The influence of maternal obesity on placental fatty acid composition, synthesis of inflammatory mediators and pregnancy outcomes

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Declaration

All the work presented within this thesis was completed by the author. All studies were carried out under the supervision of Dr Matthew Elmes, Division of Nutritional Science, School of Bioscience, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire, LE12 5RD.

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Abstract

Introduction: Obesity is a global issue with ever increasing prevalence, having detrimental effects on the health of the population. Maternal obesity has been identified to significantly increase the risk of prolonged and dysfunctional labour, labour induction and emergency caesarean delivery. An established rodent model of diet induced obesity has been utilised to unravel the potential mechanisms behind uncoordinated myometrial contractile function labour. In this model a high-fat, high-cholesterol (HFHC) diet successfully induces maternal obesity, significantly altering maternal fatty acid status leading to un-coordinated contractions that significantly prolongs labour. Potential mechanisms identified to be behind dysfunctional labour is a decrease in the uterine expression of key contractile associated proteins (CAPs), an increase in progesterone concentrations, the hormone that suppresses expression of the contractile proteins, and decreased circulating PGF2α that stimulates myometrial contractions during labour.

Aims: The aim of this thesis was to first evaluate the impact the HFHC diet may have on the placenta in the maternal obesity model, focusing on its role in the initiation of labour. The second aim was to assess if the human placenta from obese women had the same characteristics. The third aim was to evaluate how these fatty acid profile changes may affect trophoblast cell's ability to produce pro-labour mediators, in vitro. Finally, the last chapter aimed to evaluate non-communicable disease and diet alongside obesity on pre-term birth, mode of delivery and birthweight, in a setting where obesity, NCDs and adverse pregnancy outcomes are extremely prevalent. Two of the most common NCDs associated with obesity and placental pathologies, hypertension and type 2 diabetes were evaluated alongside obesity, looking at how these effect pregnancy outcomes. The final prospective study aimed to evaluate any dietary patterns associated with pregnancy outcomes.

Methods: Fatty acid profiles of the placental tissue from both the rat study and from human participants of different BMI were analysed through GCMS and RTqPCR to compare the fatty acid composition and gene expression profiles respectively. Gene expression profiles were centred around the placentas role in initiation of labour including hormone synthesis and signalling (e.g corticotrophin releasing hormone -CRH, Oestrogen receptor 1 -ER1 and cytochrome P450 family 17 subfamily A - CYP17), prostaglandin synthesis (e.g cyclooxygenase 2 - COX2), inflammatory regulators (e.g nuclear factor kappa B - NFκB and Sirtuin 1 SIRT1) and peroxisome proliferator-activated receptors - PPARs. HTR8-SVneo trophoblast cell line was treated with increasing doses of Oleic acid and pro-inflammatory cytokine interleukin 6 (IL-6) and prostaglandins PGE2 and PGF2a were measured using ELISA. Prostaglandin synthesis enzymes and PPARγ mRNA levels were also investigated to attempt to elucidate a signalling pathway. Multinomial logistic regression was used to assess the joint impact of obesity and NCDs on mode of delivery, preterm birth and birthweight. A food frequency questionnaire was also used to assess dietary patterns and multinomial logistic regression used to understand the effects they may have on pregnancy outcomes.

Results: This experiment found significant alterations in the fatty acid composition of the placenta from obese pregnant rats and women. In the obese rat placentae, there was a significant decrease in saturated fatty acids, and an increase in monounsaturated fatty acids (MUFAs) as well as decreased desaturase enzyme activity. Similar results were found in placental tissue from obese pregnant women, although to a much lesser extent. Interestingly, only one of the genes quantified was differentially expressed. Placental expression of PPARγ within the placenta was found to be significantly higher in HFHC fed rats but this was expressed any differently within the placenta of pregnant women of different BMI. In the cell culture study oleic acid at the highest physiological doses of (400μM) significantly decreased the synthesis of all the markers measured. Only one enzyme, responsible for producing PGE2 was found to decrease in a similar pattern with increasing oleic acid treatment. The retrospective cohort study found obesity to increase the risk of operative deliveries and remained significant after adjusting for confounding

variables, for emergency CS. Once hypertension and type 2 diabetes were added into the model this no longer remained significant and neither of these had any significant effect on pregnancy outcomes looked at. No dietary patterns were significantly associated with any of these.

Discussion: A diet high in saturated fats may create an anti-inflammatory response, seeing an increase in monounsaturated fatty acids, namely oleic acid within the placenta. The decrease in expression of genes associated with the prostaglandin biosynthetic pathway by high physiological levels of oleic acid may explain the reduced circulating PGF2α, and reduction in the expression of CAPs and dysfunctional contractions seen in the translational model. In conclusion the research findings from this thesis suggest further study into the effects of diet and fatty acid profiles as a risk factors or potential biomarkers for negative pregnancy outcomes are warranted.

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Abbreviations

AA	=	Arachidonic acid
ACTH	=	Adrenocorticotropic hormone
BMI	=	Body Mass Index
cAMP	=	cyclic adenosine monophosphate
CAPs	=	Contractile associated proteins
cGMP	=	cyclic Guanosine 3',5'-cyclic monophosphate
COX	=	cyclooxygenase
CRH	=	Corticotrophin-releasing hormone
CRP	=	C-reactive protein
CS	=	Caesarean section
DAG	=	diacyl glycerol
DGAT	=	Diacylglycerol Acyltransferases
DGLA	=	dihomo-gamma-linolenic acid
DHA	=	docosahexaenoic acid
EPA	=	eicosapentaenoic acid
ERK	=	extracellular signal-regulated kinase
FA	=	fatty acid
FABP	=	fatty acid binding protein
FAME	=	Fatty acid methyl ester
FATP	=	fatty acid transport protein
FFAs	=	Free fatty acids
GDM	=	gestational diabetes mellitus
GPCRs	=	G-protein coupled receptors
HDL	=	high density lipoproteins

HETE	=	hydroxy-eicosatetraenoic acids
HPA	=	Hipothalmic-pituitary-adrenal
IL	=	Interleukin
IP3	=	Inositol triphosphate
LA	=	linoleic acid
LBW	=	Low birth weight
LCAT	=	lecithin cholesterol acetyl transferase
LDL	=	low density lipoproteins
LMICs	=	Lower middle Income countries
LOX	=	lipooxygenases
LPL	=	lipoprotein lipase
LT	=	leukotrienes
MAPK	=	mitogen activated protein kinase
MCP-1	=	monocyte chemoattractant protein 1
MLCK	=	myosin light chain kinase
MMPs	=	matrix-metallopeptidases
MPF	=	maternal-placental-foetal
MUFA	=	monounsaturated fatty acid
NAFLD	=	non-alcoholic fatty liver disease
NCD	=	non communicable diseases
NLRP3	=	NOD-, LRR- and pyrin domain-containing protein 3
NO	=	Nirtric oxide
NOS	=	Nitric oxide synthase
OA	=	Oleic acid
OXTR	=	oxytocin receptor

PGI2	=	prostaglandin I2
PIP2	=	Phosphatidylinositol 4,5-bisphosphate
PKC	=	protein kinase C
PPAR	=	Peroxisome proliferator-activated receptors
PTB	=	preterm birth
PTHrP	=	Parathyroid hormone-related protein
PUFA	=	polyunsaturated fatty acid
ROS	=	reactive oxygen species
SFA	=	saturated fatty acid
TAG	=	triacylglycerol
TG	=	triglyceride
TNF	=	tumour necrosis factor
TX	=	thromboxanes
VLDL	=	very low density lipoproteins
WHO	=	World Health Organisation

1 Literature review

1.1 Obesity

Body mass index (BMI) is a crude estimate of body fat estimated through an individual's weight (in kilograms) divided by their height (in metres) squared (kg/m^2). The World Health Organisation (WHO) defines obesity as having a BMI ≥ 30 (World Health Organisation, 2000). However, this has been adjusted for those with a South Asian heritage, due to differences in their fat accumulation (Nishida *et al.*, 2004, compared in Table 1.1. South Asians with a similar BMI to white Caucasians were found to have increased visceral fat and greater frequency of associated malignancies (Nishida *et al* 2004). Obesity arises from an overconsumption of energy in comparison to energy burned leading to weight gain (Selassie and Sinha, 2011). Energy intake being 5% greater than expenditure a day results in a 5kg fat mass gain over 1 year (Jéquier, 2002). However, the situation is more complex as a combination of factors including metabolic, endocrine and nervous system signals are also involved (Masood and Moorthy, 2023). Additionally extrinsic factors such as increased sedentary employment and general inactivity, easier access to highly processed fast food and longer working hours, all contribute to obesity, creating an obesogenic environment (Selassie and Sinha, 2011).

Classification	*Normal BMI range*	*South-Asian BMI range*
Underweight	≤18.5	≤18.5
Normal	18.5-24.99	18.50-22.99
Overweight	25.00-29.99	23.00-26.49
Obese	≥30	≥27.5

Table 1.1 Body Mass Index (kg/m2) classifications for the normal World Health Organisation and adjusted South Asian values (Nishida et al. 2004)

Obesity is characterised by an over-accumulation of triglycerides (TGs) and lipid droplets, particularly within adipose tissue which acts as a buffer for excess lipids (Krahmer, Farese and Walther, 2013). The primary role for adipocytes is energy storage but they also form a metabolically active tissue, through the secretion of adipokines which act in an endocrine, paracrine and autocrine manner to regulate homeostasis throughout the whole body (Jacobi, Stanya and Lee, 2012). These can induce low grade chronic inflammation, insulin resistance and atherogenic effects (Leal and Mafra, 2013). Obesity induced inflammation is characterised by an increase in plasma levels of Tumour necrosis factor (TNF) and C-reactive protein (CRP) and release of pro-inflammatroy cytokines including interleukin-6 (IL-6) and monocyte chemoattractant protein 1 (MCP1) from adipose and infiltrating macrophages (Stienstra et al., 2007).

Free fatty acids (FFAs) play a major role in the initiation of chronic inflammation in white adipose tissue (Jacobi, Stanya and Lee, 2012). This is regulated through the nutrient sensing nuclear receptors which have transcriptional regulatory activity (Jacobi et al., 2012). Peroxisome proliferator-activated receptors (PPARs) are activated through dietary fats and their metabolites and play a key role in adipogenesis, controlling genes involved in fatty acid uptake, lipid storage and energy homeostasis (Jacobi, Stanya and Lee, 2012). These act through recruitment of large protein complexes which modify histones, altering the structure of chromatin (Jacobi, Stanya and Lee, 2012). PPARs in particular, heterodimerize to the nuclear receptor RXR and bind to PPAR response elements in the promoter regions of their target genes (Stienstra et al., 2007). PPARs have been implicated in obesity in a number of tissues not just adipose but are often found to have anti-inflammatory actions (Stienstra et al., 2007).

1.2 Obesity trends

WHO reported 890 million adults were classed as obese in 2022, and over 390 million adolescents (aged 5-19) classed as overweight or obese (World Health Organisation, 2024), showcasing how this problem is persisting through generations. In the US,

projections based on current trends estimate that in 2030 obesity will be the most common BMI category in women nationwide (Ward et al., 2019). In England the 2021 Health survey found 26% of women to be obese (Baker, 2023). This is not confined to western countries either, obesity rates are increasing much more rapidly in Lower-middle income countries (LMICs) (Ford, Patel and Narayan, 2017). Obesity has become the most prevalent medical condition affecting pregnant women (Poston et al., 2016). A Romanian study found rates of overweight and obesity to exceed 40% in the second trimester of pregnancy and greater proportions of overweight and obesity in multiparous women (Ursache et al., 2023). Similar trends are seen in other countries. In the US the distribution of BMI in mothers shifted upwards from 2011 to 2015 and 8% increase in obesity specifically was seen (Deputy, Dub and Sharma, 2018). A significant increase in obesity was also seen in Ireland from 2010 to 2017, with the greatest increase seen in nulliparas (Reynolds et al., 2019).

1.3 Non-communicable diseases (NCDs)

Non-communicable diseases (NCDs) are now responsible for 71% of all global deaths, and disproportionately affects LMICs. Some of the world's highest rates of NCDs are in South Asian countries and is described as an epidemic (Waqas et al., 2020). The most common NCDs globally are cardiovascular disease, cancer, diabetes, hypertension and chronic respiratory disorders (Mendis, 2010). This widespread occurrence of NCDs not only affects older generations but spans the entire population including women of reproductive age (Hussein, 2017).

Within the literature, NCDs affecting pregnant women are not well defined (Firoz et al., 2022). This scoping review found a wide range of conditions be classed as NCDs with no common definition of framework within pregnancy so could include pregnancy specific conditions (Firoz et al., 2022). 65% of maternal deaths are associated with NCDs during pregnancy and 75% of these occur within LMICs (World Health Organisation, 2016). The maternal burden of NCDs is proportionately increasing despite the decreases seen in maternal mortality (Hussein, 2017) and NCDs generally tend to worsen during pregnancy

(Olusoji Edward, Monday and Kola, 2019). The International Diabetes Federation has estimated 382 million people, 184 million of those women, to have diabetes and this is expected to exceed 592 million by 2035 (Kapur, 2015). The global prevalence of hyperglycaemia in pregnancy is estimated 16.9%, with 90% of cases in LMICs and the highest prevalence found in South-East Asian regions (Guariguata et al., 2014). Hypertension has been identified as the most common NCD within pregnant women in multiple studies (Olusoji Edward, Monday and Kola, 2019; Kumari et al., 2022), alongside diabetes and obesity occurring both alone and in combination (Berger et al., 2019). These estimates are consistently increasing along with the rising prevalence of overweight, obesity and other metabolic disorders (Kapur and Hod, 2020).

1.4 Obesity and NCDs

Obesity is closely linked with many NCDs. A large multi-centre European study found obesity is associated with a significant reduction in disease free years of adults, regardless of sex, smoking status, activity levels and socioeconomic status (Nyberg et al., 2018). South Asian countries are seeing a rapid increase in obesity related NCDs such as diabetes type 2 and hypertension among others (Misra and Khurana, 2011). Obese individuals are at increased risk of developing some of the most common NCDs including hypertension and heart disease (Selassie and Sinha, 2011). Several studies have found obese pregnant women to be at an increased risk of having hypertension and diabetes than those of a normal BMI (Sebire et al., 2001; Bhattacharya et al., 2007; Briese et al., 2011). In the US approximately 90% of people with type 2 diabetes have a BMI classifying them as obese and risk of this increases linearly with BMI (Selassie and Sinha, 2011). Those with chronic hypertension during pregnancy have been found to have significantly higher weight regardless of parity (Ursache et al., 2023). Obesity increases the risk of transmitting NCDs across generations and increases the risk of pregnancy complications such as gestational diabetes and pre-eclampsia (Hanson et al., 2019).

