agreement with Kirwan et al that stated that circulating maternal TNF-α levels are raised in GDM, and TNF-α is a good predictor of insulin insensitivity[58].

As there was no difference in the intra- or extra-cellular concentrations of IL-1β or TNF-α in the placental explants of the lean and obese participants, and glucose stimulation had an inhibitory effect, it appears that placentae from obese women behave similarly to placentae from lean women with regard to TNF-α production.

Obese women are known to experience transient periods of hyperglycaemia. Therefore, the fact that intra- and extra-cellular levels are decreased by raised glucose is intriguing. One might speculate that the raised glucose levels may exacerbate the inflammatory environment within the placentae, such as occurs in GDM; however it appears that the opposite is true. As glucose levels climb, the production of the inflammatory cytokines IL-1β, IL-6, and TNF-α is suppressed. This suggests that raised glucose levels are involved in the regulation of cytokines by down-regulating cytokine

gene expression. Why the placentae of some obese women may do this, or the mechanism involved in this regulation, is unknown. It is hard to speculate why some obese women's placentae appear to exhibit a protective response whereas other women's placentae exacerbate the inflammatory situation aiding the development of GDM.

However, a study by Lappas et al, needs to be considered. Lappas et al cultured placental explants for 18h from lean women, and women diagnosed with GDM. Glucose stimulation at 10mM, 20mM, and 40mM was found to increase the release of leptin in the placental explants of lean women but not in the explants from women with GDM[112]. Therefore, as the placental explants from the obese participants appear to behave similarly to explants from the lean participants under glucose stimulation, it could be speculated that, the increasing concentrations of glucose could induce increased release of leptin. As leptin has been linked to foetal growth, this could potentially represent a mechanism how maternal obesity mediates increased foetal growth. In order to assess whether this was true, an ELISA for leptin would have to be performed, which is perhaps an area for future research.

Of particular importance is the finding that although cytokine production of IL-6 was decreased by glucose stimulation; intra- and extra-cellular concentrations of IL-6 levels were still raised in the placental explants of obese participants compared to lean participants. It is of particular importance as a recent study by Jones et al has reported a link between IL-6 and foetal adiposity in the placentae of obese women. System A amino acid transport activity, and protein expression for the system A transporter isoforms, were measured in cultured human primary trophoblastic cells, and both were found to be increased by IL-6. The isoform SNAT2 was found to be up-regulated in the MVMs of placentae from obese women who delivered LGA neonates. Thus, it was speculated that

the increased system A activity, secondary to IL-6 stimulation, was implicated in the development of increased foetal adiposity[74].

This study's results are in conjunction with Jones et al. Raised fat mass has been reported in the neonates of obese participants, and raised IL-6 concentrations have been demonstrated within their placentae. Although this study has made no attempt to investigate the activity or expression of system A, it could be speculated that the raised IL-6 concentrations observed in the placentae of obese participants have resulted in altered system A activity and potentiated increased neonatal adiposity via this mechanism.

It would be interesting to correlate the maternal circulating IL-6 levels in the obese participants to neonatal birth weight, and fat mass, and determine whether these variables demonstrate any positive correlation. However, as maternal circulating IL-6 levels were not measured, this is impossible in the case of this study. This is perhaps an area of future research.

4.3.2.2. Leptin Stimulation:

Leptin stimulation did not appear to have any effect on intra- or extracellular levels of any of the cytokines.

As mentioned earlier leptin is known to be implicated in the development of macrosomia in the context of GDM[71] and umbilical leptin levels have been correlated to foetal birth weight[70]. These two findings suggest that leptin is involved in the regulation of foetal growth; however the mechanism is currently unclear. The results of this study would suggest that leptin is not implicated in the development of foetal macrosomia, in the context of obesity, by affecting cytokine production within the placenta.

One possible reason for the lack of effect of leptin on the production of cytokines could be leptin resistance. The concentration of leptin used for stimulation was 100ng/ml. In lean women, circulating levels at term are approximately 10-30ng/ml, and even in the obese women circulating levels at term are only approximately 30-50ng/ml[58]. Lepercq et al demonstrated that in obese placentae raised levels of circulating leptin induce down-regulation of leptin receptors within the syncytiotrophoblast mediating leptin resistance[76]. Thus, it could be speculated that the levels used were so high that they induced down regulation of the receptors, or completely saturated the receptors, leading to a decreased effect of leptin stimulation.

Farley et al have hypothesised that the association of leptin and foetal macrosomia may be attributable to increased system A activity[75]. This was based on the finding that leptin has been shown to stimulate system A activity in the placentae of lean women[72]. However, they found that SNAT 4 expression is actually decreased in the placentae of obese women, and they also reported no differences in the expression of the system A isoforms, SNAT 1 and 2, in the

placentae of lean and obese women[75]. That work, suggests that leptin is involved in the regulation of system A, but due to the high levels of circulating maternal leptin, leptin resistance is instigated preventing leptin regulation of system A in the obese placentae.

It is, therefore, tempting to speculate that due to leptin resistance, leptin does not alter the activity of system A in the placentae of obese women via the production of IL-1β, IL-6, or TNF-α. However, once again as no information was gathered on the activity of system A this is just speculation.

4.3.2.3. TNF-α Stimulation:

TNF-α stimulation affected only IL-6 production and similar effects were exhibited in placental explants from lean and obese participants. Placental explants from obese participants were demonstrated to contain higher intra-cellular concentrations of IL-6.

This is in agreement with a study by Turner et al, which demonstrated that TNF-α stimulation increases secretion of IL-6 in human placental explants over four hours incubation time. However, TNF-α was also found to increase IL-1β secretion, a finding this study did not show.[80] In this study, TNF-α stimulation appeared to have no effect on IL-1β production in the placental explants from lean and obese participants. It may be speculated that IL-1β is not involved in the regulation of altered foetal growth. Further research is however needed to confirm this speculation.

In this study, TNF-α appeared to not only increase secretion of IL-6, but also increase overall production of IL-6 (intra-cellular levels+extra-cellular levels). This is in conjunction with another study which showed that in isolated cytotrophoblastic cells that TNF-α has a pronounced effect on IL-6 secretion[113].

Similar concentrations of TNF-α were used as in the study by Turner et al, which stated that a TNF-α concentration of >100pM (approximately 26ng/ml) was needed to elicit a sustained response. In this study, three doses of TNF-α were used 1ng/ml (~3.9pM), 10ng/ml (39pM) and 30ng/ml (~116pM). IL-6 production was found to exhibit a dose response effect to 10ng/ml and 30ng/ml but not 1ng/ml. These concentrations are significantly greater than fasting circulating levels found in pregnant lean women (2.13pg/ml) and pregnant obese women (2.80pg/ml) with normal glucose tolerance[58]. They are even greater than fasting levels found in pregnant obese

women with GDM, who are known to have significantly raised circulating levels of TNF-α. This study has shown that maternal obesity is associated with changes in responsiveness to TNF-α and thus to regulatory processes within the placenta.

This finding could potentially explain why placentae of obese women, and women diagnosed with GDM differ. TNF-α is known to be significantly raised in GDM, but not in obese pregnancy, which may potentiate increased IL-6 production. As IL-6 has now been shown to increase system A activity this suggests a mechanism how increased foetal adiposity may be so closely associated with GDM. A TNF-α dependent effect of IL-6 on system A may be more important in GDM than maternal obesity without GDM. Why women with GDM initially have raised levels of TNF-α in comparison to obese women, is however unknown.