1.5 Pregnancy outcomes and obesity

There is a large body of evidence demonstrating that maternal obesity increases the risk of several negative pregnancy outcomes. Multiple studies from across the world have found significantly increased frequencies of caesarean sections (CS) in obese women

(Kerrigan and Kingdon, 2010; Hermann et al., 2015), increased risk of CS (Briese et al., 2011; Denison et al., 2008; Graves et al., 2006; T. S. U. Kiran et al., 2005), for both elective and emergency (Sebire et al., 2001; Bhattacharya et al., 2007; Pettersen-Dahl et al., 2018). This has been demonstrated across ethnicities (Fyfe *et al.*, 2011) and without other conditions such as hypertension and diabetes (Sheiner *et al.*, 2004). It has also been demonstrated that increased BMI reduced the success of vaginal birth following a prior CS, particularly if their BMI increased from their first pregnancy (Durnwald, Ehrenberg and Mercer, 2004). Research carried out in Northwest England found 17.7% of pregnant women to be classed as clinically obese who had significantly higher rates of CS, as well as prolonged first stages of labour (Kerrigan and Kingdon, 2010). An increased risk of induction of labour has been shown for overweight (Sebire et al., 2001; Graves et al., 2006), obese (Hermann et al., 2015; T. S. U. Kiran et al., 2005) and morbidly obese women (Bhattacharya *et al.*, 2007). Along with a reduced risk of successful induction (Durnwald, Ehrenberg and Mercer, 2004; Arrowsmith, Wray and Quenby, 2011; Zelig et al., 2013). It has been shown that babies of women with a BMI over 30 are at a significantly higher risk of birth trauma, admission to the neonatal unit and were more likely to need assistance with body temperature (Usha Kiran *et al*., 2005). These factors can lead to an increased burden on the NHS with extra care creating an increased cost, accounted for by the delivery method and increased hospital stays (Solmi and Morris, 2018).

There are 2 main theories for how obesity affects CS rates. The first is the obstetrical dilemma. This is the theory that the babies' head has become too large for a woman to give birth without medical assistance (Wells, 2017). Obesity has shown to be associated with foetal macrosomia (Ehrenberg, Mercer and Catalano, 2004; Sheiner et al., 2004; Arrowsmith, Wray and Quenby, 2011; Briese et al., 2011) so the babies are large for their gestational age – this could mean larger heads so much harder for the woman to give birth to vaginally. Obese women tend to have a greater fat mass so this could narrow the birth canal making it more difficult and medical assistance being required (Zhou *et al.*, 2019). However, when looking at reasons for emergency CS it was found that obstructed labour was not associated with increasing BMI but ineffective uterine contractility and

foetal stress (Cedergren, 2009). Other studies have found obese women are more likely to have a CS due to arrest or delay in the first stages of labour (Kerrigan and Kingdon, 2010; Hirshberg, Levine and Srinivas, 2014), higher incidences of arrest (Verdiales, Pacheco and Cohen, 2009) and reduced dilation and longer labour (Kominiarek *et al.*, 2011; Maged *et al.*, 2017). Despite other studies not finding increased length of labour for obese women (Hirshberg, Levine and Srinivas, 2014; Ellekjaer, Bergholt and Løkkegaard, 2017), these studies still strongly suggest an altered mechanism or physiology involving uterine contractions. The differing contraction strengths or mis regulation could lead to the need for medical assistance and so could explain the increase in CS. It is therefore important to understand how or why this is occurring in order to provide effective solutions to midwives and mothers.

1.6 Pregnancy outcomes and NCDs

Multiple studies have found NCDs to be associated with negative pregnancy outcomes globally (Amjad et al., 2020; Bonham et al., 2018; J. L. Kitzmiller et al., 2018; Malhamé et al., 2021; Rasool et al., 2021). Hypertension is a significant contributor to pregnancy-related complications and complicate 5-10% of pregnancies worldwide (Kapur and Hod, 2020). Globally, 1 in 6 pregnancies are associated with hyper glycemia and the Asia-pacific region accounts for about half the global burden (Gan, International Diabetes Federation. and World Diabetes Foundation., 2003). NCDs will have effects on the short-term pregnancy outcomes but can be considered multipliers of NCDs globally as poor maternal health increases the risk of subsequent generations to developing NCDs (Kapur and Hod, 2020). Mothers with NCDs are at greater risk of delivering prematurely and in turn these premature offspring are at greater risk of NCDs in later adult life including diabetes and hypertension and giving birth prematurely themselves, creating an intergenerational cycle (Howson et al., 2013). As well as the perinatal complications associated with both NCDs and obesity during pregnancy risks to the health of the mother and offspring in the future are evident (Hawley, 2016).

1.7 Implications of adverse pregnancy outcomes

Adverse pregnancy outcomes such as preterm birth (PTB), CS and low birthweight (LBW) all have negative impacts on the mother, her offspring and the wider population. CS can affect current and future pregnancies as well as having implications for the offspring. Several cohort studies have found an increased risk of severe acute maternal morbidity (including haemorrhage, hysterectomy, uterine rupture, and obstetric shock) (Sandall *et al.*, 2018). Subsequent pregnancy following a CS is also associated with an increased risk of stillbirth, premature delivery, uterine rupture, and abnormal placentation (Sandall *et al.*, 2018). Offspring born via CS compared to those delivered vaginally also face significantly different short term and long-term health outcomes. Short term risks include altered immune development, allergy, asthma and reduced gut microbiome diversity (Sandall et al. 2018). In later adult life, those born via CS are at greater risk of developing adiposity, hypertension, altered liver function and neurological and stress related problems (Sandall *et al.*, 2018). The WHO has stated that CS does improve pregnancy outcomes for women, but no additional benefits are found above CS rates of 10-15% (Maciej Serda *et al.*, 2010). A 2008 study found that 50% of 137 countries had CS rates greater than 15% and estimated to reach 30% globally within the next 8 years (World Health Organisation, 2018b).

The definition of PTB is those born before 37 weeks and this further categorised into extreme preterm (before 28 weeks' gestation), very preterm (28-32 weeks) and moderate to late preterm (32-37 weeks) (World Health Organisation, 2018a). Infants born premature are more likely to die during the first 28 days and first year, with mortality rates increasing with increased gestational age (Institute of Medicine (US) Committee, 2007). PTB is the second leading cause of death under 2 years of age worldwide and is a major concern to global policy makers (Howson *et al.*, 2013). Developmental immaturity is the key issue for preterm infants and can have both short-term consequences and effects over their entire lifespan (Institute of Medicine (US) Committee, 2007). Compared with infants born at term, those born preterm are at a greater risk of respiratory distress, hypoglycaemia and rehospitalisation and these risks increase with the severity of preterm (Story *et al.*, 2019). PTB significantly contributes to

child neurodevelopment disorders including cerebral palsy, attention deficit disorder and learning disabilities (Schieve *et al.*, 2016). The financial impacts have been estimated around £14,614 for infants born extreme preterm and £11,958 for those born very preterm within the first 5 years of life within the UK (Petrou *et al.*, 2003).

Low birthweight is defined as birthweight below 2500g (CHEN *et al.*, 1991). Low birthweight is associated with hypertension, heart disease, and type 2 diabetes among other health issues (Waqas *et al.*, 2020). Very low birth weight (defined as below 1500g) has been associated with cognitive problems and brain structure abnormalities (Farajdokht *et al.*, 2017). Within the US both preterm birth and low birthweight are the leading causes of infant mortality (Institute of Medicine (US) Committee, 2007).

1.8 Normal Labour

In terms of parturition pregnancy can be broken down into four phases (Vannuccini *et al.*, 2016). The first is uterine quiescence – a state of inactivity or dormancy. The uterus is a primarily muscular organ consisting of 2 basic components, the body and it's endometrium and the uterine cervix (Leppert, 1995). The uterus body is mostly smooth muscle with extracellular matrix between the cells and the cervix approximately 10-15% smooth muscle with the rest connective tissue (Leppert, 1995). During this first quiescent phase, the cervix is reasonably firm with 1-2cm dilation during the final few weeks of gestation (Leppert, 1995). The myometrial tissue is in a proliferative and hypertrophic state to establish the required number and size of myocytes to allow for effective contractions at birth (L. Liu et al., 2013; Shynlova et al., 2009). This is maintained by progesterone, prostaglandin I_2 (PGI_2), Parathyroid hormone-related protein (PTHrP), calcitonin gene-related protein, vasoactive intestinal peptide and nitric oxide (Vannuccini *et al.*, 2016).

The second phase is activation which involves priming of the myometrium, including changes in contractile associated proteins (CAPs) (Vannuccini *et al.*, 2016). Following from this, is stimulation is the third phase. Uterotonics including prostaglandins (PGs),

oxytocin and Corticotropin releasing hormone (CRH), parallel to an increase in cytokines resembling an inflammatory reaction, generate contractions (Vannuccini *et al.*, 2016). The final stage is the expulsion of the foetus and placenta (Vannuccini *et al.*, 2016).

Labour itself is defined as 2 distinct stages. The first stage occurs until the point of full cervical dilation at 10cm (Friedman, 1995), stretching through the force of the uterine contractions (Leppert, 1995). The second stage then starts where the mother can actively push in line with their contractions to deliver the foetus (Friedman, 1995).

1.8.1 Processes

The key processes involved in a successful labour are cervical softening, myometrial priming and strong, phasic uterine contractions.

1.8.1.1 Cervical softening

Cervical softening (or ripening) occurs as the cervix changes from a supporting role for the foetus to the birth canal (Stables and Rankin, 2010). This occurs through an increase in water and non-collagen compounds within the cervix, decreasing collagen concentration and dispersing and disorganising fibres (Vidaeff and Ramin, 2008). This is coincided with an increase in matrix-metallopeptidases (MMPs), MMP2 and MMP9 which are involved in the degradation of extracellular matrix (Stygar *et al.*, 2002).

1.8.1.2 Myometrial priming

Myometrial priming is the transition from a quiescent state to an active state. These changes include regulation of contractile associated proteins (CAPs), structural changes and electrophysiological changes. Structural changes occur as there is an increase in the formation of gap junctions and associated proteins such as Connexin-43 which allow for co-ordinated contractions (Kota *et al.*, 2013 and Cluff *et al.*, 2006). Along with increases in cyclooxygenase (COX) 2, oxytocin receptors (OXTR) and PG receptors (Norwitz, Robinson and Challis, 1999). Electrophysiology changes involve changes in ion channels, the switching off of relaxation pathways such as cyclic adenosine monophosphate (cAMP) and cyclic Guanosine 3',5'-cyclic monophosphate (cGMP) which promote smooth

muscle relaxation through various intracellular reactions; the increase in stimulatory G-protein coupled receptors (GPCRs) and coupling of these (López Bernal, 2003). These signal through the hydrolysis of PIP2, generating inositol 1, 4, 5 – triphosphate, which releases sarcoplasmic reticulum calcium, and diacyl glycerol which activates protein kinase C (PKC) and downstream mitogen activated protein kinase (MAPK) signalling (López Bernal, 2003). There is also a decrease in nitric oxide (NO), an endothelium relaxing factor, mediated by a sharp decline in NO synthase (NOS) at term (Vidaeff and Ramin, 2008). Once the myometrium is primed contractions can occur through stimulation by uterotonics (Norwitz, Robinson and Challis, 1999).

1.8.1.3 Myometrial contractions

Contractions occur in the myometrium, mediated by cellular elements of the uterine myocytes predominantly regulated by calcium (Pehlivanoğlu, Bayrak and Doğan, 2013). The myosin and actin interactions are regulated through myosin light chain kinase (MLCK). Following an increase in cellular calcium, calcium binds to calmodium (forming calmodulin complex) which activates MLCK and in turn phosphorylates the regulatory light chain of myosin, so it interacts with actin forming the functional structure that is capable of producing force (López Bernal, 2003). Calcium influxes through L-type calcium channels and releases form the sarcoplasmic reticulum (Wray, 2007, Wray et al., 2003).

1.8.2 Endocrinology of parturition

These processes are brought about through hormonal profile changes and inflammation. The key changes and their impacts will be discussed here. Many of these create multiple positive feedback loops, rapidly reinforcing and increasing the presence of each other, these relationships are demonstrated in Figure 1.1. Although in humans, it is not entirely clear the exact start point for these cascade of events, the accumulation of several drivers' results in the altered physiology and subsequent activity.

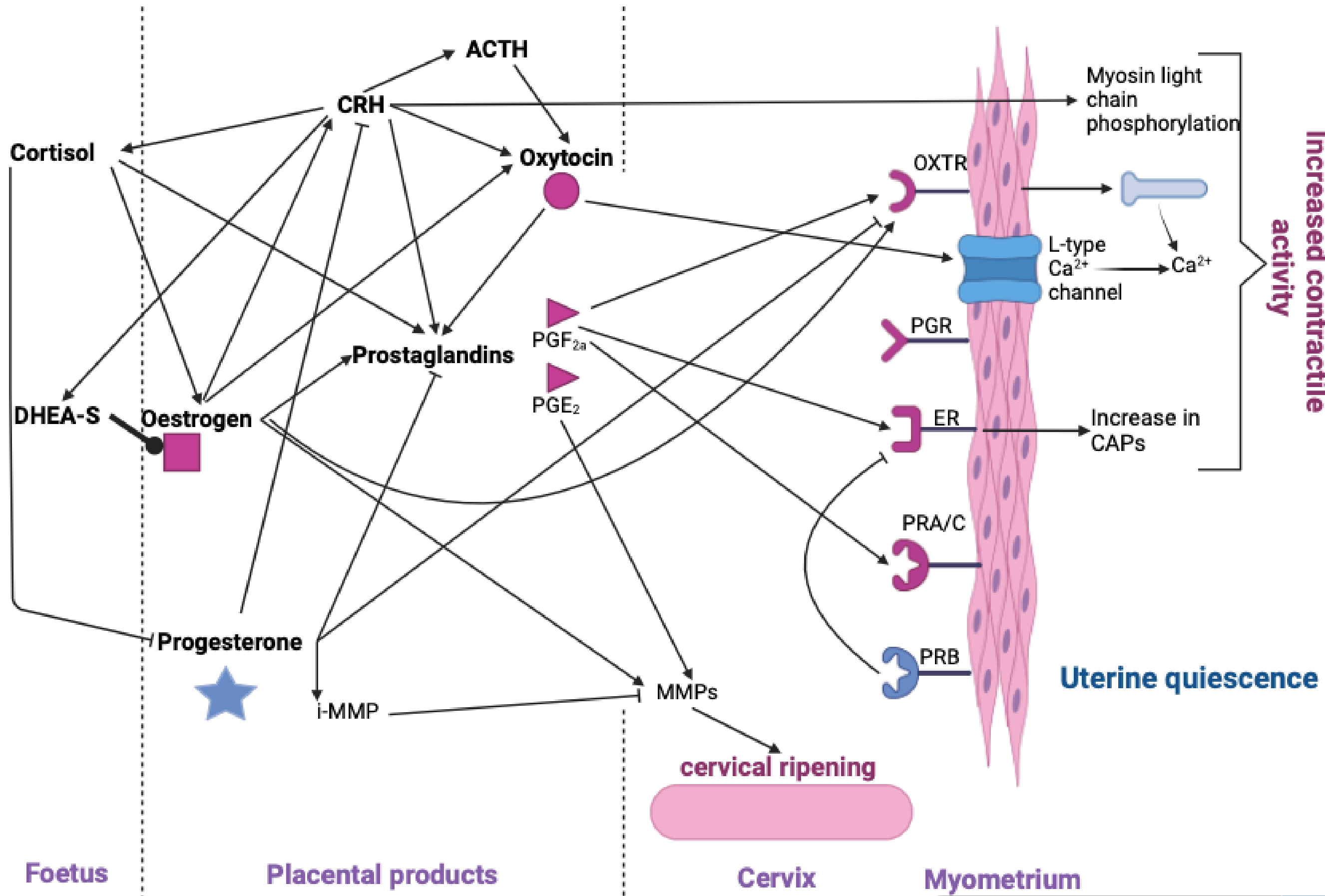

Figure 1.1 The initiation of labour in humans, showing the interactions and actions of the major contributors of parturition .The pink represents those that stimulate contractions and blue for those that promote uterine quiescence. The arrows represent stimulation or an increase in production. OXTR= oxytocin receptor, PGR = prostaglandin receptor, ER = oestrogen receptor, PR = progesterone receptor, MMP = metallioproetin matrix, CAPs= Contractile associated proteins. Briefly: The increase of Oestrogens stimulates an increase in contractile associated proteins, oxytocin receptor, prostaglandin, oxytocin and CRH production. CRH increases foetal cortisol release the inhibits progesterone and increases oestrogen and prostaglandins. CRH also stimulates PGs and oxytocin directly and oestrogen through the production of the precursor DHEA-s in the foetus. In the myometrium CRH increases myosin light chain phosphorylation. Oxytocin is a direct stimulant of myometrial contractility through the increase in L-type calcium channels and releasing intracellular calcium from the endoplasmic reticulum. Oxytocin also increases PG production which creates a positive feedback loop. PGs increase receptors of oxytocin, oestrogen and Progesterone receptor A/C, the inactive form, contributing to the switch to oestrogen dominance. Finally, PGs and Oestrogen increase matrix metallioproteinase (MMP) which causes cervical ripening. Created with BioRender.com

1.8.2.1 Progesterone and Oestrogen

An alteration from progesterone to oestrogen dominance occurs. This shift is a very distinct and clear change in a lot of animals at the start of labour, through the hypothalamic-pituitary-adrenal (HPA) axis. A surge in cortisol, leads to CYP17 activation which is responsible for the pregnenolone to oestradiol conversion (Kota *et al.*, 2013), resulting in a large increase in serum oestrogen. However, in humans' oestrogen is seen to rise steadily over pregnancy but there is no distinct rise at term (Lamming, 1993). There is evidence of a localised reduction of progesterone and an increase in oestrogen resulting in an oestrogenic environment from metabolomics study (Birchenall, Welsh and López Bernal, 2022). Further to this, progesterone is also not seen to reduce in circulation at term (Vidaeff and Ramin, 2008). There are theories an increased level of deactivation occurs or alterations in receptors. In humans a reduction in progesterone receptors, but no change in oestrogen receptors is observed in labouring tissue, suggesting an increase in oestrogen receptor to progesterone receptors which may lead to a functional change in signalling at a local level (Arruda *et al.*, 2013). There are 3 types of progesterone receptors A, B and C which when progesterone binds, dimerize and translocate to the nucleus to bind specific motifs in the promoter region of progesterone responsive genes (Zakar and Hertelendy, 2007). Within the steady-state myometrium it is proposed that oestrogen receptors and inflammatory signalling balance out signalling which upregulates progesterone receptors A and B (Zakar and Hertelendy, 2007). GPCRs (e.g PGI_2) stimulate adenyl cyclase activity and increases cAMP, in turn upregulating progesterone receptors A and B (Zakar and Hertelendy, 2007). At term there is a decreased responsiveness to progesterone as the inactive receptor A is increased in comparison to B. This is suggested to be due to an increase in oxytocin, PGF_{2a} and PGE_2 that act through GPCRs to stimulate PKC and decreasing cAMP, thus, favouring isoforms A and C (Zakar and Hertelendy, 2007). The decrease in progesterone activity stimulates oestrogen receptor α and enhancing oestrogen responsive genes (Zakar and Hertelendy, 2007).

Progesterone is a driver of uterine quiescence, generally opposing the actions of oestrogen (Lamming, 1993). In the myometrium progesterone decreases contractile

activity through an increase in NO synthesis; downregulation of prostaglandins; reduced expression of CAPs and suppression of inflammation (Abel and Baird, 1980; Byrns, 2014). Generally opposing the action of oestrogen. In the cervix, progesterone increases MMP inhibitors (Kota *et al.*, 2013), preventing cervical softening and ripening.

Oestrogen itself does not directly stimulate contractions but has signalling properties that promote uterine activity and promotion of labour (Lamming, 1993). One mechanism is through enhancing oxytocin (Lamming, 1993) and increasing OXTR (Arrowsmith, Wray and Quenby, 2011). Oestrogens can further aid contractions within the myometrium by increasing enzymes responsible for muscle contraction (MLCK and calmodium), connexin-43 synthesis and gap junction formation (Vannuccini *et al.*, 2016). Oestrogen has also been seen to increase prostaglandin production (Abel and Baird, 1980) and increasing arachidonic acid (AA) liberation through phospholipase A activity, an opposing role to progesterone (Ababneh and Troedsson, 2013). Within the cervix oestrogen also contributes towards the rearrangement and dispersion of collagen in the cervical ripening process (Vannuccini *et al.*, 2016).

1.8.2.2 Corticotropin-releasing hormone

Corticotrophin-releasing hormone (CRH) is a peptide hormone. Levels of CRH are detectable form the second trimester of pregnancy and rise exponentially through pregnancy (Mclean *et al.*, 1995) increasing 50-fold in the third trimester (Lamming, 1993). CRH binding protein binds CRH and prevents it from acting and is seen greatly in excess throughout pregnancy until around 20 days before delivery as CRH levels are much higher and then decreases significantly 10 days pre-delivery (Mclean *et al.*, 1995). Combined, these lead to a surge in active CRH, acting as one of the main drivers form labour initiation. CRH acts directly and indirectly on contractility of the myometrium (Iliodromiti *et al.*, 2012). Indirectly CRH is involved in the regulation and feedback loops of hormones and other physiologically active compounds. ACTH drives oestrogen synthesis by increasing Dehydroepiandrosterone (DHEA) in the foetal adrenal gland (Lamming, 1993) which is converted to oestrogen in the placenta. Adrenal cortisol is also stimulated and creates a positive feedback loop, producing more CRH production within

the placenta (Vannuccini *et al.*, 2016). When added to placental explants increases in PGE_2 and $PGF_{2\alpha}$ are observed (Jones and Challis, 1989). CRH has been shown to increase COX2 and PGE_2 receptor subtype 1 expression, along with increasing oxytocin production from placental cells (Karteris *et al.*, 2001). Direct effects of CRH on myometrial contractility are mediated through the type of receptors expressed. CRH receptor 1 maintains the relaxed state of the myometrium through activation of adenyl cyclase and cAMP but at term, an increase in receptor 2 is seen and binding of CRH stimulates PLC/inositol triphosphate, extracellular signal-regulated kinase (ERK) 1/2 and RhoA to increase myosin light chain phosphorylation and promote contractility (Vannuccini *et al.*, 2016).

1.8.2.3 Oxytocin

Oxytocin is a neuropeptide synthesised by the hypothalamus and peripheral tissues including the lining of the uterus, corpus luteum, amnion and placenta (Arrowsmith and Wray, 2014). Although not a huge increase is seen in serum levels, its actions are believed to be due to an increase in oxytocin receptors within the uterine cavity (Lamming, 1993). Oestrogen signalling and myometrium stretching lead to increased production of oxytocin (Arrowsmith, Wray and Quenby, 2011). Progesterone has an opposing effect limiting the synthesis of oxytocin and it's receptor (Challis *et al.*, 2000). Oxytocin is the most potent natural stimulant for uterine contractions (Lamming, 1993). Methods of promoting contractions include stimulating PG production, increasing L-type calcium channels within the myometrium and calcium release (Kota *et al.*, 2013). Oxytocin has been shown to increase PG production in multiple tissues and this activity is increased in labouring tissues (Lundin-Schiller and Mitchell, 1990), which may involve the MAPK system (Arrowsmith and Wray, 2014). Oxytocin receptors are rhodopsin-type class 1 GPCRs. In the myometrium, activation of these receptors leads to $G\alpha/11$ coupling to PLC-beta, this hydrolyses Phosphatidylinositol 4,5-bisphosphate (PIP2) to Inositol triphosphate (IP3) and diacylglycerol (DAG) which activates PKC and controls mobilisation of calcium from the sarcoplasmic reticulum (Arrowsmith, Wray and Quenby, 2011) , releasing calcium and causing contractions as described in the processes of labour section.

1.8.2.4 Prostaglandins

PGs are a type of eicosanoids, a family of bioactive compounds derived from polyunsaturated fatty acids (PUFAs) (Lands, 1992). There are 3 series of PGs, series 1, 2 and 3 based on which PUFA they are derived from. Series 1 and 2 are derived from omega 6 FAs, with series 1 a metabolite of DGLA and series 2 a metabolite of AA. The third series are derived from EPA, an omega 3 PUFA (Bergström, Danielsson and Samuelsson, 1964; Lands, 1992). Series 2 are said to be the most biologically active and the most common of these are PGE_2 and $PGF_{2\alpha}$ (Bergstrom, Danielsson and Samuelsson 1964). They are produced in the placenta and fetal membranes (described in section1.10.7). PGs have a distinct and important role in labour. During labour, a rise of 2-series prostaglandins is seen in amniotic fluid (Lamming, 1993) and an increase in PG synthesis enzymes in membranes, decidua and myometrium (Vidaeff and Ramin, 2008). In human pregnancy PGE_2 administration can induce labour (Thiery *et al.*, 1974) and PG inhibitors such as indomethacin, a COX inhibitor, has been shown to prevent preterm labour (Lundin-Schiller and Mitchell, 1990).

PGs are ubiquitous inflammatory mediators (Vidaeff and Ramin, 2008) and have various effects on the uterine cavity. These actions are mediated through G-protein coupled receptors (GPCRs), classified according to highest affinity for their ligand. PGE_2 receptors are EP_1, EP_2, EP_3 and EP_4 and $PGF_{2\alpha}$ has FP (Breyer *et al.*, 2001). These have differing signalling effects due to differential coupling of these receptors to G_s ro G_i proteins (A. H. Khan et al., 2008). There are 2 categories of PG receptors, stimulatory which include EP_1, EP_3, FP and TP (thromboxane) and relaxatory DP, EP_2, EP_4 and IP (Olson, 2003). Activation of G_s stimulates adenyl cyclase, increases cAMP and induces smooth muscle relaxation (A. H. Khan et al., 2008). G_i proteins act in the opposite manner, inhibiting adenyl cyclase and thus reducing cAMP and increasing intracellular calcium, initiating contractions of smooth muscle (A. H. Khan et al., 2008). FP receptors also increase the mobilisation of calcium within the cell (Olson, 2003). Alteration of expression of these receptors can modulate uterine contractility or quiescence. In humans decreases of EP_3 and FP have been seen to be down regulated initially in pregnancy compared to a non-pregnant

uterus and then significantly increase at term labour (Olson, 2003). In the baboon decreased expression of EP_2 have been observed during labour (Olson, 2003). Progesterone has demonstrated to increase EP_2 and antagonist of progesterone to decrease EP_2 and increase FP mRNA expression in rats and in culture IL-1β has induced EP_4 expression (A. H. Khan et al., 2008).

In the myometrium PGs cause an increase in L-type calcium channels (Kota *et al.*, 2013), influencing contractions as described in processes section 1.8.1.2. PGE_2 is primarily involved in cervical ripening and collagen degradation (Kota *et al.*, 2013). This is achieved through increasing collagenase activity such as MMP-8 and 9, increasing an inflammatory influx of cells and cytokine production, and increasing progesterone catabolism and increasing CRH production (Li et al., 2021). $PGF_{2\alpha}$ upregulates oxytocin receptors (creating another positive feedback loop) and gap junctions within the myometrium (Kota *et al.*, 2013). Increased $PGF_{2\alpha}$ production within feto-placental tissues induces a functional withdrawal of progesterone through the increased expression of progesterone receptor A in myometrium (Merlino *et al.*, 2007). Both these prostaglandins also seem to be involved in the remodelling of the extracellular matrix of the foetal membranes to weaken them allowing breakage and the birth of the foetus (Li et al., 2021).

1.8.2.5 Inflammation

Inflammation is also a major driver for parturition with labour being described as a pro-inflammatory event (Bollopragada *et al.*, 2009). This inflammatory cascade within the cervix may occur well before the initiation of contractions (Vidaeff and Ramin, 2008). Before and during labour within the cervix there is an invasion of leukocytes and resident macrophages increase 10-fold (Vidaeff and Ramin, 2008). Labour has been shown to cause inflammatory changes to the uterus and inflammation has a suggested to have a role in labour. Leukocytes have been shown to infiltrate in labouring myometrium predominantly in the lower but also in the upper uterine segment (Thomson *et al.*, 1999). Neutrophils are abundant during but not before labour starts in the lower uterine section and macrophages are present before labour and increase equally in the upper and lower segments during labour (Thomson *et al.* 1999). T-lymphocytes increase in the

upper segment during labour (Thomson *et al.*, 1999). Macrophages were also seen to infiltrate the decidua before labour in mice – increasing in 12h before and during labour, whereas in the myometrium no macrophages were found before but increased during labour (Hamilton *et al.*, 2012). Biopsies from women who had spontaneous labour had significantly more inflammatory cells, specifically macrophages and neutrophils, compared to those who delivered by CS pre-labour and non-pregnant tissue (Thomson *et al.*, 1999). Placental macrophages are believed to contribute to intraplacental signalling as well as extraplacental release (Pavlov *et al.*, 2020).

Cytokines are small, secreted proteins, released by cells which act in an autocrine, paracrine or endocrine manner both synergistically or antagonistically one another (Zhang and An, 2007). There is a shift in cytokine production from a reduction in anti-inflammatory cytokines such as IL-10 and increase in pro-inflammatory cytokines including IL-1 and IL-6 at parturition (Kyathanahalli, Snedden and Hirsch, 2023). NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) has been implicated as a major inflammatory regulator at term, thought to increase the expression of pro-inflammatory cytokines, chemokines (cytokines with chemotactic activities) and adhesion molecules (Lim and Lappas, 2018). IL-10 supresses tumour necrosis factor (TNF) production (Vidaeff and Ramin, 2008). It has also been shown to inhibit TNF and IL-1 induction of COX2 and PGE_2 release within the placenta and foetal membranes (Rice, 2001).