4.3.2.4. Insulin Stimulation:

Insulin stimulation was not found to affect the production of any of the measured cytokines. This suggests that insulin is not involved in the regulation of placental cytokine production of IL-1β, IL-6, or TNF-α, and is not implicated in foetal growth through this mechanism.

Previous studies conducted on placental explants cells have demonstrated that insulin stimulation is associated with an increased production of leptin, however the demographics of the participants involved were not given[114]. This could be occurring in the context of obesity as obese women have been shown to have slightly elevated circulating insulin levels in comparison to lean women. However, it is more likely that this is of more significance in the context of GDM as women with GDM are known to have significantly raised insulin levels[58]. Raised insulin levels could thus mediate raised leptin levels potentiating increased placental, or foetal, growth. However, once again this is just speculation, as the mechanisms of how these interactions occur are currently unknown.

4.4. Overall - Implications of Results to Overall Model of Obesity:

The finding that glucose has an inhibitory effect on placental cytokine production in placentae from lean and obese participants is intriguing. Unlike the situation in GDM, which has been shown to up-regulate genes regulated to inflammatory response, it appears that in the non-diabetic individual raised glucose concentrations cause down-regulation of cytokines, well at least IL-1β, IL-6, and TNF-α. Thus it can be speculated that the transient periods of hyperglycaemia experienced by many obese women are insufficient to induce foetal overgrowth by inducing increased expression of placental cytokines.

Leptin stimulation was not shown to alter cytokine production in placentae of obese or lean participants, which could, perhaps, be attributed to leptin resistance. Although obese pregnancy is associated with raised levels of leptin compared to lean pregnancy, it is unsure how, or if, leptin is involved in foetal growth in the context of obesity. As stated earlier, leptin is known to be implicated in macrosomia associated with GDM; however, it is unknown whether this is true for macrosomia associated with obesity. From these results, it can be concluded that leptin is not implicated in foetal growth by modifying cytokine production within the placenta.

TNF-α was not shown to be elevated in the placentae of obese participants compared to lean participants. It was thought that if raised levels were reported in placentae of obese participants then this could potentiate foetal adiposity by; a) directly increasing SNAT1 and 2 activity, or, b) indirectly increasing IL-6 levels leading to increased SNAT2 activity, or, c) increasing lipolytic pathways increasing the amount of PUFAs which can be used in foetal adipogenesis.[71] However, as TNF-α levels were not elevated in

placentae of obese participants compared to lean participants it can be concluded that TNF-α in our obese cohort was not implicated in the development of the increased foetal adiposity.

In contrast to this, it is interesting that TNF-α production is not elevated in the placentae of the lean participants. Circulating TNF-α levels are known to be increased in non-pregnant obese women, however in the pregnant obese women TNF-α levels have been shown to be similar to that of lean pregnant women. As shown by our data, placental explants from lean participants did not secrete significantly more TNF-α than obese participants. This is consistent with the overall situation in pregnant obese women. This begs the question, why do obese women who become pregnant not continue to have raised TNF-α levels in comparison to lean women? It can be hypothesised that an inhibitory feedback loop is instigated when an obese woman becomes pregnant which suppresses WAT production of TNF-α leading to the similar circulating TNF-α levels as the lean individual. However, it is also impossible to measure the amount of TNF-α that WAT produces in isolation from the placenta during pregnancy. Nonetheless, this is definitely an enticing area for future research.

IL-6 basal production was shown to be increased in placentae of obese participants and production was shown to be stimulated by TNF-α and inhibited by glucose. Therefore, in the context of obesity, IL-6 may be raised independently, as TNF-α levels are known to be not elevated, and in the context of GDM, IL-6 may be raised as a result of raised TNF-α levels. Thus the raised levels of IL-6 associated with obesity puts it as a strong contender in the development of foetal adiposity.

However, probably the most intriguing fact which has failed to be mentioned, until now, is that while placentae of obese participants have been shown to have elevated levels of IL-6, there is no evidence of inflammation on light microscopic examination. It would,

therefore, appear that the cytokines are not affecting the overall morphological appearance of the placentae. Also the speculations, that the placentae of obese participants may behave similarly to placentae of women with GDM, which were made in the introduction, have been shown to be false. Placentae from women with GDM have been shown to up-regulate TNF-α, IL-1, and leptin receptors resulting in altered angiogenesis and overall disruption of placental structure. However, there was no statistical difference between placental histological classification of lean and obese participants. As no other studies, to date, have examined the placentae of obese women in isolation from any other maternal morbidity, these results cannot be compared to other findings.

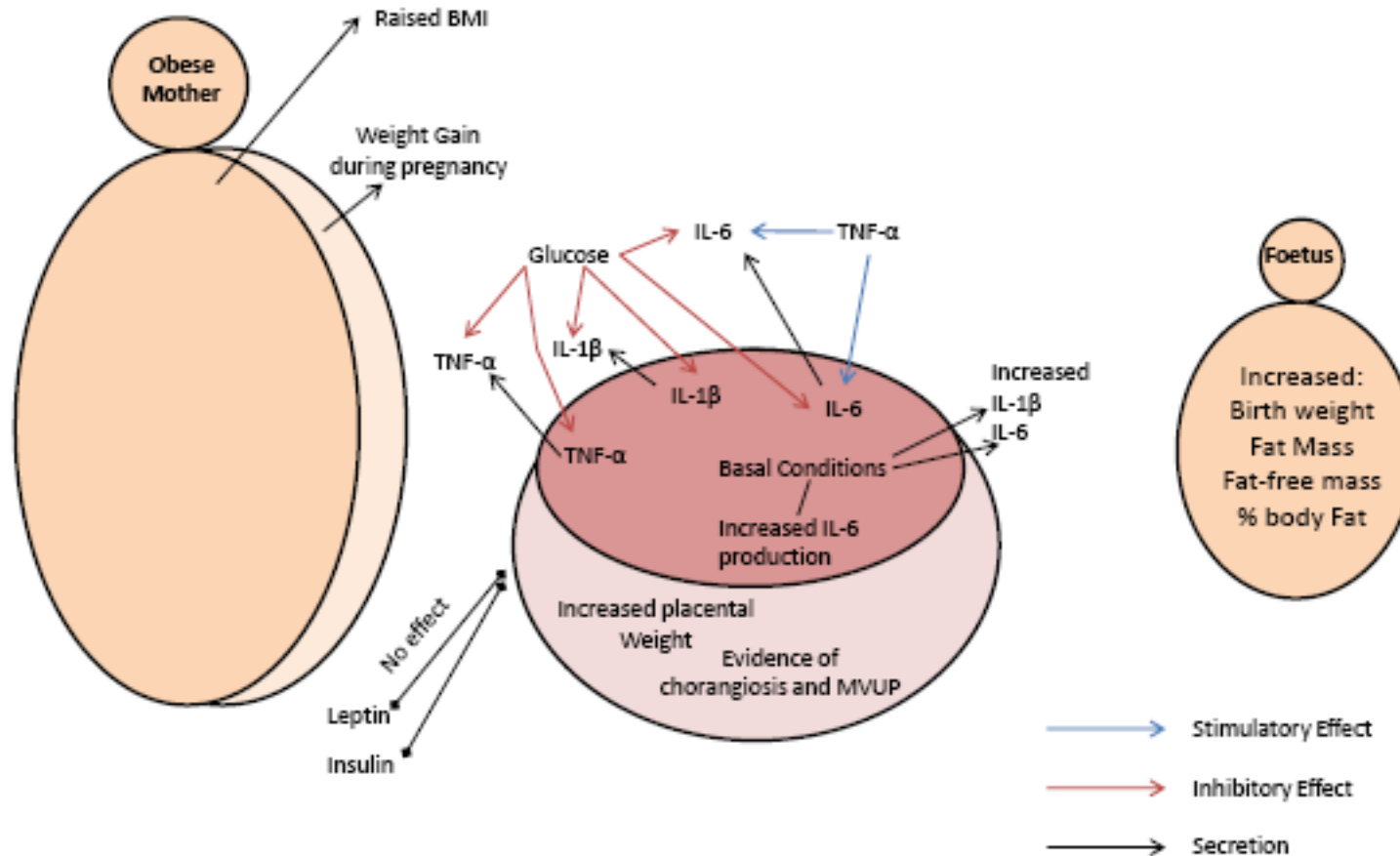In summary, the study's results show:

Figure 4-1- Diagram to show summary of study's findings for the maternal, neonatal, and placental variables investigated.