In the third trimester basal secretions of TNF and IL-8 from placental macrophages increased by nearly 3-fold, whereas IL-11 and IL-17 significantly decreased compared to first trimester (Pavlov *et al.*, 2020). TNF and IL-1β have a feedforward mechanism by which they can increase inflammatory environment by increasing chemokine production, and cellular adhesion molecules, increasing the recruitment of leukocytes to the myometrium (Lim and Lappas, 2018). Both of these bind to a family of receptors which share the toll/IL-1 receptor (TIR) domain which facilitates a cascade of protein interactions involving IRAK kinases (Hauguel-de Mouzon and Guerre-Millo, 2006). TNF levels increase at labour and induces PGE_2 production from chorion, amnion and decidua through the increased expression of COX2 and phospholipase A_2 (Rice, 2001). MMPs are

also increased in the cervix and myometrium through TNF stimulation (Rice, 2001). IL-1 induces collagenase production, stimulates PG production and NFκB production (Iliodromiti *et al.*, 2012). IL-1 induces COX2 mRNA expression, PGE_2 release and the translocation of phospholipase A_2 from the cytosol to cell membrane where it is active (Rice, 2001). In the cervix, IL-1β induces expression of MMPs and cell adhesion molecules (Rice, 2001). An increase in COX2 and PGE_2 production is seen with decreased PGDH in trophoblasts (Challis, Patel and Pomini, 1999). IL-6 increases at term in placental tissue, chorion, amnion and lower uterine segments, correlated with contraction strength (Rice, 2001). This signals through a different cytokine receptor, dimerizing with gp130 and JAK-STAT pathway signalling (Hauguel-de Mouzon and Guerre-Millo, 2006). IL-6 enhances endometrial CRH production and MMPs (Iliodromiti *et al.*, 2012). In cultured fetal membranes IL-6 has been demonstrated to increase PGE_2 production (Kent *et al.*, 1993). MAPK acts a signalling convergence for these pro-inflammatory cytokines suggesting amplification between IL-6, TNF and adipokines such as leptin (Hauguel-de Mouzon and Guerre-Millo, 2006). Platelet activating factor, promotes calcium influx and eicosanoid and collagenase production (Vidaeff and Ramin, 2008).

NFκB signalling has been shown to be particularly important. In amnion cells, NFκB has been shown to be constituently active and increased the expression of COX2 in association with labour (Allport *et al.*, 2001). Furthermore, NFκB signalling if though to contribute to the functional withdrawal of progesterone due to repressing progesterone receptor function (Allport *et al.*, 2001). NFκB plays a functional role in progesterone withdrawal, stimulates PG synthesis and the expression of MMPs, connexin-43 and oxytocin (Iliodromiti *et al.*, 2012). IL-1β increases NFκB DNA binding (Allport *et al.*, 2001). As fetal tissues attain senescence at term, generation of a collection of cytokines and chemokines are characterised as senesce-associated secretory phenotype (SASP), which induce NFkB signalling (Kyathanahalli, Snedden and Hirsch, 2023).

1.9 Role of the placenta

1.9.1 Anatomy

The placenta is a foetal organ with maternal blood vessels forming spiral arteries, fetal blood vessels, and connective tissue including trophoblasts, forming a single thick dish attached to the uterine wall (Lamming, 1993). The chorionic plate faces the foetal side with the umbilical cord and the basal plate is attached at the maternal endometrium with an intervillous space in between (Burton and Fowden, 2015). Trophoblast cells initially form around the blastocyst to provide nutrients but then have 2 distinct cell types. First are the undifferentiated cytotrophoblasts and the second are the differentiated syncytiotrophoblasts, a specialised layer of cells that are in contact with maternal blood and are the main area of maternal-foetal communication and endocrine function (Wang and Zhao, 2010). These spiral arteries, originate from the basal plate, release maternal blood onto the trophoblasts, which have elaborately branched villous trees of foetal blood vessels forming globular lobes around each spiral artery (Burton and Fowden, 2015). Throughout pregnancy it's main role is transfer, moving water, amino acids and fatty acids (Lamming, 1993). The placenta is also a transient endocrine organ and central regulator of maternal-placental-foetal (MPF) physiology (Vannuccini *et al.*, 2016). There are 5 main classes of placental products which can all act in endocrine, paracrine and autocrine functions throughout the MPF unit via multiple receptors at different sites (Iliodromiti *et al.*, 2012). At term, it plays a key role in synchronising foetal maturation with parturition, influencing myometrial activation and stimulation via combined actions of multiple placental products (Iliodromiti *et al.*, 2012). The most likely mechanism behind the initiation of labour is in the paracrine relationships of the foetal membranes, placenta, and maternal decidua (Lamming, 1993).

1.9.2 Steroid hormones

The first class of placental product is steroid hormones. The foetal-placental unit can produce all the active steroids such as oestrogens and progestins which are important for a successful pregnancy and these enzymes change in numbers and activity throughout gestation (Smith, 2001). Progesterone is initially produced by the corpus luteum but later

in pregnancy the placenta becomes the predominant producer (Vannuccini *et al.*, 2016). Oestrogen and progesterone are produced from cholesterol via a series of enzymatic reactions as shown in Figure 1.2. The placenta is the primary source of oestrogens (Vannuccini *et al.*, 2016). Production of oestrogen in human placenta relies on androgens (Lamming, 1993). Esterone and 17b-estradiol are derived from maternal C19 androgens (testosterone and androstenendione) and estriol from C19 oestrogen precursor DHEA-S which is produced in the foetal adrenal glands (Vannuccini et al., 2016). This process is called aromatisation through the enzyme aromatase cytochrome P450 and so is essential in oestrogen synthesis (Kota *et al.*, 2013). Both DHEA and oestradiol increase towards term, despite no distinct rise in oestrogen at the start or labour (Vannuccini *et al.*, 2016). Glucocorticoids have been shown to increase oestrogen synthesis in animal models and within human placenta have been shown to increase aromatase production (Li et al., 2014).

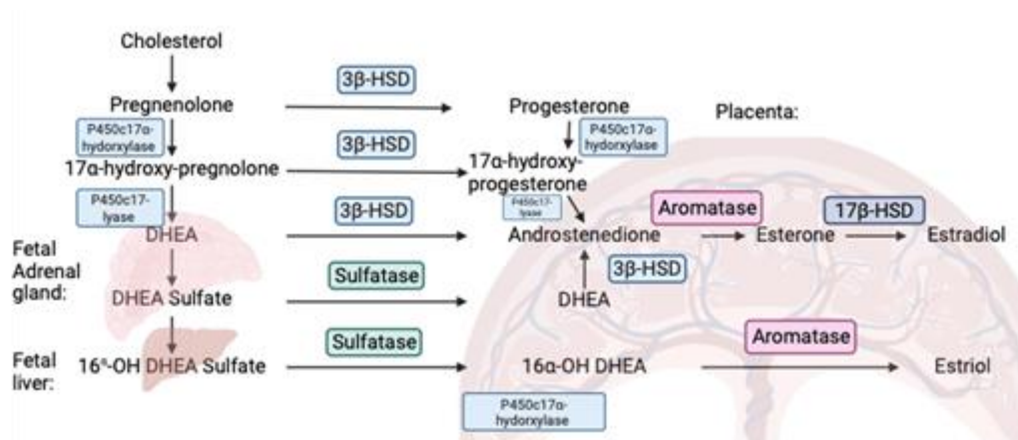

Figure 1.2 Synthesis of oestrogen and progesterone in the human (adapted form Li et al 2014). In the human cholesterol is converted to steroid precursors through a series of enzymatic reactions. DHEA is converted to DHEA sulfate within the foetal adrenal gland and then within the foetal liver it's converted to 16^{α}-OH DHEA-S. In the placenta sulfatases then remove the sulfate and DHEA is converted to esterone through a 2 step process involving aromatase which can then be converted to estradiol through 17β-HSD. 16^{α}-OH DHEA is converted to estriol through aromatase. Progesterone is also converted to esterone/estradiol through a 4 step process. Figure created in Biorender.com

1.9.3 Hypothalamic-pituitary hormones

Second are hypothalamic-pituitary hormones, including CRH and oxytocin. CRH is secreted in cytotrophoblasts, and is present in foetal blood (Lamming, 1993). It's stimulated by glucocorticoids and an increased sensitivity is seen with oxytocin (Lamming, 1993). Oxytocin production and receptors both increase in the placenta during pregnancy (Iliodromiti *et al.*, 2012). In the amnion and chorion extracellular protein kinase signal transduction pathway increases PGE_2 production and COX2 production (Arrowsmith, Wray and Quenby, 2011). Within the decidua MAPK signalling increases $PGF_{2\alpha}$ production (Arrowsmith, Wray and Quenby, 2011). There is also some evidence showing oxytocin can increase AA release (Vidaeff and Ramin, 2008). Oxytocin upregulation of $PGF_{2\alpha}$ in the decidua specifically, then acts on the myometrium to upregulate oxytocin receptors and gap junctions (Vannuccini *et al.*, 2016).

1.9.4 Growth factors

Third are growth factors including TGF, EGF and Activin-A. TGF and EGF can increase PG production although there is no clear rise in concentration of these near term (Iliodromiti *et al.*, 2012). Activin-A is seen to increase at term and enhances the production of oxytocin and PGs in a paracrine manner (Iliodromiti *et al.*, 2012).

1.9.5 Neuropeptides

Fourth, are neuropeptides NPY and NKB both of which are found to increase at labour and then fall drastically after. NPY is involved in blood flow and uterine contractility, whilst NKB causes relaxation of placental blood vessels as well as similar effects of NPY (Iliodromiti *et al.*, 2012).

1.9.6 Cytokines

Finally, the last class of placental products are cytokines. The placenta has been shown to be capable of producing all cytokines and acts as an integrative organ, relaying and enhancing (Hauguel-de Mouzon and Guerre-Millo, 2006). Syncytiotrophoblasts are the major synthesis site of IL-6 and leptin (Hauguel-de Mouzon and Guerre-Millo, 2006). White blood cells in the placenta secrete IL-6, IL-1 and TNF and IFN (Iliodromiti *et al.*,

2012). IL-1 is also produced in the decidua and endothelial stromal cells (Iliodromiti *et al.*, 2012).

1.9.7 Prostaglandins

PGs are produced in the placenta, foetal membranes, and decidua. The synthesis pathway of PGs can be seen in Figure 1.3. Phospholipids are cleaved by phospholipases (in the sn-2 position) releasing lysophospholipid and free PUFA (E. Herrera & Ortega-Senovilla, 2023). Conversion of these n3 and n6 PUFAs occurs through cyclooxygenase (COX) enzymes (E. Herrera & Ortega-Senovilla, 2023). There are 3 isoforms of COX enzyme, COX1 is constitutively expressed, COX2 has inducible expression and COX3 shares the catalytic activity of COX1 but only 20% of the activity (Chandrasekharan *et al.*, 2002). The formation of 2 series PGs is via AA conversion to PGH_2 by COX1 and COX2. From here 4 main PGs are produced. PGI_2 via the PTGIS enzyme, PGE_2 by the PTGES, 2 and 3 enzymes, $PGF_{2\alpha}$ by AKR1B1 and AKR1C3 enzymes or from PGE_2 via CBR1 and finally PGD_2 via PTGDS and HPGDS (Phillips, Fortier and López Bernal, 2014). Similar patterns of prostaglandin synthesis enzymes are seen within decidual cells, extravillous trophoblasts and syncytiotrophoblasts of the placenta, demonstrating their ability to produce PGE_2 and $PGF_{2\alpha}$ (Phillips, Fortier and López Bernal, 2014). The primary limiting step of this synthesis pathway is releasing AA which is mediated by 2 phospholipases but is also dependent on reincorporation of AA into the glycerophospholipids' through lysophosphatidic (Lamming, 1993). However, in vitro, the addition of AA does not increase the synthesis rate and so suggesting COX enzymes have a rate limiting step also (Thorburn, 1991). HPGD and CBR1 enzymes are responsible for the inactivation of PGs and SLCO2A1 for transport (Phillips, Fortier and López Bernal, 2014). PGDS (gene name HPGDS) oxidises and degrades PGE_2 and so acts as a protective barrier, not allowing PGs to reach the decidua or myometrium during pregnancy, with progesterone stimulating expression (Jones and Challis, 1989). At term, however, these levels are modestly decreased, with $PGF_{2\alpha}$ displaying negative effects on HPGDS expression (Jones and Challis, 1989).

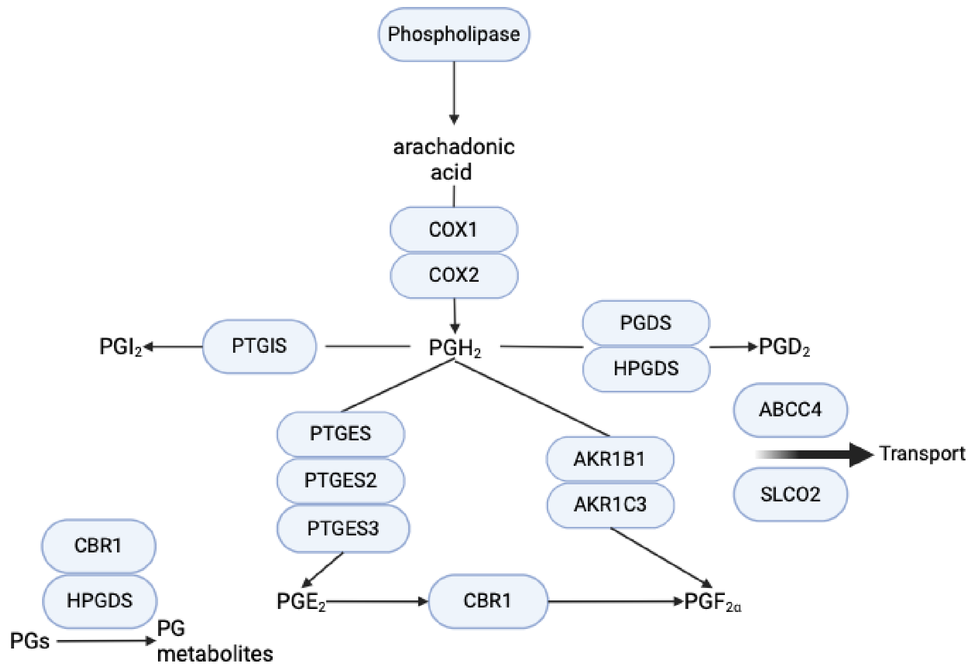

Figure 1.2 Prostaglandin series 2 synthesis, transport and inactivation. Phospholipases release arachidonic acid from the cellular membrane. COX enzymes then convert this into PGH_2. From here there are 4 possible products. PTGIS converts to PGI_2; PGDS and HPGDS convert to PGD_2; PTGES, PTGES2 and PTGES3 convert to PGE_2 and $PGF_{2\alpha}$ con be formed through CBR1 from PGE_2 or through AKR1B1 and AKR1C3 from PGH_2. HPGDS and CBR1 inactivate PGs into their metabolites. ABCC4 and SLCO2 are involved in PG transportation. Figure is adapted from Phillips et al (2014) and created in BioRender.com.

1.10 Rat

1.10.1 Anatomy

Rats have litters of around 10-16 pups, each with their own placenta (Aguilera *et al.*, 2022). Gestation tends to last from 19 to 21 days and they give birth to altricial neonates who have underdeveloped sensory and locomotor systems (Aguilera *et al.*, 2022). Trophoblasts invade into the uterine wall to facilitate blood flow into the placenta and in rats the placenta is hemotrichorial as there are 3 layers cells between the fetal and maternal blood space (Aguilera *et al.*, 2022). The rat placenta consists of three sections. The labyrinth zone which receives maternal blood and functions as the exchange site, the junctional zone which participates hormones synthesis and the yolk sac (Aguilera *et al.*, 2022). The labrynth zone sits on top of the chorionic plate and consists of maternal sinusoids, felt capillaries and trophoblastic septa which consists of the outer trophectoderm in contact with the maternal blood (cytotrophoblasts) and underneath 2 layers of syncytiotrophoblasts (Furukawa, Tsuji and Sugiyama, 2019). The junctional zone consists of spongiotrophoblasts and trophoblastic giant cells which form just below the labyrinth zone (Furukawa, Tsuji and Sugiyama, 2019). Finally, the yolk sac is an extraembryonic membrane surrounding the embryo comprised of epithelial and mesodermal cells (Furukawa, Tsuji and Sugiyama, 2019). The human and rat placenta are compared in figure 1.4. Steroidogenesis is also different between the rat and human placenta. In humans the placenta converts androgens to oestrogen in the placenta, however, in the rat the placenta provides the androgens, but these are converted into oestrogen in the ovaries (Lamming 1993).

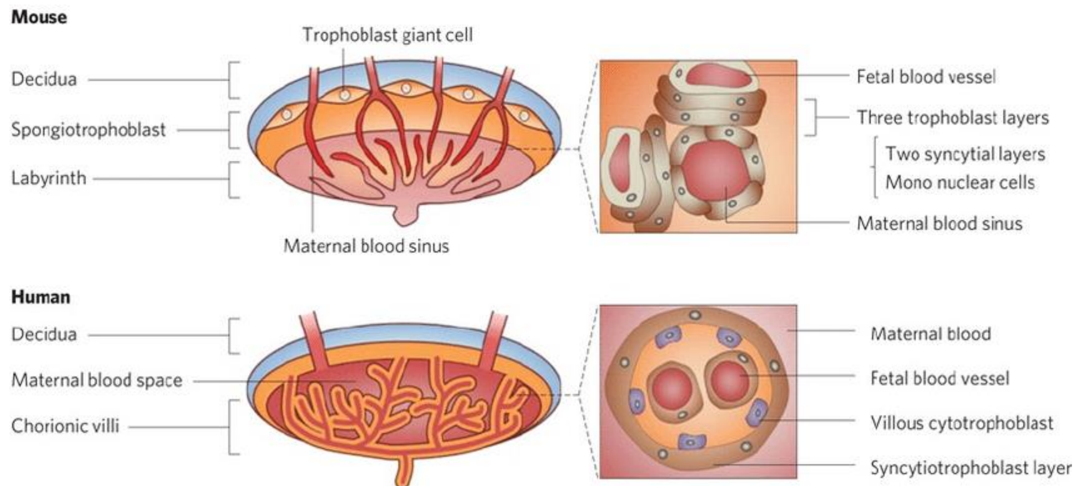

Figure 1.3 Comparison of the structure of rat and human placenta from (Sun et al.*, 2013)*.

1.10.2 Normal labour

In the rat the process of parturition is fairly clear cut and well documented. Labour is preceded by a fall in P4 production which is produced in the corpus luteum. Oestrogen synthesis occurs in the ovaries here, with the placenta providing the androgens needed for synthesis. Relaxin levels fall 24h before labour. 6-8h before, the sensitivity to oxytocin arises in the myometrium and although levels are low initially, they increase moderately when the first pup arrives. $PGF_{2\alpha}$ is the normal luteolytic signal that induces labour, and this is enhanced by oestrogen (Lamming, 1993). A study in rats identified electrical impulses that originate from each placental unit that has a specialised interface with the myometrium (Lutton *et al.*, 2018). These channels allow contractions from the placental site to the cervix through timing activations and although exact regions of these haven't been identified in humans, some potential fibres that could form similar conduits have been found (Lutton *et al.*, 2018).

1.11 Obesity potential effect on labour

As discussed in section 1.5 obesity has a demonstrated impact on labour and CS rates in particular. There are 2 main theories for how obesity affects CS rates. The first is the obstetrical dilemma. This is the theory that the babies' head has become too large for a woman to give birth without medical assistance (Wells, 2017). Obesity has shown to be associated with foetal macrosomia (Ehrenberg, Mercer and Catalano, 2004; Sheiner et al., 2004; Arrowsmith, Wray and Quenby, 2011; Briese et al., 2011) so the babies are large for their gestational age – this could mean larger heads so much harder for the woman to give birth to vaginally. Obese women tend to have a greater fat mass so this could narrow the birth canal making it more difficult and medical assistance being required (Zhou *et al.*, 2019). However, when looking at reasons for emergency CS it was found that ineffective uterine contractility and foetal stress were the main issues not obstructed labour (Cedergren, 2009). Other studies agree and have found obese women are more likely to have a CS due to arrest or delay in the first stages of labour (Kerrigan and Kingdon, 2010; Hirshberg, Levine and Srinivas, 2014) , higher incidences of arrest (Verdiales, Pacheco and Cohen, 2009) and reduced dilation and longer labour (Kominiarek *et al.*, 2011; Maged *et al.*, 2017) . Despite other studies not finding increased length of labour for obese women (Hirshberg, Levine and Srinivas, 2014; Ellekjaer, Bergholt and Løkkegaard, 2017), these studies still strongly suggest an altered mechanism or physiology involving uterine contractions. The differing contraction strengths or mis regulation could lead to the need for medical assistance and so could explain the increase in CS. It is therefore important to understand how or why this is occurring in order to provide effective solutions to midwives and mothers.

1.11.1 Normal diet vs obese diet

Optimal nutrition is also important for successful and healthy pregnancy outcomes. Pregnancy increases the mothers nutritional demand to help meet the physiological adaptations of pregnancy itself and to maintain foetal growth and development. Total energy intake is suggested to increase by 340kcal in the second and 450kcal third

trimester of pregnancy to keep up with total energy expenditure and weight gain (Picciano, 1997). Activity levels, pre-pregnancy BMI and metabolic rate all need to be accounted when considering appropriate energy consumption during pregnancy (Mousa, Naqash and Lim, 2019). This may help avoid insufficient or excessive gestational weight gain that can result in macrosomia and the obstetric complications shoulder dystocia and operative vaginal delivery associated with it (Mousa, Naqash and Lim, 2019). Protein is essential for many physiological functions such as structural support and functionally in the form of enzymes, protein transporters and hormones as well as the formation of new tissue so sufficient intake of bioavailable complete protein sources is essential. An estimated 21g/day extra is needed during the second and third trimester (Picciano, 1997), with total protein accounting for 10-25% of energy intake (Mousa, Naqash and Lim, 2019). Fat intake is important during pregnancy as essential fatty acids (FAs) are key structural components of cell membranes and are vital for tissue formation (Mousa, Naqash and Lim, 2019), along with essential fatty acids being precursors for eicosanoids which play an extremely important role in pregnancy and parturition (Mcgregor *et al.*, 2001). FAs are depleted by 40% in pregnancy so adequate intake of essential FAs are important as lack of these can result in issue such a premature birth and pre-eclampsia (Mousa, Naqash and Lim, 2019).

Several studies have looked at dietary patterns and obesity to identify patterns. These have been done across several countries, so results vary due to variations within traditional diets seen. However, some key patterns are found. A 'meat-fat' dietary pattern has been positively correlated with obesity suggesting diets high in meat and oil to be associated with obesity (Maskarinec, Tasaki and Novotny, 2000; Cho, Shin and Kim, 2011). Soft drinks, sweets and refined grains have also been positively associated with obesity within women specifically (Rezazadeh and Rashidkhani, 2010). Even in a relatively lean young Japanese female population 'Western' dietary patterns, defined as high intakes of meat, fats, oils, processed meats and eggs, was related to an increased BMI (Okubo *et al.*, 2008). This could be dependent on genetic predisposition as significant associations with a western diet (using similar definitions) was only seen within those that had a familial history of obesity but not those who did not (Paradis *et al.*, 2009).

1.11.2 Lipids

Lipids are a group of primarily hydrocarbon containing organic compounds which can be classified into simple lipids which are mostly neutral in charge and complex lipids which are polar (Sikorski and Kolakowska, 2010).

FAs are simple lipids and are characterised by their long chain of carbon atoms (Sikorski and Kolakowska, 2010). These can be either saturated or unsaturated depending on the presence of double bonds they possess and are named in this manner using the IUPAC system. This system names based on number of carbon atoms (as the first number), followed by number of double bonds being separated by a colon (Sikorski and Kolakowska, 2010). The location of the double bond is then described as an n number from the methyl end of the carbon chain or Δ if describing from the carboxyl end (Sikorski and Kolakowska, 2010). FAs can be saturated (SFA) with no double bonds, monounsaturated (MUFAs) with one double bond and polyunsaturated (PUFAs) with more than one double bond. Within this thesis we refer to double bond location from the methyl end, so using n numbers and the most common and frequently mentioned are n3, n6 and n9.

Complex lipids include acylglycerols. These consist of up to three fatty acids (acyls) as mono (one), di- (two) or tri- (three) glycerides attached to a glycerol back bone (Sikorski and Kolakowska, 2010). They represent the most common form of FAs in food and mono- or di- are often the precursors or degradation products of triacyclglycerols (TAGs) or phospholipids (Sikorski and Kolakowska, 2010). Phospholipids are similar in structure but contain only 2 acyls attached to the glycerol backbone plus phosphate group containing a variable alcohol group thus forming amphipathic molecules with both a polar and neutral portion (Sikorski and Kolakowska, 2010). Most cells have the capacity to create TAGs from fatty acids for storage with adipose, liver and intestine being most adept (Lehner and Quiroga, 2016). These are synthesised via Diacylglycerol Acyltransferases (DGAT) enzymes within the endoplasmic reticulum of cells and deposited into lipid droplets

(Lehner and Quiroga, 2016). Lipid droplets are composed of a phospholipid monolayer with a neutral lipid (mainly composed of TAG) centrally (Lehner and Quiroga, 2016).

Fats within the diet are solubilised by bile acids, transported into the intestine mucosa and broken down completely into FAs (Deuel, 1951). FAs are also present within the blood from either lipolysis of white adipose tissue or circulating lipoproteins (Lehner and Quiroga, 2016). Uptake into cells is predominantly through membrane proteins such as CD36 and fatty acid transport (FATPs) or binding (FABPs) proteins (Lehner and Quiroga, 2016). Transport around the body is mediated through lipoproteins in the blood. These structures are a complex of lipids and proteins that solubilise cholesterol, TAGs and other lipophilic molecules (G. A. Francis, 2016). These are then classified based on their density with high density lipoproteins (HDL) being the smallest and containing the most protein and low-density lipoproteins (LDL) being larger and less dense (G. A. Francis, 2016). These lipoproteins are formed in the liver and are secreted as very low-density lipoproteins (VLDL) which contain endogenous triglycerides and nascent HDL (Smith, Pownall and Gotto, 1978). Following their secretion, these undergo rapid modification through lipid and apoprotein transfer via the enzymes lecithin cholesterol acetyl transferase (LCAT) and lipoprotein lipase (LPL) producing LDL and HDL (Smith, Pownall and Gotto, 1978). These are catabolised through physical transfer and exchange of individual components or taken up as a whole into cells (either receptor mediated or by passive endocytosis) (Smith, Pownall and Gotto, 1978).

Cholesterol is a sterol which can be produced in animals and humans in a 6-step process form acetyl coenzyme A (Brown and Sharpe, 2016). It can also be taken up into cells from LDLs via LDL receptors and exported out of cells through cholesterol pumps (Brown and Sharpe, 2016). Cholesterol is important for cell membrane integrity and signalling cascade domains and is a precursor for steroid hormones (Wild and Feingold, 2023).

Most FAs in the human are obtained from diet but there is some de novo synthesis within lipogenic tissues (liver, adipose and lactating mammary glands) from excess carbohydrates to form Palmitic acid and Mystiric and Stearic to a much lesser extent

(Lehner and Quiroga, 2016). FAs can also be modified within the endoplasmic reticulum of cells to produce 16+ carbon SFAs, MUFAs and PUFAs (Bond *et al.*, 2016). Elongation is the addition of 2 carbons at a time to the backbone of the fatty acid and this is done through ELOVL enzymes, followed by a reduction, dehydration and further reduction step (Bond *et al.*, 2016). Animals have the ability to introduce double bonds into fatty acyl chains but only in a species and tissue dependent manner (E. Herrera & Ortega-Senovilla, 2023). Desaturation (insertion of double bonds at positions 4, 5, 6 and 9) take place in the endoplasmic reticulum of cells (E. Herrera & Ortega-Senovilla, 2023). Different desaturase enzymes catalyse the desaturation at different points. Δ9 desaturase, also known as SCD, is used for the conversion of SFAs to n9 MUFAs, most commonly Palmitic and Stearic acid to palmitoleic and oleic acid respectively (Bond *et al.*, 2016). Δ5 and Δ6 desaturase enzymes, also known as FADS1 and 2, catalyse the synthesis of n3 and n6 PUFAs (Bond *et al.*, 2016). Animals can only insert these double bonds at the carboxyl end of the fatty acid chain and so are unable to alter the n number of FAs. The specific production of n3 and n6 LCPUFAs are made via elongation and desaturation of 18 Carbon essential fatty acids, therefore plants and algae are needed for sources of essential fatty acids as they have the ability to insert these double bonds (E. Herrera & Ortega-Senovilla, 2023). These processes can be modulated by diet, age and hormones. Biosynthesis pathways can be shown in figure 1.5.