4.5. Limitations

4.5.1. Design of study

There are several limitations to this study. Firstly the sample was not a population based sample. Sample size was determined by the willingness of women within the Liverpool area to consent and comply with the FFB study. The study was also restricted by the amount of resources available. Members of the team were constricted to the amount of hours they could input and the whole study was restricted by funding.

With regard to placental explants, there are also limitations to the study design. A similar study, conducted by Varastehpour et al, demonstrated that placentae of women who delivered obese infants had raised TNF-α gene expression and protein content. This current study differs to the study by Varastehpour et al as placentae of obese women were cultured, irrespective of whether the neonate was obese.[85] As maternal obesity is associated with IUGR and macrosomia, on analysis the findings associated with IUGR may obscure the findings related to macrosomia, ultimately resulting in no significant findings, and thus is a limitation of this study. It would be advantageous to categorise neonatal adiposity prior to analysis to prevent one pathological mechanism masking another.

4.5.2. Implementation of study

For the candidate, the implementation of the study posed the biggest issue. There were multiple problems throughout the study, which were individually addressed in the most appropriate manner in order to solve the problems effectively.

Primarily, the most obstructive problem to the study was participant consent and attendance to the FFB clinic. Consent of participants relied on the cooperation of the midwives, at the various antenatal centres, to invite eligible women to partake in the study. However,

due to several reasons, such as time constraints, or unawareness of the study, not all women that were eligible for the study were consented by the midwives. There was also confusion as to the procedure for booking appointments to the FFB clinic. Midwives thought it was the responsibility of the FFB administration team, and the administrators thought that the midwives were booking the appointments. As a result many women that were eligible for the study either were not consented, or those that were consented, were not given appointments to the FFB clinic. To date, fifty nine women, who were consented to the study, never attended a FFB clinic. Ultimately this led to much data being irretrievably lost. Several participants who had been consented but not invited to clinic commented on the inefficiency of the study due to lack of correspondence and the disappointment they had incurred as a result.

Of the participants that were successfully consented and given an appointment to the FFB clinic, not all participants attended. When asked as to the reason why, many participants said they forgot about their appointment, or that the appointments lasted too long and they did not have the time to attend. Again, this led to loss of data.

4.5.2.1. Problems with Obtaining Placentae and Neonatal anthropometric Measurements:

During the ten month period, seventy four placentae were obtained out of a possible one hundred and thirty six participants consented, giving a success rate for obtaining placentae following deliveries of 54.4%. Out of the seventy four placentae obtained, thirty eight corresponding neonatal anthropometric measurements were obtained, giving a success rate of 51.4%. As the numbers demonstrate, there were multiple problems experienced with regard to obtaining placentae and neonatal anthropometric measurements.

The first problem experienced was participants not having a reminder form, clearly visible, in their hand-held maternity notes. Without

this form, midwives would not be aware of the participant's involvement in the FFB study. Initially, distribution of these forms relied on participant attendance to the FFB clinic. Therefore, as mentioned earlier, problems were experienced as participants were either not given appointments to the clinic, or participants did not attend their appointment. This issue was addressed by obtaining ethical consent to contact participants by post, and forms were then sent out by post. This then relied on participants placing the forms within their notes themselves. The only opportunity for the candidate to ensure that the form was not only placed in the notes, but also clearly visible to the midwives, was on the coincidental chance that the candidate checked the ward at the same time as the participant was admitted. Many placentae were obtained through this method, however many were not, and thus data was lost.

Problems encountered with actually physically obtaining the placentae, and notification of participant delivery to the candidate so that the subsequent anthropometric measurements could be performed, were mainly attributed to time constraints of the midwives. It was recognised that the midwives have a highly stressful and busy job, and when women's lives may be in jeopardy, helping with a study may not be top of their priorities. Therefore, every effort was made, by the candidate, to check the maternity wards daily in case a delivery had occurred that they had not been notified about but, due to prior commitments, such as laboratory work, this did not occur at the same frequency or time each day, and thus due to swift discharge times, participants, and their infants, were missed.

Also there was no policy outlined for when the candidate was unavailable to measure a neonate. Despite the candidate's best efforts, there were several occasions when this was unavoidable, and therefore with no alternative, neonatal data was regrettably lost.

As a result of the multiple problems incurred by this study, a significantly smaller sample size was obtained. At the outset, it was hoped that one hundred and fifty placentae would be obtained from participants with a BMI of greater than thirty five. Therefore, with only seventy four placentae obtained, the study has half the number of placentae that were initially expected. However, it should be remembered that the FFB study is a two year project and there is still a further twelve months for obtaining placentae and neonatal anthropometric measurements. Thus, it is speculated that the desired number will be achieved by the end of the two year period. However, even with half the expected final sample size, many of the results shown were significant, which is promising for when the final statistical analysis is performed.

4.6. Future work

4.6.1. Within this study

Previous studies examining immunohistochemistry have revealed that the syncytium is a source of IL-1β and IL-6, and Hofbauer cells have also been shown to exhibit signals for both cytokines[90]. From this study, no conclusions can be drawn about which cell types are contributing to the intra- and extra-cellular cytokine concentrations. Therefore, the next step in this study is immunohistochemical staining of the incubated placental tissue fragments. This is scheduled to be conducted over the next coming year.

Also future work is planned for investigating macrophages within the placentae of obese participants. This hopes to support, or dismiss, the finding by Challier et al that there are increased numbers of macrophages within the placentae of obese women. Immunohistochemical staining for CD14 and CD68, both macrophage markers, has already begun and image analysis is scheduled over the next coming months.

If raised numbers of macrophages are found within the placentae of obese participants, further work is planned to determine whether these macrophages contribute to elevated cytokines witnessed in the placentae of obese participants. This will again be conducted by immunohistochemical staining over the course of the next year, once image analysis of the CD14 and CD68 stained slides has been completed.

4.6.2. Beyond this study

To elucidate the mechanisms leading to, not only increased foetal growth, but also decreased foetal growth, in association with obesity, future research needs to segregate uncomplicated maternal obesity from obese mothers with GDM. Further segregation of neonates

into increased, normal, and decreased adiposity then needs to be done. It would then be interesting to conduct in-depth maternal, neonatal, and placental analysis within the different categories. In each category the following variables could be examined; maternal circulating hormonal and cytokine levels; neonatal anthropometry; placental histopathology; placental responsiveness to factors raised in obesity and the effect this has on cytokine production and nutrient transporter activity; and finally immunohistochemistry on the placental explants. This would give a detailed analysis of the individual mechanisms resulting in each of the neonatal outcomes, and then comparisons between the mechanisms could then be made. Without doing so there are too many confounding factors which stand to conceal important associations.