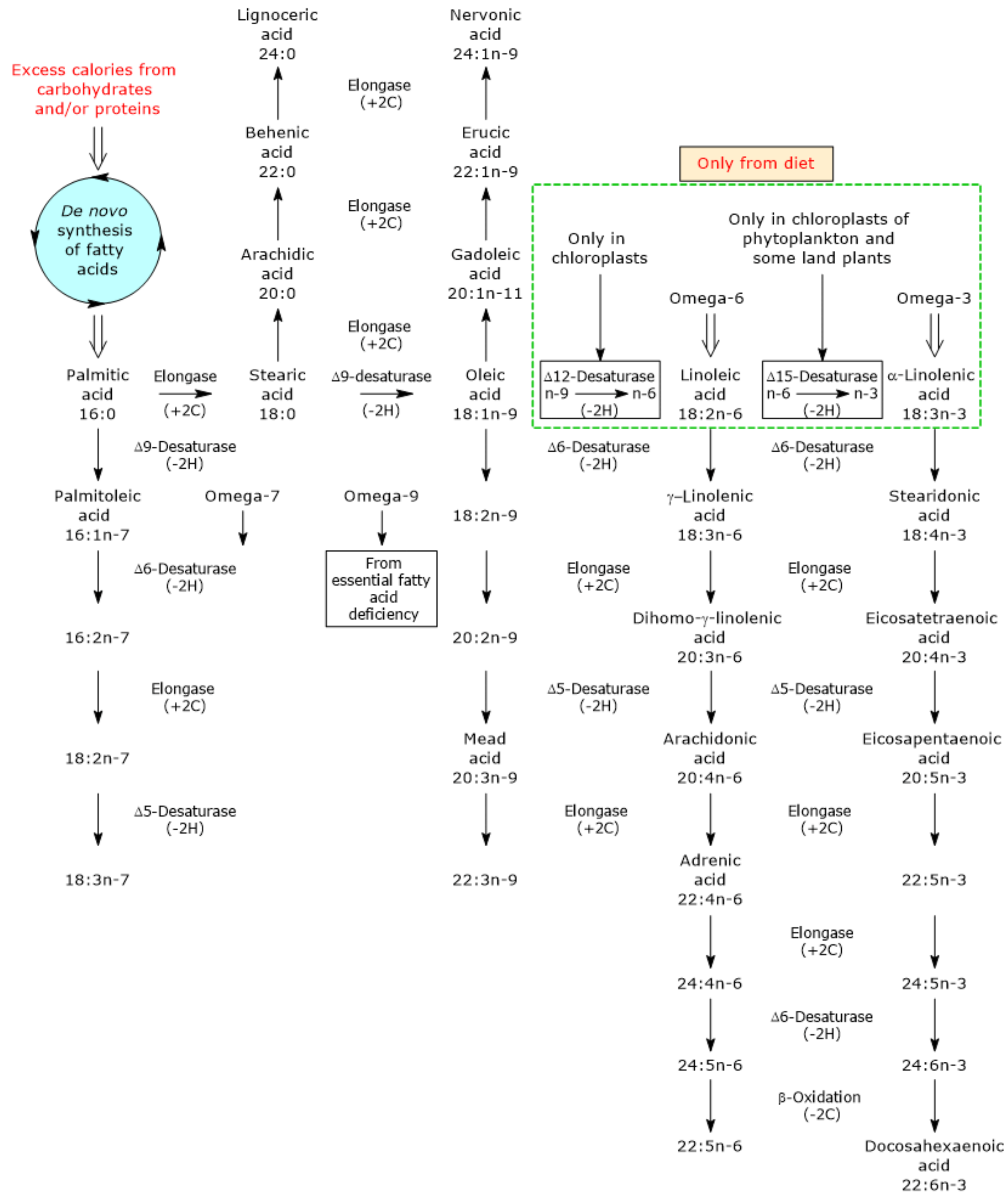

Figure 1.4 Fatty acid de novo synthesis pathways from tuscanydiet.net2013/02/03/long-chain-fatty-acid-synthesis.

1.11.2.1 Lipid metabolism during pregnancy

During the first and second trimester an increase in fat deposits and storage is seen (E. Herrera & Ortega-Senovilla, 2023), in both women and rats (E. Herrera, 2002). Glyceride glycerol and FA synthesis both contribute to FA accumulation in adipose tissue (E. Herrera, 2002). LPLs are expressed in the capillary endothelium of adipose tissue and hydrolyse TAGs within lipoproteins (E. Herrera et al., 2006). These FA and glycerol

products are then readily taken up into the adipose tissue (E. Herrera et al., 2006). During the third trimester there is a marked change in lipid metabolism. The second and third trimester sees an increase in total cholesterol (TC), LDL-C, HDL-C, TC/HDL, with lipoprotein A and AIP increasing only in the third (Lippi *et al.*, 2007) and late pregnancy sees a state of hyper triglyceridemia and smaller increases in phospholipids (E. Herrera, 2002). This shift is primarily caused by a decrease in uptake and increase in lipolytic activity within adipose tissue. Decreased LPL activity at adipose tissue reduces the uptake of those hydrolytic products (E. Herrera, 2002). The increased lipolytic activity releases non esterified fatty acids (NEFAs) and glycerol which are transported to the liver (E. Herrera et al., 2006), these NEFAs are then converted into acyl-coA and glycerol into glycerol-3-phosphate, then re-esterifed for triglyceride synthesis and incorporated into VLDL s for release into circulation (E. Herrera, 2002). Triglycerides are also seen to increase in HDL and LDL which isn't as normal (E. Herrera, 2002). Late pregnancy lipid metabolism is shown in Figure 1.5.

1.11.2.2 Transfer of lipids to the placenta

The transfer of fatty acids from maternal circulation to the foetus is via the placenta. Circulating lipids are converted into FFA by the placenta for uptake and processing (Wild and Feingold, 2023). The permeability of the placenta for fatty acids varies between species (Lamming, 1993). Generally, there is non-selective transfer except for some FATPs specific for PUFAs (Álvarez *et al.*, 2021). The rate of transfer is predominantly based on concentrations within maternal and foetal compartments and availability of carrier proteins (Lamming, 1993). Preferential uptake of long chain PUFAs have been demonstrated within placental membranes as follows: arachidonic acid> linoleic acid > a-linoleic acid > oleic acid (Campbell, Gordon and Dutta-Roy, 1996). Placental tissue expresses LPL, scavenger receptors for VLDL, LDL and HDL, LDL receptor related proteins, PLA2 as well as intracellular lipase proteins (E. Herrera et al., 2006). Placenta expresses LPL which hydrolyses VLDL in maternal circulation, releasing NEFAs (Álvarez *et al.*, 2021). These free FAs are moved into cells via flipflop mechanisms involving FABPs (Amusquivar and Herrera, 2003). This is demonstrated in Figure 1.5. The site of exchange in humans is the syncytiotrophoblasts, contained within the choronic villi, which at term are the only

continuous cell layer in contact with maternal and foetal blood (Kelly, Powell and Jansson, 2020; Álvarez et al., 2021). These cells have the apical membrane in contact with maternal blood and the basal membrane connected with foetal capillaries (Álvarez *et al.*, 2021). Fatty acid transporters FABP 1, 3, 4 and 5 are expressed which account for FA uptake into trophoblasts (Myatt and Maloyan, 2016; Lewis and Desoye, 2017). Macrophages within placental tissue also have triglyceride hydrolytic capabilities (Bonet *et al.*, 1992).

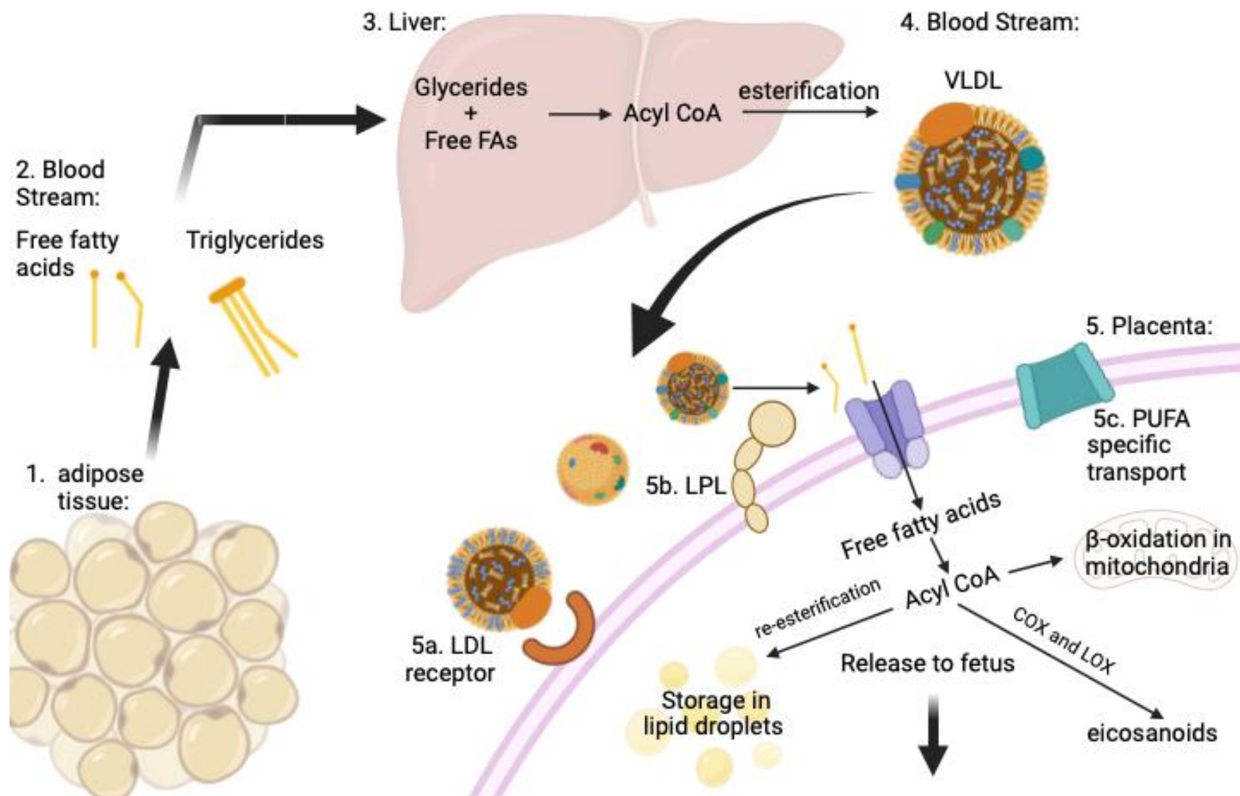

Figure 1.5 Lipid metabolism during late pregnancy. Briefly, 1. During later pregnancy there is an increase in lipolysis of adipose tissue releasing free fatty acids and triglycerides into circulation. 2. These are transporter to the liver in the blood stream. 3. Free fatty acids are combined with glycerides and form Acyl CoA before being re-esterified. 4. These are then released into the blood stream as very low density lipoproteins (VLDL). 5. Once they reach the placenta these are absorbed. 5a. this occurs through Low density lipoprotein (LDL) binding to the LDL receptor. 5b lipoprotein lipase (LPL) hydrolyses Lipoproteins, releasing free fatty acids which are then transporter into the cells through flipflop proteins or 5c. through specific PUFA transporter proteins. Upon entering placenta cells free fatty acids are then converted into Acyl CoA and re-esterified into lipid droplets for storage, release to the fetus, metabolised by COX and LOX enzymes to form eicosanoids or oxidised at the mitcohondria to produced energy. Figure created with Biorender.com

Once FAs have crossed the plasma membrane, they are ligated with co-enzyme to form acyl-coA, enter the metabolic pool. From here they have 4 possible fates: storage, energy, biological activity or transfer (Figure 6) (Lewis and Desoye, 2017). Transfer is the primary role of the placenta mediated by the hydrolysis of glycerides intracellularly and FFA diffuse into the foetal blood stream, where they are bound to alpha-fetoprotein, transported to the liver to be esterified and released again (E. Herrera, 2002). There is a biased transfer of n3 fatty acids with DHA being the most readily transferred followed by α-Linolenic and Linoleic acid, facilitated by membrane proteins (E. Herrera, 2002). Transfer can also be bi-directional so some release back into maternal blood stream is seen (Lewis, Childs and Calder, 2018). It is estimated 6% of fatty acids are immediately transferred to the fetus and rates of transfer can be affected by cellular metabolism, endothelial barrier and blood flow (E. Herrera, 2002; Lewis et al., 2018). Storage of fatty acids occurs through re-esterification to form lipid pools (E. Herrera, 2002). Within synctiotrophoblasts, lipid droplets consist of mainly triglycerides mostly with SFAs and MUFAs (Gázquez et al., 2018; Lewis, Childs and Calder, 2018). β-oxidation is the degradation pathway for long chain fatty acids in the mitochondria, producing Acetyl-CoA, fuelling the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (Houten *et al.*, 2016). β-oxidation occurs mostly in syncytiotrophoblasts due to their larger energy requirement (Shekhawat *et al.*, 2003). The bioactivity of lipids is through the conversion of FAs into eicosanoids and other FA derivatives (Lewis and Desoye, 2017). The main enzymes responsible for this are COX, lipooxygenases (LOXs) and cytochrome P450, producing PGs, thromboxanes (TX), leukotrienes (LTs) and hydroxy-eicosatetraenoic acids (HETEs) (Duttaroy and Basak, 2020). These can go on to elicit a variety of functions dependent on the precursor FA they are produced from, acting as pro- or anti-inflammatory and as ligands to PPARs influencing expression of genes involved in processes including fatty acid synthesis and oxidation, insulin sensitivity and inflammatory responses (Duttaroy and Basak, 2020). There is also evidence of de novo synthesis within the placenta for example desaturation of LA to AA, so the placenta has the ability to adapt the FFA patterns before transfer to foetus (Lamming, 1993).

1.11.2.3 Alterations to lipid metabolism with obesity

High maternal BMI has been found to alter lipid processing and metabolism systemically in pregnant women. Obese women are found to have lower TG levels in the second and third trimester and nearer to term lower cholesterol and LDL levels compared to lean women (Kelly, Powell and Jansson, 2020). Lipid oxidation is also found to be increased in mothers with greater BMI in the second and third trimester so the switch from glucose metabolism to lipid oxidation starts earlier (Bugatto *et al.*, 2017). However, the placenta of obese women has been shown to have decreased lipid oxidation and increased levels of de novo synthesis (Saben *et al.*, 2014). Further changes in lipid transfer and metabolism have also been observed in the placenta (Dubé *et al.*, 2012). Rats fed a high fat diet were found to have an increased triglyceride content of the placenta (Qiao et al., 2015; Song et al., 2017) and this is corroborated in human studies of placenta from obese women (Hirschmugl et al., 2017; Lewis and Desoye, 2017). This is further confirmed in cell-based studies, treating with NEFAs enhanced lipid droplet formation, FA esterification into triglycerides by up to 100%, and a further 10-20% into DAG, coupled with a reduction into phospholipids, glycerol and reduced oxidation rates (Pathmaperuma et al., 2010; Gázquez et al., 2018).

Maternal dyslipidaemia can alter placental function due to FAs being important for membrane synthesis and precursors for hormones, inflammatory mediators and PGs (Lewis, Childs and Calder, 2018; Louwagie et al., 2018). The availability of these free FAs for the placenta is determined by maternal plasma through current diet and past diet via the mobilisation of fat stores (Lewis, Childs and Calder, 2018). Manipulations of n3 and n6 fatty acid levels within the diet altered they FAs in membrane lipids and lipid droplets within adipose tissue (Amusquivar and Herrera, 2003). In sheep manipulation of FAs impacted steroid hormone synthesis (Mattos, Staples and Thatcher, 2000). The balance of FAs in the diet is so important due to the modulating effect LCPUFAs have on each other. N3s, in particular, have a large impact on AA metabolism through displacement and reduction in synthesis via competitive inhibition of Δ6 desaturase enzyme (Amusquivar & Herrera, 2003; E. Herrera, 2002; Mattos et al., 2000). The reverse is also

true where excessive linoleic acid can inhibit the synthesis of DHA from linolenic acid (E. Herrera, 2002).