Future research could also be conducted on the role of leptin in foetal growth. As mentioned earlier, leptin may be involved in cellular regulation opposed to overall foetal growth. It would therefore be particularly intriguing to perform a prospective longitudinal study investigating the relationship between maternal circulating leptin levels and the chance of offspring developing metabolic syndrome, and determine whether leptin has any effect on the long term outcome of the infant.

4.7. Conclusions

Maternal obesity and increased weight gain during pregnancy have been previously shown to be associated with unfavourable neonatal outcomes, with some neonates achieving sub-optimal growth and others achieving excessive growth. Many factors are believed to be implicated in the development of these outcomes; however, they are far from understood. Maternal obesity leads to alterations within the placenta, instigating a pro-inflammatory state, characterised by an excessive accumulation of macrophages, which is not apparent on morphological examination. This pro-inflammatory state is unlike acute inflammation, which is characterised by neutrophil accumulation, suggesting that a similar situation, as occurs in WAT, may also be occurring within the placenta. More importantly, these placental alterations are not only thought to cause short term neonatal problems, but also cause detrimental changes to the long term development of the offspring, impacting upon its entire lifetime.

Therefore, areas for research may be into the association of nutritional intake on foetal outcome, and a possible intervention could, perhaps, be the implementation of a specific diet tailored for obese pregnant women. This could potentially limit excessive weight gain during pregnancy and reduce the risks to the developing foetus. The implementation of a tailored diet will, however, involve a drastic lifestyle change for the women, which will be notoriously hard to enforce and monitor. However, it is hoped that as more and more research is conducted into the adverse foetal consequences of maternal obesity, the associated risks will become more publicised. With increased publication, it is anticipated that this will encourage women to lose weight prior to conception of a planned pregnancy.

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Appendix I

Patient information sheets

Liverpool Women's Hospital

Crown Street

Liverpool L8 7SS

Tel 0151 702 4101

Principal Investigators: Dr Siobhan Quenby & Dr Andrew Weeks

_____

INFORMATION SHEET (PLACENTAL CONTROLS)

We would like to invite you to take part in a research study.

Please take time to read this information carefully and discuss it with others if you like.

Ask us if there is anything that is not clear.

Take time to decide whether or not you wish to take part.

Why are we doing this research?

Fit for Birth is a large study in which 3000 overweight women are being recruited. It will examine the effect of the mother's weight on pregnancy and its outcome.

As part of that study after delivery we will be examining the placentas of a few women who are very overweight. We want to compare these placentas with women with a normal body weight – and this is where you come in.

We would like to take your placenta away for examination after you deliver. The placenta will be cut up in the laboratory and looked at under the microscope to see if there are any abnormalities. The health of your placenta (and the rest of the placentas from the 'normal weight group') will be compared with those from the overweight group to see the effect of body weight.

Why have I been chosen?

You have been chosen to take part because you have a normal body mass index.

Do I have to take part?

No, it is up to you to decide. You are still free to change your mind at any time and without giving a reason. This would not affect your care – you would receive normal care.

What will happen if I take part?

You will notice no difference to your care. After your placenta is delivered, instead of being disposed of, it will be taken away to a laboratory for examination. Parts of the placenta may be kept

for future reference. The remainder will be disposed of in the normal way. A special trained midwife would like to measure round your baby's arm and also to measure the thickness of a fold of skin.

What are the possible disadvantages of taking part?

None.

What are the possible advantages of taking part?

There are a few placental abnormalities that are important for the management of future pregnancies. These are very rare, but would be picked up at this examination. If we did find one of them, then we may be able to improve the outcome of any future pregnancies that you have.

What if something goes wrong?

In the unlikely event of anything going wrong with the treatment, or if you wish to complain, or have any concerns about the way you have been approached or treated during the course of this study, then the normal NHS complaints system will be available to you. If you are harmed due to someone's negligence, then you may have grounds for a legal action. There are, however, no special compensation arrangements in place in case of problems with the research.

Will my details be kept confidential?

All information that is collected about you during the course of the research will be kept confidential. The completed forms with your name on them will be kept in the central trial office in Liverpool and your name will be removed before the data is transferred to the computer database for analysis. If you agree to take part in the research, the researching doctors may look at any of your medical records to analyse the results. They may also be read by people from regulatory authorities to check that the study is being carried out correctly.

What will happen to the results?

The study is planned to finish in late 2010. The results will then be analysed and published in a medical journal. You will not be identified in any publication. If you would like to receive a copy of the final publication, then please indicate this on the consent form.

Who is funding the research?

The research is funded by Liverpool Primary Care Trust through MerseyBeat, a project of the University of Liverpool. The doctors conducting the research are not being paid any extra for it.

Who has approved the study?

This study has been approved by the Liverpool Women's Hospital Research and Development Department, and the Liverpool Adult Research Ethics Committee.

Contact for Further Information

For further information about the study please contact:

Mr David Rycroft - Fit for Birth administrator,

> School of Reproductive and Developmental Medicine, Liverpool Women's Hospital, Crown
>
> Street, Liverpool L8 7SS
>
> Tel: 0151 702 4179; e-mail David.Rycroft@liv.ac.uk

OR go to our website at www.fitforbirthstudy.org.uk

Fit for Birth

Liverpool Women's Hospital

Crown Street

Liverpool L8 7SS

Tel 0151 702 4101

Principal Investigators: Dr Siobhan Quenby & Dr Andrew Weeks

Patient Hospital Number:

CONSENT FORM (PLACENTAL CONTROLS)

Title of Project: Fit for Birth: a prospective cohort study

Name of Researchers: Dr Siobhan Quenby & Dr Andrew Weeks, University of Liverpool

Please initial box

1.   I confirm that I have read and understand the information sheet dated May 2009.

2.   I understand that my participation is voluntary and that I am free to withdraw at any

time without my medical care or legal rights being affected.

3.   I understand that sections of my medical notes and data collected during the

study may be looked at by individuals from the University of Liverpool,

from regulatory bodies 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, and

my baby's, records.

4.   I agree to take part in the above study.

5.   I agree to my GP being informed of my participation in the study.

6.   Do you wish to receive a final copy of the results when they are published?   Yes / No

_____   _____   _____

Name of Patient   Date   Signature

_____   _____   _____

Researcher   Date   Signature

1 copy to be kept with handheld notes, 1 copy to be kept in brown hospital notes

Appendix II

Placental Reminder Form

This Patient has consented for the *'Fit for Birth'* Study:

PLEASE SEND PLACENTA FOR HISTOLOGY

Please text/leave voicemail:

immediately to notify the researcher that the woman has delivered, e.g. Sarah Jones has delivered on Mat 1, as the baby also requires measuring for the study (Call anytime - 24/7).

The relevant histology form is attached behind.

Thank you for your help ☺

Appendix III

Covering Letter to Patients with FFB Reminder Form

Dear

I am writing to you with regard to the 'Fit For Birth' study that you consented to at the beginning of your pregnancy. As part of the study we would like to examine your placenta once your baby has been born. In order to help us obtain your placenta for examination please place the two enclosed forms at the **very front** of your hand-held notes. These forms will highlight to your midwife that you are part of the 'Fit For Birth' study and that your placenta requires sending for histology. Having your placenta sent for histology will not affect your treatment during your stay in hospital and your participation within this study is greatly appreciated.

If, however, you have changed your mind and no longer wish to have participate in the 'Fit For Birth' study then do not place these forms with your notes and your placenta will not be sent for examination.

Thank you very much for cooperation with our study,

Yours Sincerely,

The Fit for Birth Team

Appendix IV

FFB Reminder Poster to the Midwives

THIS PATIENT HAS CONSENTED FOR THE *'FIT FOR BIRTH'* STUDY

PLEASE SEND PLACENTA FOR HISTOLOGY

PLEASE QUOTE: 'FIT FOR BIRTH STUDY' AS REASON FOR HISTOLOGY

Thank you for your help

NOTE – Appendix V-VIII are not available electronically as part of this thesis.

Appendix V

FFB Histology Form

Appendix VI

Technique used for Neonatal Skin fold thickness measurement using Harpenden Callipers

Appendix VII

Control Patient Information Sheets

Appendix VIII

Control Consent Forms

Appendix IX

Bulk Birth Weight Centile Calculator

Example of bulk birth weight centile calculator used.

Appendix X –

Protocols for Laboratory work

- ○ **Advance Preparation:**

The following items were prepared in advance of a placenta arriving at the laboratory for culturing:

1. For sterilisation, the following items were autoclaved at 121°C for fifteen minutes:
    i.   Glass dish
    ii. 3 sets of forceps
    iii. 3 sets of scissors
    iv. 1ml pipette tips
    v.   250µl pipette tips

2. 10M, 2M and 3M NaOH:

- 10M = 100ml distilled H_2O + 40grams NaOH

- 2M = 200ml distilled H_2O + 20 grams NaOH

- 0.3M = 1 litre distilled H_2O + 12grams NaOH

3. 10mM HCl:

- NB. Laboratory stock of HCl = 10M solution

- 10mM = 50 µl stock HCl + 50ml distilled H_2O

4. 20mM Tris:

- 20mM Tris = 100ml distilled H_2O + 0.24grams Trizma base.

- Adjust to pH 8.0 with HCl.

- Filter prior to use.

5. Primary Buffering solution (PBS) / 0.1% Bovine serum albumin (BSA):

- 1 litre of PBS = NaCl   8 g
                   KCl   0.2 g
                   $Na_2HPO_4.2H_2O$ 1.5 g
                   KH_2PO_4 0.2 g

    - Dissolve and adjust to pH 7.4 with 2M NaOH.

    - Defrost 10% BSA from -20°C freezer.

    - In a sterile universal tube prepare:

    - 20 ml PBS / 0.1% BSA = 200 µl 10% BSA + 19.8 ml PBS.

- Mix by inversion / vortex and store at 4 °C.
- Filter prior to use.

6. 5 litres 5mM Tyrodes solution:

- 5 litres Tyrodes solution =

135 mM	NaCl		39.45 g
5 mM	KCl		1.86g
1.8 mM	$CaCl_2$ ($2H_2O$)		1.32g
1 mM	$MgCl_2$($6H_2O$)		1.02g
10 mM	HEPES		11.92g
5 mM	Glucose		4.5g

- Dissolve in 1-2 litres water, and adjust pH 7.4 with 10M NaOH.
- Add water to make up to 5 litres.
- Store in 1 litre glass bottles and filter prior to use.

7. 1 litre of each; 15mM and 25 mM glucose Tyrodes:

- First 2 litres of Tyrodes solution was made up =

135 mM	NaCl	15.78g
5 mM	KCl	0.74g
1.8 mM	$CaCl_2$ ($2H_2O$)	0.53g
1 mM	$MgCl_2$($6H_2O$)	0.41g
10 mM	HEPES	4.77 g

- Dissolve in 2 litres dH_2O, and transfer to 2 1litre bottles.
- Label 1 bottle 15 mM and add 5.4 grams of glucose, dissolve by stirring with a magnetic stir bar, and adjust to pH 7.4 with 10M NaOH.
- Label 1 bottle 25 mM and add 9 grams of glucose, dissolve by stirring with a magnetic stir bar, and adjust to pH 7.4 with 10M NaOH.
- Filter prior to use.

8. Stimulant preparation:
   a. **Leptin** - Only one working stock concentration was needed for leptin.

- Leptin (398-LP) was supplied by R&D Systems as a lyophilized powder and was stored at -20°C to -70°C.
- It was reconstituted at a concentration of 1mg/ml in sterile 20 mM Tris-HCl = 1ml 20 mM Tris-HCl to the 1 mg leptin supplied.
- pH was adjusted to 8.0, using 10M NaOH
- After reconstitution 1 mg/ml leptin is stable for 1 month at 2-8 °C and 3 months at -20 to -70°C. Freeze thaw was avoided.
- Working stock solution of 40 µg/ml was prepared =

    20 µl 1mg/ml leptin + 480 µl diluent.
- It was stored in 25 µl aliquots at -20°C to -70°C.

    b. TNF-α - Three working stock concentrations were required for TNF-α; 12 µg / ml, 4 µg/ml, 0.4 µg / ml.

- TNF-α (210-TA) was supplied by R&D Systems as a lyophilized powder and was stored at -20 to -70°C.
- It was reconstituted at a concentration of 100 µg / ml in sterile filtered PBS / 0.1% BSA, by adding 100 µl PBS / 0.1% BSA to the 10 µg TNF-α supplied.
- After reconstitution 100 µg/ml TNF-α is stable for 1 month at 2-8°C and 3 months at -20 to -70°C. Freeze thaw was avoided.
- Working stock solutions of the following concentrations were prepared:

12 µg/ml TNF-α=90µl 100 µg/ml TNF-α+660 µl sterile filtered PBS/0.1% BSA.

4 µg/ml TNF-α=150 µl 12 µg/ml TNF-α+300 µl sterile filtered PBS/0.1% BSA.

0.4 µg/ml TNF-α=50 µl 4 µg/ml TNF-α+450 µl sterile filtered PBS/0.1% BSA.

- 3 aliquots of each concentration were stored in 40 µl aliquots and the remainder in 25 µl aliquots at -20°C to -70°C.

    c. Insulin – four working stock concentrations were needed for insulin; 400nM, 40 nM, 40 µM and 400µM.

- Insulin (I0259) was supplied by Sigma-Aldrich as a powder and was stored at -20°C.

- It was reconstituted at a concentration of 20 mg/ml in 10 mM HCl =

    250 mg insulin + 12.5 ml 10 mM HCl.

- A working stock solution of concentration 230µg/ml was prepared:

10 µl 20 mg/ml stock solution + 850 µl sterile filtered PBS/0.1% BSA.

- Further working stock solutions of the following concentrations were prepared by serial 10x dilution:

23 µg/ml = 100µl 230µg/ml stock + 900 µl sterile filtered PBS/0.1% BSA

2.3 µg/ml = 100 µl 23 µg/ml stock + 900 µl sterile filtered PBS/0.1% BSA

0.23 µg/ml= 100 2.3µl µg/ml stock + 900 µl sterile filtered PBS/0.1%BSA

- Each concentration was stored in labelled 25 µl aliquots at -20°C.

- ○ **Advance Preparation for Each Placenta Received:**

1. 2 sets of scintillation vials were labelled; both sets were labelled 1-11. One set was for protein assays to be performed on, and the second set was for immunohistochemistry to be performed on. They were placed in two separate racks.

- 4ml of filtered control Tyrodes solution was pipetted into vial 1 and 4-11 and recapped.

- 4ml of 15mM Tyrodes solution was pipetted into vials labelled 2 and recapped.

- 4ml of 25mM Tyrodes solution was pipetted into vials labelled 3 and recapped.

- All vials were placed in the fridge.

2. A further set of scintillation vials were labelled 1-11. Each vial was also labelled with a 'W', to denote that it contained water, not Tyrodes solution.

- Approximately 36 ml of distilled H_2O was fast pipetted in a sterile universal tube.

- 18µl of protease inhibitor cocktail (PI) was added to the distilled H_2O (10µl PI per 20ml distilled H_2O). The solution was inverted to mix.