High fat diets also effect the gene expression within the placenta. Transcriptomic studies have found alterations in the expression of around 400 genes in rats involving pathways for apoptosis, angiogenesis, lipid metabolism and anatomical size and structure regulation (Nitert et al., 2013; Lin et al., 2019; Wang et al., 2021). Obese humans also had 288 genes differentially expressed, also involving pathways of angiogenesis and lipid metabolism (Saben *et al.*, 2014). A huge range of genes including FATPs, FABPs, processing and storage genes have been looked at in tissues and cells, both in humans and different animal species with some conflicting results. LPL was demonstrated to increase in rats with a high saturated fat diet (Mazzucco *et al.*, 2013). FATPs in humans see a general increase/ positive association with BMI for FATP 1, 3 (Hirschmugl *et al.*, 2017) and FATP 2 (Lager *et al.*, 2016). However, this is not consistent across other species with FATP1 found to be reduced in rats fed a high fat diet (Ye et al., 2017; Louwagie et al., 2018). FATP4, on the other hand, is generally seen to decrease in both human (Hirschmugl *et al.*, 2017) and rat (Ye *et al.*, 2017), despite one study finding no correlation with BMI (Lager *et al.*, 2016). FABPs have been shown to be decreased in obese sows (Tian *et al.*, 2018) and FABP3 reduced in high fat fed rats (Louwagie *et al.*, 2018). However, an alternative study feeding high fat diets to mice saw an increase in FABP3 and FABPpm (Qiao *et al.*, 2015). CD36 lipid transporter was also found to decrease in high fat fed rats (Ye et al., 2017; Louwagie et al., 2018). There is also evidence, that obesity induced inflammation can regulate the expression, activity and location of FA transporters but studies show inconsistent results (Álvarez *et al.*, 2021). There is concurrent evidence that genes involved in lipid droplet formation CGI-58 and PLIN2 are increased with obesity in both tissue and cells but ACAT2 is not (Hirschmugl *et al.*, 2017). Proteomics has further backed up changes in increased lipid synthesis and energy production in obese placentas (Kelly, Powell and Jansson, 2020).

There is also some evidence maternal nutrition can affect the epigenetic status of tissues. Maternal obesity has been shown to increase DNA methyl transferase enzymes within

adipose tissue that correlates with gene repression (Myatt and Maloyan, 2016). Evidence of methylation differences has been observed specifically in third trimester placentas from women of different BMIs (Myatt and Maloyan, 2016). These changes within lipid availability, storage and utilisation will have differing effects on the placentas ability to produce lipid mediators such as prostaglandins that have implications for signalling cascades involved with labour at term. Differential gene expression in response to FA changes may also affect expression of enzymes and other proteins involved with term labouring.

1.11.3 Cholesterol

Cholesterol has been shown to have a direct effect on myometrial contraction (Shmygol, Noble and Wray, 2007; Zhang et al., 2007). Furthermore, cholesterol is important throughout pregnancy, including in the placenta, as it is part of every membrane and enriched in lipids rafts, the origin for a number signalling cascades (Woollett, 2011). Specifically, cholesterol has direct effects on oxytocin receptor function through alterations in membrane fluidity and direct binding effects (Gimpl and Fahrenholz, 2001) and this has been demonstrated directly in the myometrium (Klein, Gimpl and Fahrenholz, 1995). Cholesterol is also essential for steroid hormone synthesis (Li et al., 2014). Despite obese diets commonly being associated with increased cholesterol, several studies have failed to find differences within pregnant mothers in either circulating levels or placental membranes (Lassance *et al.*, 2015) and some finding decreased cholesterol in comparison to lean women (Kelly, Powell and Jansson, 2020). No association between elevated cholesterol levels in early pregnancy and risk of caesarean sections has been found in overweight and obese women (Fyfe *et al.*, 2013). In mice fed a high fat high cholesterol diet, total cholesterol levels within the placenta were not found to be affected and was hypothesised to be due to the increased hydrolytic activity of neutral and cholesterol ester lipases to upregulate cholesterol shuttling to foetus (Kuentzel *et al.*, 2022).

1.11.4 Uterine and cervical changes

There is evidence that the myometrium and its contents have been altered by obesity. Obese women were found to have significantly lower protein and significantly higher TG contents which could indicate a reduced muscle content in the myometrium (Gam *et al.*, 2017). However, no significant differences have been found in smooth muscle cell numbers or extracellular matrix in biopsies from normal, overweight and obese women (Sweeney *et al.*, 2014), so this effect is not clear. Differences in CAPs has also been observed in obesity including gap junction and lipid rafts proteins and COX2 in myometrial tissue from a rat model of diet induced maternal obesity (M. J. Elmes et al., 2011; Muir et al., 2016). Within obese women a reduced effectiveness of PGs was seen in cervical ripening treatment for induction of labour, suggesting an alteration in the cervix responsiveness to PGs (Beckwith *et al.*, 2017). These changes may mean the frequency and force of contractions are reduced as the myometrial tissue is less adapted for labour.

1.11.5 Hormonal changes with obesity

An alteration to progesterone production was seen in rat model of obesity. M. J. Elmes et al. (2011) found increased progesterone levels in non-labouring obese rats and Crew, Mark and Waddell (2018) found the normal cyclical decrease of progesterone overnight did not occur in obese rats but remained constant. Lower circulating levels of progesterone and oestrogen have also been demonstrated in obese women (Lassance *et al.*, 2015). Another study also found obese women to have lower progesterone in the first and third trimester when compared to lean women but in a foetal sex dependent manner (Maliqueo *et al.*, 2017). If progesterone is already lower, then a functional decline at term would be smaller and could make this change insignificant.

Insulin sensitivity is decreased towards the end of gestation to increase the supply of glucose to the foetus and increasing lipolysis to increase free FA supply (Jeve, Konje and Doshani, 2015). Blood glucose and insulin changes in pregnancy are known to be exacerbated in overweight and obese women (Roland *et al.*, 2020). Obese rat models have been found to have reduced insulin sensitivity (Bazzano *et al.*, 2018). Increased insulin and impaired glucose tolerance is also seen rats fed a HFHC diet (Song et al.,

2017). Insulin has a myriad of effects but in terms of lipid metabolism it drives fatty acid storage (Elchalal et al., 2005; Lewis and Desoye, 2017) and has an impact of VLDL production in the liver (E. Herrera, 2002). Insulin resistance has been hypothesised to further disrupt normal lipid metabolism, increasing VLDL and small dense LDL formation ectopic fat accumulation (Bozkurt *et al.*, 2016). Increased insulin resistance may exacerbate issues with altered fatty acid profiles including inflammation and reactive oxygen species (Carlson, Hernandez and Hurt, 2015).

An increase in hepatocyte growth factor has been observed in the amniotic fluid of obese women, this again leads to increased esterification and de novo synthesis with a decrease in oxidation in cells (Visiedo *et al.*, 2015). Adipose tissue secretes adipokines and with increases in adipose tissue with obesity, this can become dysfunctional and can have demonstrated impacts on smooth muscle function (AlSaif, Mumtaz and Wray, 2015). Increased leptin but decreased adiponectin throughout pregnancy was also demonstrated in obese rats (Song et al., 2017). Leptin has a potential influence during labour as receptor mRNA has been found to increase with pregnancy in placenta (Zhao *et al.*, 2004) and myometrium (Chien *et al.*, 1997). In myometrial contractility bath work leptin was found to induce a small inhibitory effect in both spontaneous and oxytocin induced contractions (Moynihan *et al.*, 2006). Leptin was found to increase pro-inflammatory cytokines and PGs in both placenta and maternal adipose (Lappas, Permezel and Rice, 2005). Adiponectin receptors have been found in myometrium of both mouse and human and in organ bath work activation of these potently inhibited contractility (Vyas *et al.*, 2019) and Vistafin has reduced both the amplitude and area under the curve in in vitro studies of human myometrial contractility, more potently than leptin (S. Mumtaz et al., 2015). Apelin has demonstrated inhibitory effects on myometrial contractions in vitro from pregnant tissue (Hehir, Glavey and Morrison, 2008). Grehlin is inversely related to BMI, modulatory effects have been demonstrated on myometrial contractility, however these are very inconsistent and so inconclusive (AlSaif, Mumtaz and Wray, 2015).

For labour specific placental hormones, a decrease in placental CRH was found in obese women (Saben *et al.*, 2014). As CRH is a major contributor to the initiation of labour, having effects on oxytocin, PG production and the myometrium (Iliodromiti *et al.*, 2012), lower levels could result in alterations in this process. A reduced sensitivity to oxytocin is also found with obesity. Muir et al. (2016) found obese rats required greater doses of oxytocin to elicit the same contractile response as their lean counterparts. In humans, obese women were more likely to need oxytocin (Walsh, Foley and O'herlihy, 2011; O'Dwyer et al., 2013) and in significantly higher doses (Pevzner et al., 2009; Carlson, Corwin and Lowe, 2017). Walsh, Foley and O'herlihy (2011) also identified that greater BMI reduced success with oxytocin used for induction of labour. This suggests a reduced responsiveness of obese women to oxytocin. The mechanism behind this is unclear as studies have found no alterations in oxytocin receptor mRNA or protein expression in myometrium between different BMI groups (Grotegut *et al.*, 2013). This is also not a clear-cut issue as maximum oxytocin doses did not change in obese women, instead was suggested they just need more time to progress through first stage of labour (Chin *et al.*, 2012). Oxytocin is a potent contractile stimulator (Iliodromiti *et al.*, 2012) this may also contribute to reducing contractile functionality and thus prolonging labour.

1.11.6 Placental structure changes with obesity

As previously discussed, rats fed a high fat diet saw altered expression of genes involved in placental size and structure (Wang et al., 2021). The morphology of the rat placenta in obesity has been studied frequently. Obese dams typically have decreased labrynth zone (Song et al., 2017; Wang et al., 2021) and increased junctional zones (Lin *et al.*, 2019). Peng et al (2022) also found a high fat diet to negatively impact the vascularity of the placenta, finding a reduced number of capillaries with uneven distribution within sparse and disordered trophoblasts (Peng *et al.*, 2022). This could all impact the nutrient uptake and exchange within the placenta as the junctional zone is the site of exchange within the rat. Some have also reported heavier placentas with reduced efficiency (Borengasser *et al.*, 2014), however, others have found no change (Wang et al., 2021; Rodríguez-González et al., 2022). There is also some evidence of altered mitochondrial dynamics of fusion and fission (Borengasser *et al.*, 2014). Altered uptake of nutrients, particularly

fatty acids, may affect the precursors available to the placenta that could impact its ability to produce molecules and signals essential for preparing the uterus for labour.

1.11.7 Inflammatory changes with obesity

Obesity is often associated with an increase in systemic inflammation; however, the literature is inconsistent in pregnant women. As reviewed by Kelly, Powell and Jansson (2020), there are a number of reports finding no significant elevation in maternal circulating cytokines in obese pregnant women, suggesting this may not be a general phenomenon. The placenta has been described as particularly susceptible to inflammation and oxidative stress (Zhang et al., 2023), however, this is not consistent in all studies with large variation in responses with foetal sex and gestational age (Crew, Waddell and Mark, 2016). In rats a high fat diet has been commonly seen to increase IL-6, IL-1β and TNFα within the placenta between gestational day 18-21 (Hsu et al., 2020; Ludidi et al., 2023; Zhang et al., 2023). A cafeteria diet, feeding highly palatable foods, observed no increase in inflammation in maternal, placental or foetal compartments near term despite increased adiposity (Crew, Waddell and Mark, 2016). In sheep an increase inflammation was seen in the placenta suggested to be the result of TLR4 and free FAs (Zhu *et al.*, 2010). In humans a trend towards increased inflammation was seen in obese placentas, however, this was insignificant (Saben *et al.*, 2014). Maternal BMI has been shown to be positively correlated with MCP-1 and TNF in circulation and with IL-1b expression in the placenta (Aye *et al.*, 2014). Cytokines in maternal plasma were also found to be elevated with increased BMI and potentially activate p38-MAPK and STAT3 in the placenta (Aye *et al.*, 2014). Some studies suggest an increase in TNF from excess adipose tissue to be the perpetuator of this increased inflammatory state (M. Yuan et al., 2001). This has also been suggested in rats, as a result of a high fat diet (Song et al., 2018). An inflammatory surge is seen at the onset of labour and is important in many positive feedback loops involved in parturition so alterations to inflammatory regulation during pregnancy may impact this clear and distinct change.

1.11.8 Reactive oxygen species (ROS)

A lipotoxic environment is often associated with reactive oxygen species (ROS), alongside inflammation (Mandò *et al.*, 2018). Free FAs in the blood can generate ROS when oxidised (Eastman *et al.*, 2021). This is also suggested to come from an excess of intracellular TGs which impact mitochondrial efficiency leading to an accumulation of electrons to form superoxide radicals (Jarvie *et al.*, 2010). Ordinarily antioxidants within a cell would neutralise these but with increased FAs this process can be overwhelmed and cause cellular and DNA/RNA damage (Carlson, Hernandez and Hurt, 2015). Oxidative stress in the placenta can affect vascularity, trophoblasts apoptosis, proliferation and differentiation (Myatt and Cui, 2004). Insulin resistance can exacerbate this further due to the increased drive in storage, so in pregnancy and obesity this may be heightened (Carlson, Hernandez and Hurt, 2015). Lipoperoxidation was increased in obese dams but not ROS (Rodríguez-González *et al.*, 2022). Obesity has been shown to have mitochondrial disfunction in several tissues and is often tied to oxidative stress (Eastman *et al.*, 2021). Human studies have found an increase of oxidative stress within the placenta of obese women (Hastie and Lappas, 2014; Mele et al., 2014).

It has been demonstrated obesity has distinct changes on a body wide and cellular scale. Increased adipose tissue, as a results of increased energy intake induces hormonal changes which increase systemic inflammation. The majority of work so far has focused on the direct changes within the myometrium due to the obvious alterations to contractility. As detailed in the section 1.9, the placenta plays a key role in the initiation and continuation of labour predominantly through the production of hormones, cytokines, and PGs. Altered FA intake has been seen to alter the circulating lipids as well as how these lipids are taken up, stored and utilised, particularly within the placenta. Understanding the effect obesity has on these, may elucidate further mechanisms and potential therapeutics, to identify and aid women with greater BMIs who may experience difficult labours.

1.12 Aims and hypothesis

The overall aim of this thesis is to evaluate the impact obesity has on the placenta. It is hypothesised alterations in the maternal environment will alter the physiology of the placenta resulting in disruptions to the normal initiation and progression of labour.