- 2ml of the distilled H_2O/PI was pipetted into each scintillation vial and recapped.

- All vials were placed in the fridge.

    NB. This was only done on the day of culturing to avoid PI degradation.

3. A further set of scintillation vials were then labelled 1-11. Each vial was also labelled with 'NaOH', to denote that it contained NaOH, not Tyrodes solution or water.

- Approximately 10ml of 0.3M NaOH was pipetted into each vial and recapped.

4. 160 microcentrifuge tubes were labelled using the following coding:

- Letter – denoted the order in which placentae were received (A-M) e.g. 1=A, 2=B etc.

- Number – corresponded to stimulant condition (1-11) – see stimulant table above.

- E – denoted that the supernatant was extracellular – 6 tubes were labelled per stimulant

- I – denoted that the supernatant was intracellular – 4 tubes were labelled per stimulant denotes intracellular

    NB. Only tubes for the first set of vials were labelled e.g. 1-11, 11x6 extracellular and 11x4 intracellular. No microfuge tubes were needed for the second set.

5. 11 sterile bijou tubes were labelled with the date, corresponding placental letter, and numbered 1-11. They were

filled with approximately 5ml of 4% neutral buffering formalin (NBF).

6. The microbiological safety class II (MCII) cabinet was cleaned using 1:100 TriGene Advance solution and dry with absorbent towels.

On the day of culturing, prior to receiving the placenta:

1. The water bath switched on to heat up to 37°C. The water level was checked and filled up using distilled water if it was deemed too low.

2. The following items were placed on a clean plastic tray in the MCII cabinet:

- Sterile glass dish

- 2 sets of sterile forceps

- 2 sets of sterile scissors

3. The following items were placed within the MCII cabinet but set to one side:

- 2x10cm Petri dishes containing filtered 5mM Tyrodes solution.

- 1x20cm Petri dish containing filtered 5mM Tyrodes solution.

- Both sets of labelled scintillation vials containing Tyrodes solution.

- 3 sterile pots containing PBS labelled 1, 2, 3.

- Sharps discard pot containing 1:100 TriGene Advance Solution.

- Defrosted stimulants placed on ice.

4. An equipment decontamination container was prepared (Large plastic container filled to 50% capacity with 1% Virkon).

o **Villous tissue Preparation:**

1. Once the placenta was received from theatre, it was placed in the sterile glass dish.

2. The maternal and foetal aspects of the placenta were photographed prior to commencing excision of tissue.

3. The placenta was positioned with the maternal aspect facing upwards.

4. 3x1cm^3 full thickness samples were cut from the placenta. The samples were cut from 3 different areas of the placenta to ensure a representative sample was taken. Samples were cut from the belly of a cotyledon, avoiding the area directly below the cord and the periphery.

5. The samples were placed in the pot 1 containing PBS. They were then subsequently placed in pot 2, and then pot 3. This was to remove any excess blood from the samples.

6. The 2 samples were then placed in the two 10cm Petri dishes (the third remained in the PBS to avoid drying out).

7. Using the forceps and scissors the chorionic plate and the decidual edge were dissected from the remaining villous tissue.

8. Each sample was then dissected into approximately 0.25 cm^3 fragments which were then placed in the 20cm Petri dish. This was to ensure that all the fragments from the three samples were mixed.

9. 6 fragments were then placed in each scintillation vial.

10. 10 µl of the working stock solutions of the stimulants were then added to the appropriate scintillation vials (according to the stimulant table).

11. The vials were then placed, uncapped, in the shaking water-bath for 3hours.

12. The placenta was then placed back in the histology bucket and returned, along with a completed histology form, to delivery suite to be sent for histology.

13. After 3 hours, the vials were removed from the water-bath.

14. Using forceps, the fragments from the first set of scintillation vials (1-11) were quickly placed into the corresponding scintillation vials containing 2ml distilled H$_2$O/PI and recapped.

15. The scintillation vials containing the supernatant were then placed on ice.

16. The supernatant from each vial was then aliquotted into the corresponding, labelled microfuge tubes, NB. Also labelled 'E' (extracellular). Approximately 660μl of supernatant was placed in each microfuge tube. Tubes were placed on ice until all tubes had been aliquotted. This was to avoid degradation of cytokines within the supernatant.

17. Aliquots were stored at -80°C. Freezer plans were then filled in.

18. The fragments from the second set of scintillation vials were placed into the bijou tubes containing 4% NBF. The bijou tubes were then placed in the fridge overnight to fix in the NBF.

19. The scintillation vials now containing the fragments were then incubated for ≥ 18 hours at 4°C.

20. After 18-20 hours, the fragments were then removed and placed in the scintillation vials labelled 1-11 'NaOH', containing 10ml of NaOH. The vials were recapped and placed in the water-bath at 37°C ≥ 6 h.

21. The vials containing the supernatant were each placed on ice after the fragments had been removed.

22. The supernatant from each vial was then aliquotted into the corresponding, labelled microfuge tubes NB. Also labelled 'I' for intracellular. Approximately 500 μl of supernatant was placed in each microfuge tube. Tubes were placed on ice until all tubes had been aliquotted. This was to avoid degradation of cytokines within the supernatant.

23. Aliquots were stored at -80°C. Freezer plans were then filled in.

24. After the 6 hour incubation of the scintillation vials containing the 0.3M NaOH were placed in the fridge.

o <u>Methodology for Processing of Fixed Samples and Wax Embedding</u>

Due to time constraints on the candidate, the wax embedding was conducted by Lisa Heathcote and Jo Drury.

1. After the fragments in the bijou tubes had fixed in the 4% NBF for 24 hours, samples were placed in mesh bags (in 2 groups of 3 fragments) and placed in corresponding, labelled plastic cassettes.

2. The cassettes were then put in a processing basket and placed in the Shandon Citadel Processor to be dehydrated, cleared and impregnated with paraffin wax. In summary the following process is followed:

   - 4% formalin in neutral buffer   45 minutes
   - 60% Ethanol   1 hour
   - 70% Ethanol   1 hour
   - 90% Ethanol   1 hour
   - 100% Ethanol   1 hour
   - 100% Ethanol   1 ½ hours
   - 100% Ethanol   2 hours
   - Xylene 1   1 hour
   - Xylene 2   1 ½ hours
   - Xylene 3   2 hours
   - Wax 1   2 ½ hours
   - Wax 2   3 ½ hours

3. Once finished, the blocks were placed into heated reservoir in the embedding machine.

4. A metal mould was then taken from the heated unit and half filled with wax.

5. A cassette was then taken and placed on the heated area by the wax tap to prevent solidification of the wax.

6. The sample was then removed from the cassette and transferred to the wax-filled mould using hot forceps.

7. The mould was then transferred to the cold area of the embedding unit. Hot forceps were then used to position the tissue in the mould. Once position the sample was covered with a plastic cassette with the label facing upwards.

8. The sample was then topped up with wax, and placed on cooling block.

9. After 30 minutes the cassette was removed from the metal mould.

10. All the blocks were then stored in at room temperature.

   o **ELISA Methodology - Advance Preparation:**

1. Antibodies:
- R&D Systems supplied the ELISA duo-sets for all the cytokines (catalogue reference DY201 (IL-1β), DY206 (IL-6) and DY208 (TNF-α)).
- Each duoset contained a bottle of:
   a) Monoclonal capture antibody:
      - Capture antibodies for all cytokines were reconstituted in 1ml sterile PBS to give a stock concentration of 720µg/ml.
      - Capture antibodies can be stored for 60 days at 4°C or frozen at -20°C to -70°C for 6 months.
   b) Secondary Polyclonal Biotinylated goat anti-human antibody:
      - Secondary antibodies for IL-6 and IL-1β were reconstituted in 1ml PBS/1% BSA, giving a stock concentration of: IL-6 = 36 g/ml, and IL1β = 18 µg/ml.

-Secondary antibody for TNF-α was reconstituted in 1ml of PBS/0.1% BSA giving a stock concentration of 54μg/ml.

- Secondary antibodies can be stored for 60 days at 4°C or frozen at -20°C to -70°C for 6 months.

c) Detection agent (Streptavidin horseradish peroxidise (HRP))

d) Pre-determined concentrations of cytokines to be used for preparing the standard curves:

- IL-1β stock concentration = 40ng/ml

- IL-6 stock concentration = 45ng/ml

- TNF-α stock concentration = 310ng/ml

(TNF-α was reconstituted in 0.5ml of PBS/0.1% BSA, giving a diluted concentration of 31ng/ml).

The following solutions were made up prior to commencing an ELISA run:

1. 5litres of PBS:
- 5 litres of PBS = NaCl   40 g
                  KCl   1.0 g
                  $Na_2HPO_4.2H_2O$ 7.5 g
                  KH_2PO_4 1.0 g

  - Dissolve in 1 litre of distilled H_2O and adjust to pH 7.4 with 2M NaOH.
  - Make up volume to 5 litres using distilled H_2O

2. PBS/1%BSA:
  - Defrost 10% BSA from -20°C freezer.
  - In a sterile universal tube prepare:
    - 20 ml PBS/1% BSA = 2ml 10% BSA + 18 ml PBS.
    - Mix by inversion / vortex.

3. PBS/0.1% BSA:

- Defrost 10% BSA from -20°C freezer.
- In a sterile universal tube prepare:
  - 20 ml PBS/0.1% BSA = 200 μl 10% BSA + 19.8 ml PBS.
  - Mix by inversion / vortex.

4. Wash buffer:
  - Approximately 2 litres should be made up prior to each run
  - 2 litres of wash buffer=
      2ml of Tween 20 + 2litres of PBS
  - Store at room temperature.

5. Blocking buffer:
  = 90ml PBS + 10ml 10% BSA + 5g sucrose + 250μl 20% sodium azide
      - Store in the fridge at 4°C in an opaque glass bottle.

4.7.1.1. The ELISA Run:

This method has been written as if three plates were running simultaneously, one for each of the cytokines assayed.
NB.
The diluent used throughout the run for IL-6 and IL-1β was PBS/1% BSA
The diluent used throughout the run for TNF-α was PBS/ 0.1% BSA

Initially, the diluent for TNF-α contained 0.05% Tween 20, however this was omitted after several unsuccessful ELISA runs using this diluent.

1. A plan detailing where the samples to be assayed were to be placed on the microtiter plate was prepared prior to the ELISA run. Samples were run in duplicate and an average of the two concentrations was taken.

2. Antigen coating the plates:

    - The primary antibodies for each cytokine were prepared:

        - For each cytokine:

            55μl of capture antibody was added to 9945μl of appropriate diluent.

        - (Final concentrations: IL-6 = 200ng/ml, IL-1β = 100ng/ml and TNF-α = 4μg/ml).

    - 3 ELISA plates were labelled; IL-1β, IL-6 and TNF-α.

    - Each plate was then coated with 100 μl per well of primary antibody and covered with plate sealer.

    - The plates were then incubated at room temperature overnight.

3. The plates were then washed using a wash bottle filled with wash buffer. The wells were filled forcefully and once the entire plate was full, the buffer was decanted into the sink and the blotted vigorously on paper towels. This was repeated 3 times in total.

4. The wells were then filled with 300 μl of blocking buffer (which was at room temperature) and incubated for 1 hour at 37ºC in a Dynatech Varishaker Incubator.

5. The samples to be assayed were then removed from the freezer and allowed to defrost in ice.

6. A separate standard curve for each cytokine to be assayed was prepared:

    ▪ IL-1β:

      The highest concentration of IL-1β on the standard curve was 500pg/ml=

      10μl of the supplied IL-1β standard stock + 990 μl PBS/1% BSA

      All other standard curve concentrations were then prepared by serial dilution using PBS/1% BSA as follows:

Code for plate plan	Final concentration of IL-1β(pg/ml):	Volume of previous IL-1β standard curve	Volume of PBS/1% BSA

		concentration (µl)	(µl)
S1	500.00	n/a	990
S2	250.00	300	300
S3	125.00	300	300
S4	62.50	300	300
S5	31.25	300	300
S6	15.63	300	300
S7	7.81	300	300
S8	3.91	300	300

Table 4.1 - Table to show how standard curve for IL-1B was produced.

- IL-6:

The highest concentration of IL-6 on the standard curve was 600pg/ml=

10µl of the supplied IL-6 standard stock + 740µl PBS/1% BSA

All other standard curve concentrations were then prepared by serial dilution using PBS/1% BSA as follows:

Code for plate plan	Final Concentration of IL-6 (pg/ml):	Volume of previous IL-6 standard curve concentration (µl)	Volume of PBS/1% BSA (µl)
S1	600	n/a	740
S2	300	300	300
S3	150	300	300
S4	75.0	300	300
S5	37.5	300	300
S6	18.75	300	300
S7	9.38	300	300
S8	4.69	300	300

Table 4.2 - Table to show standard curve for IL-6 was prepared.

- TNF-α:

The highest concentration of TNF-α on the standard curve was 1000pg/ml=

37µl of the supplied TNF-α standard stock + 963µl PBS/0.1% BSA

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All other standard curve concentrations were then prepared by serial dilution using PBS/0.1% BSA as follows:

Code for plate plan	Final Concentration of TNF-α (pg/ml):	Volume of previous TNF-α standard curve concentration (µl)	Volume of PBS/1% BSA (µl)
S1	1000	n/a	963
S2	500	300	300
S3	250	300	300
S4	125	300	300
S5	62.5	300	300
S6	31.25	300	300
S7	15.6	300	300

Table 4.3 - Table to show how standard curve for TNF-a was prepared.

NB. Blank diluent was used as S0 for all cytokines.

7. 1:2 dilutions of the extracellular supernatants were prepared = 150µl sample + 150µl diluents

This was done because when samples were run undiluted the generated cytokine concentration was greater than the highest concentration on the standard curve i.e. '>max'

8. 1:4 dilutions of the intracellular supernatant were prepared = 60µl sample + 180µl diluents

The reasoning for this was the same as for dilution of extracellular supernatant.

NB. All the samples were vortexed thoroughly prior to use to ensure a representative sample was used for the ELISA.

9. After one hour, the plates were removed from the incubator and washed three times with wash buffer (repeat step 3).

10. 100 µl of each standards, sample or diluent was pipetted into the plate according to the plate plans. The plates were then incubated for 1 hour at 37ºC in a Dynatech Varishaker Incubator.

11. The secondary antibodies were then prepared by performing a 1:180 dilution =

      55µl antibody + 9945µl diluent

    The final concentrations of each cytokine were as such:

        IL-1β = 100ng/ml

        IL-6 = 200ng/ml

        TNF-α = 0.3µg/ml

12. After one hour, the plates were removed from the incubator and washed three times with wash buffer (repeat step 3).

13. 100 µl of the secondary antibody was then added to each well and covered. The plates were then incubated for 1 hour at 37ºC in a Dynatech Varishaker Incubator.

14. The plate plan was then entered onto the Multiskan Ascent plate reader. Appropriate sample identification and any dilutions were inputted onto the plate plan and saved under a new file name.

15. A 1:200 dilution of the detection agent, Streptavidin HRP, was then prepared =

      50µl Streptavidin HRP + 9950µl diluent

16. After one hour, the plates were removed from the incubator and washed three times with wash buffer (repeat step 3).

17. 100 µl of Streptavidin/HRP was added to each well and incubated for 30 minutes at room temperature.

18. After 30 minutes, the plates were removed from the incubator and washed three times with wash buffer (repeat step 3).

19. 100µl of TMB/H_2O_2 was added to each well. The solution was inspected prior to use and was discarded if it already appeared blue. The plates were then incubated, in the dark, at room temperature, for 6-7 minutes, or until there was an adequate colour gradient along the standard curve.

20. 50 µl of 1M H_2SO_4 was then added to each well to inhibit any further enzyme activity.

21. The plate was then placed on the plate reader, ensuring there was no cover on the plate.

22. The optical density of the wells was then read between the intensities of 450nm and 540nm.

23. A graph, plotting cytokine concentration against net absorbance, was then generated, giving a standard curve. A line of best fit was then applied and the concentration of the cytokine being assayed within each sample was automatically generated using the standard curve.

24. If any results were '>max' i.e. the concentration of cytokine within that well exceeded the highest concentration on the standard curve, then, if possible, this result was repeated on another run.

25. If any results were '<min' i.e. the concentration of cytokine within that well fell below the lowest concentration on the standard curve, then, if possible, this result was repeated, undiluted, on another run.

o **Protein Concentration Analysis:**

The following solutions were made up prior to commencing a Bradford Protein Assay run:

1. 900ml of 0.3M HCl =

    27ml 10M HCl + 873ml distilled H_2O

2. 1 litre of 0.3M NaOH =

    12g NaOH + 1 litre distilled H_2O

3. 400 µl 0.25mg/ml BSA:

    - Stock BSA concentration = 2mg/1ml

    - 400 µl 0.25mg/ml BSA =

        50µl Stock BSA + 350µl 0.3M NaOH

- Final concentration of standard solution = 0.25mg/ml (1:8 dilution).

4.7.1.2. The Bradford Protein Assay Run:

1. A plan detailing where the samples were to be placed on the microtiter plate was prepared prior to the run. Samples were run in duplicate and an average of the two concentrations was taken. As there are only eleven samples per placenta, two placentae can be assayed simultaneously.

2. A standard curve was then prepared as follows:

Code used on plate plan	Final Protein Concentration (µg/ml)	Volume of 0.3M NaOH to be added (µl)	Volume of 0.25mg/ml BSA (µl)
S0	0	100	0
S1	12.5	95	5
S2	62.5	75	25
S3	125.0	50	50
S4	187.5	25	75
S5	250.0	0	100

3. 20µl of each standard was then pipetted into each well according to the plate plan. 180µl of PBS was then added, giving a total volume of 200µl.

4. 10µl of each sample was then pipetted into each well according to the plate plan. 190µl of PBS was then added, giving a total volume of 200µl. A smaller volume of sample was added, otherwise if 20µl was added the protein concentration generated was greater than the highest concentration on the standard curve i.e. '>max'
   NB. All samples were vortexed prior to pipetting.

5. 50µl of Bradford's dye reagent (at room temp) was then added to each well.

6. The plate plan was then entered onto the Multiskan Ascent plate reader. Appropriate sample identification and a dilution

factor of ten was applied to all samples as they were diluted in 10ml of NaOH.

7. The microtiter plate was then placed on the plate reader and mixed for 30seconds. The plate was read at an absorbance of 595nm.

8. A graph, plotting protein concentration against net absorbance was then generated, giving a standard curve. A line of best fit was then applied and the concentration of protein within the samples was generated automatically using the standard curve.
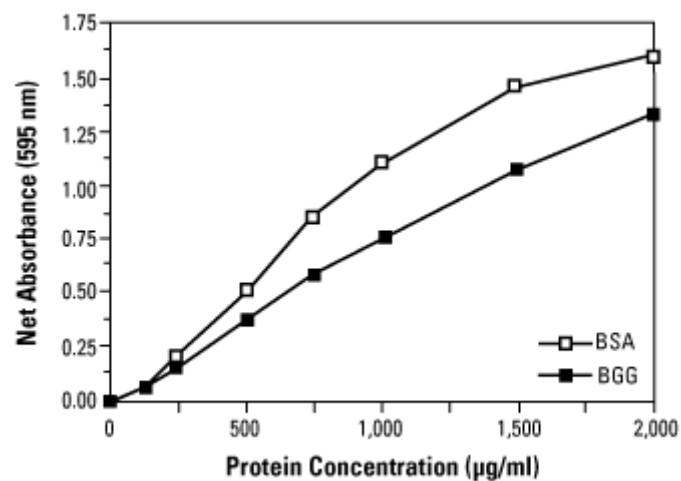

Figure 4-2 - A Typical standard curve for BSA and bovine gamma globulin (BGG) in the Pierce Coomassie Plus Protein Assay.[104]

9. Once the protein content of the villous tissue had been determined, the corresponding cytokine concentrations of the samples were divided giving a final cytokine concentration in pg/mg of protein.

Appendix XI

Conclusion for PCT

This project was commissioned by Liverpool PCT with the following aims:

> 1. *'To determine the scale of the problem of obesity in pregnancy, in the Liverpool PCT area.'*
>
> 2. *'To generate information that can be used to develop services and plan further research.'*

With respect to Aim two, the PCT were particularly interested in whether deployment of existing resources to obese women in pregnancy would be likely to impact on pregnancy outcomes. For example, if maternal weight change in pregnancy was clearly linked to foetal size it might be worth targeting dietetic advice to obese pregnant women. Although preliminary, the results presented here argue against the suggestion that redeployment of existing services to target obese pregnant women would improve neonatal outcomes. Firstly, in general obese pregnant women had recommended weight gains during pregnancy. Simple dietary modifications would be unlikely to be relevant. Secondly, neonatal body composition in this group was different from that observed in previous reports. Specifically, fat-free mass was higher in babies born to obese women compared to babies born to lean women. This suggests that women (and their foetuses) in Liverpool are nourished differently from women in previous cohorts. Interventions developed in other settings may not be directly applicable in Liverpool. Thirdly, obese pregnant women were taller than lean pregnant women. This may indicate that differences in nutrition between the two groups were longstanding. Height reflects growth until epiphyseal closure. Nutrition can influence height during childhood. Short term changes in nutrition may not affect maternal obesity. Fourthly, there is evidence that the state of chronic inflammation found in obesity is also found in pregnant obese women (albeit with some modification with respect to TNF-). We have speculated that this inflammatory state may

influence fetal nutrition (via an effect of IL-6 on System A). The inflammatory state appears to be a consequence of excess WAT which will be part of the baseline physiology when obese women become pregnant. Modifying this inflammatory process during pregnancy is not possible at present and defining suitable interventions will require a considerable research effort. Reducing the prevalence of obesity would be a more promising approach. Fifthly, even an observational study of obese pregnant women required considerable resources and effort. Supporting obese women during pregnancy would need considerable investment.

This constellation of findings might help the PCT decide where to target their resources. Dietetic intervention in obese pregnancy would introduce women to that service but would be unlikely to affect neonatal outcomes. The PCT may have more appropriate priorities. Several of the findings of this project point to the benefits of reducing obesity before pregnancy. Thus population-level campaigns are more likely to succeed than campaigns targeted to pregnant obese women.

In a world of imperfect public health information, the results presented here may contribute to rational resource allocation in the NHS as well as identify research questions.