Chapter 3 aims to quantify alterations in the FA and gene profile of the placenta in response to a HFHC diet in the rat model of diet induced maternal obesity. Following this to see if the changes observed in the rat are a good model for what is seen in obese mothers. It is hypothesised that significant alterations to the fatty acid profile would be induced and that these changes would negatively affect important processes in labour such as prostaglandin production and pro-inflammatory responses.

The findings in chapter 3 saw a significant increase in MUFAs, particularly Oleic acid in response to the HFHC fed rats with increased enzyme activity ratios seen in both rat and human. Chapter 4 aimed to investigate the effects increased physiological levels of Oleic acid had on pro-labour mediator production in trophoblast cells. It was hypothesised that increased exposure to oleic acid in both quantity and time would decrease the production of prostaglandins, inflammatory cytokines and genes involved in their synthesis pathways and regulation.

Previous research has clearly demonstrated the negative implications of both maternal obesity and NCDs on pregnancy outcomes, individually. However, due to the nature of their combined prevalence, the final chapter aimed to investigate the impact this may have on the risk associated with obesity. It was hypothesised both obesity and related NCDs would have negative effects on pregnancy outcomes and that NCDs would modulate the extent of the risk of obesity, in a region where both are rife. Finally, the smaller prospective cohort study aimed to evaluate the role of maternal diet on pregnancy outcomes in humans. It was hypothesised that decreased dietary guideline adherence and increased food groups such as saturated fat would have negative effects on pregnancy outcomes.

2 Methods

Equipment	Details	Brand
Centrifuge	Microfuge 22R Centrifuge	Beckman Coulter Life Sciences, Indianapolis, USA
Vortex	Clifton Cyclone Vortex Mixer	Nickel-Electro Ltd. Weston-super-mare, UK
Microplate reader	FLUOstar Omega	BMG Labtech, Ortenburg
MARS software	V4.01 R2 2021	BMG Labtech, Ortenburg
96-well plate		
BioPulverizer	59012 MS BioPulverizer	BIOSPEC, OK, USA
Ethanol	Ethanol absolute	Fischer Scientific, Loughborough, UK
1.5ml Eppendorfs	Natural non-sterile polypropylene Graduated RNAse and DNAse free tubes	Alpha laboratories, Hampshire, UK

Table 2.1 Frequently used equipment mentioned multiple times throughout the methods.

2.1 cDNA synthesis

cDNA synthesis: All samples were stored at -80°C until required. RNA concentration and quality (260/230 ratio of 2-2.2 considered pure) was determined using a Nanodrop 2000 (Thermo Fisher Scientific, Loughborough, UK) and all RNA diluted to 100ng/ µl in RNAse free water (Thermo Fisher Scientific, Loughborough, UK) in 1.5ml Eppendorf tubes. cDNA was synthesised using a Thermo RevertAid First strand cDNA synthesis kit (ThermoFisher Scientific, Baltics, UAB) using random hexamer primers. Briefly, the kit was taken out the --80°C freezer and thawed to room temperature. A mixture of 60uM Random hexamer primers and 500ng RNA were made up to 13ul with PCR-grade water into thin walled 96-well plates (96-well semi-skirted PCR plates, Thermo Scientific). The 96 well plate was incubated for 5 minutes at 65°C then immediately put on ice. A mix of reaction buffer at 1x final concentration, 1mM Deoxynucleotide mix, 20 units RiboLock RNase inhibitor and 200 units RevertAid RT were added to each well. The plate was then incubated at 25°C for 5 minutes, 42°C for 1 hour and the reaction then terminated by heating to 70°C for 5 minutes before cooling to 4°C. All heat cycling was done on the Thermal block cycler

(GeneAmp PCR system 9700, Applied Biosystems, CA, USA). 100ul of nuclease free water was added to each well before freezing at -20°C.

2.2 Primer design

Primers: All primers were designed using Primer express software (Version 3.0.1, applied Biosystems by ThermoFisher Scientific, California, USA) using the TaqMan Quantification parameters as detailed in Table 2.2. All primers that were designed were checked for specificity and cross reactivity using a Basic Local Aligment Search Tool (NCBI BLAST, National Library of Medicine, MD, USA). Primers were ordered from Pure and simple primers, (Merck, Sigma ALderich, Haverhill, UK All primer sequences are shown in table 2.3.

2.3 Quantitative reverse transcription polymerase chain reaction (RTqPCR)

RTqPCR was carried using the LightCycler 480 II (Roche Diagnostics Ltd. West Sussex, England) and SYBR Green I detection format (Roche Diagnostics GmbH, Mannheim, Germany). The run protocol is shown in table 2.4. Reaction were set up on white LightCycler 480 Multiwell 384-well plates (Roche Diagnostics GmbH, Mannheim, Germany), with total volume of 15 µl per sample.7.5 µl SYBR Green I (2x), 0.45 µl forward and reverse primers (10uM) and 1.6 µl. All cDNA and original RNA samples were run with a reference gene (GAPDH for rat and YWHAZ for human) to check for suitability for analysis and no contamination of genomic DNA. All samples within 2 standard deviations of the average crossing point (CP) were included in a neat cDNA pool which was serially diluted up to 1:128 and used as standard curve. All cDNA samples were diluted to 1:8, to fit centrally within the curve. Samples were run with forward and reverse primers for each gene of interest with standard curve dilution, non-template control (NTC) and samples run in triplicate. Melt curves were checked for singular product and the mean CP calculated from triplicates. Stability of the reference gene was checked across all samples. All samples were normalised against the reference gene and shown as relative gene expression. For the cell study, no stable reference gene could be found so OliGreen was used as a measure of cDNA. This was stable across all treatment and time groups. All samples were subsequently normalised against OliGreen.

Primer Tm	
Min Primer Tm	58
Max Primer Tm	60
Max Difference in Tm of Two Primers	2
Primer GC Content	
Min Primer %GC Content	30
Max Primer %GC Content	80
Max Primer 3' GC's	2
Primer 3' End Length	5
Primer 3' GC Clamp Residues	0
Primer Length	
Min Primer Length	9
Max Primer Length	40
Optimal Primer Length	20
Primer Composition	
Max Primer G Repeats	3
Max Num Ambig Residues in Primer	0
Primer Secondary Structure	
Max Primer Consec Base Pair	4
Max Primer Total Base Pair	8
Primer Site Uniqueness	
Max % Match in Primer	75
Max Consec Match in Primer	9
Max 3' Consec Match in Primer	7
Probe Tm	
Min Probe Tm	68
Max Probe Tm	70
Probe Length	
Min Probe Length	13
Max Probe Length	30
Probe Composition	
Max Probe G Repeats	3

Max Num Ambig Residues in Probe	0
No G at 5' End in Probe	TRUE
Select Probe with more C's than G's	TRUE
Probe Secondary Structure	
Max Probe Consec Base Pair	4
Max Probe Total Base Pair	8
Amplicon	
Min Amplified Region Tm	0
Max Amplified Region Tm	85
Min Amplified Region Length	50
Max Amplified Region Length	150

Table 2.2 Primer design parameters input to Primer Express Software

Gene name	Sequence
RAT	
ER1	F: CCCAGCTCCTCCTCATCCTT
	R: GCACGACATTCTTGCATTTCA
CYP17	F: CCTGCACCGGAAGTTGGT
	R: GGCTTCCTGACAGATTAGCTTCTC
PPARγ	F: TGACTTGGCCATATTTATAGCTGTCA
	R: CGATGGGCTTCACGTTCAG
PPARα	F: CCCAAGTTTGACTTCGCTATGA
	R: GCCGATCTCCACAGCAAATTATA
SIRT1	F: GCAGGTTGCAGGAATCCAAA
	R: ACCGAGGAACTACCTGATTAAAAATATC
NFκB	F:GCTTACGGTGGGATTGCATT
	R: CATCACCCATGGCACCATAA
p65	F: ACCTGGAGCAAGCCATTAGC
	R: CTGCTCCTCTATGGGAACTTGAA
SCD18	F: CTGGAGTACGTCTGGAGGAACAT
	R: ATATCCCCCAGAGCAAGGTGTA
COX1	F: CATGGGAACCAAAGGGAAGA
	R: GCGGGAATGAACTCCCTTCT
COX2	F: GAAGAACTTACAGGAGAGAAAGAAATGG
	R: CAGCAGGGCGGGATACAGT
AKR1B1	F: TCTACCTTATTCACTGGCCAACTG
	R: TGCTCCATAGCCGTCCAAGT
HPGDS	F: TGGTGGATACCCTGGACGAT
	R: GTCATTGAACGTCCGCTCTTTT
PTGES	F: GCTGCGGAAGAAGGCTTTT
	R: CTGGGTCACTCCTGCAGTACTG
SLCOA1	F: TCAAGAGCAGTCTCACCACAATC
	R: TAGCGTTGCTGATCTCATTCAAAC
PTGIS	F: ACCACTCAGCCGACGTTTTC
	R: CTTTATCCCCCACTGACAAGGA
ABCC4	F: ATTGAGGCTCTCCGGCTAAGTAA
	R: CAAACTTGTTCACGTCGTTGGA
Human	
CRH	F: AGGCACCGGAGAGAGAAAGG
	R: CCTGGCCATTTCCAAGACTTC
PPARγ	F: CAACAGACAAATCACCATTCGTTAT

	R: GGATGGCCACCTCTTTGCT
PPARα	F: CAAGATCCAGAAAAAGAACAGAAACA
	R: AAACGAATCGCGTTGTGTGA
Aromastase	F: AAGGCATCATATTTAACAACAATCCA
	R: TGAGGGATTCAGCACAGACTGT
COX2	F: AATTGCTGGCAGGGTTGCT
	R: TGCGGTACTCATTAAAAGACTGGTA
PTGES	F: CGACCCCGACGTGGAA
	R: GGACCCAGAAAGGAGTAGACGAA
EDN1	F: TCCCTGATGGATAAAGAGTGTGTCT
	R: AACGTGCTCGGGAGTGTTG

Table 2.3 Forward and reverse Primer sequences for Rat and Human primers used in PCR experiments. Designed in Primer Express software. Parameters detailed in Table 2.2.

TEMP °C	ACQUISITION	HOLD	RAMP RATE °C/S	ACQUISITON PER °C
PRE-INCUBATION				1 Cycle
95	None	5 min	4.8	
AMPLIFICATION				45 Cycles
95	None	10s	4.8	
60	None	15s	2.5	
72	Single	15s	0.11	
MELT CURVE				1 Cycle
95	None	5s	4.8	
65	None	1min	2.5	
97	Cont.		0.11	5
COOLING				1 Cycle
40	None	10s	2	

Table 2.4 PCR cycle parameters for LightCycler 480 run on all PCR experiments

2.10 Statistical analysis

All data was processed on excel. GraphPad Prism was used to produce all figures. All null hypothesis were rejected when $p < 0.05$.

Tissue: Mean, median and SEM and all statistical test were calculated on SPSS version 23 (IBM). Kolmogorov-Smirnov test was used for normality testing. For rat study non-parametric t-test Mann-Whitney U were used to calculate the difference between diet groups. For human work non-parametric one-way ANOVA Kruskal Wallis test were used.

Cells: Mean and SEM and all statistical tests were calculated on Genstat. General ANOVA were used to look at effects of treatment, time and treatment*time interaction, blocked by plate. Post-hoc Bonferoni multiple comparisons were performed for treatment and time where significance was found and were displayed as letters.

3 Fatty acid analysis and gene expression analysis of rat and human placental tissue

3.1 Introduction

Maternal obesity is often accompanied by an imbalance in FA uptake commonly due to a poor-quality diet and overconsumption of foods rich in saturated fats and a reduction in seafoods (Rifas-Shiman et al., 2009; Álvarez et al., 2021). It has been demonstrated that the diet quality of overweight and obese pregnant women further declines particularly through a reduction in MUFAs and PUFAs (Moran *et al.*, 2013). This poor-quality diet correlates with FA found in the circulation and accompanied by increased fat mass (Eastman *et al.*, 2021).

Alteration in circulating FAs is not exclusively an energy change but can alter uterine, ovarian, placental function and pregnancy events through changes in steroid and eicosanoid secretion via multiple mechanisms (Mattos, Staples and Thatcher, 2000). The 20 carbon fatty acids arachadonic acid (AA), dihomo-gamma-linolenic (DGLA) and eicosapentaenoic acid (EPA) are precursors to prostaglandins and availability of these, along with their short chain PUFA precursors, will directly impact synthesis rates (Smith, Urade and Jakobsson, 2011). Furthermore, other FAs have modulating effects on PG production through inhibition, competition and activation of enzymes involved (Dong et al., 2016; Mibe et al., 1992; C. Yuan et al., 2009; Zou et al., 2012). FAs regulate the inflammatory process (Wendell, Baffi and Holguin, 2014) and can induce oxidative, endoplasmic reticulum stress and inflammasome activation (Eastman *et al.*, 2021). These FAs can also act on a cellular level through a direct impact of gene transcription (Mattos, Staples and Thatcher, 2000; Eastman et al., 2021).

Previous work on our established rat model of maternal obesity focused on the impact of a HFHC diet on the FA composition of maternal plasma, liver and uterus (Muir *et al.*, 2018). Exposure to the HFHC diet significantly increased total MUFA, decreased total PUFA and increased the n3:n6 ratio. Of key importance, oleic acid (OA) was significantly increased almost 3-fold in HFHC fed rats and the n-6 fatty acid AA and n-3

docosahexaenoic acid (DHA) levels halved. A similar pattern was seen in the liver, but total SFA was found to be significantly decreased in the HFHC group suggesting that the higher intake of saturated fat via the HFHC diet is being converted to MUFAs. Interestingly the uterus, was particularly resistant to any diet induced changes. Only total n3 PUFAs were found to be significantly lower in the HFHC group (Muir *et al.*, 2018). Other rat models have found a correlation between plasma FA levels and placental fatty acid composition indicating a dietary influence on the placenta (Amusquivar and Herrera, 2003). Genomic changes in response to high fat diets have also been demonstrated in pathways ranging from lipid processing, angiogenesis, inflammation and FA metabolism (Nitert et al., 2013; Louwagie et al., 2018; Shrestha et al., 2020).

Research on human pregnancy has focused on the FA profiles of the plasma and erythrocytes from the first trimester to the trimester third (Pinto et al., 2015; Araujo et al., 2020). A large Spanish study has looked at individual FAs in plasma and found changes associated with BMI, sedentary lifestyle and lower education to influence total SFA, MUFA and PUFAs, where obesity was specifically shown to be a factor affecting the pro-inflammatory FAs (Martín-Grau *et al.*, 2021). Maternal obesity has also been demonstrated to induce changes in lipid storage and movement within the placenta (Dubé et al., 2012; Hirschmugl et al., 2017; Draycott et al., 2020), where placentae from obese mothers are characterized by lipid accumulation, increased storage and esterification (Alvarado et al., no date; Calabuig-Navarro et al., 2017).

3.1.1 Aims

The aim of this study was to understand how maternal obesity affects the placenta. Firstly, to assess the impact on the fatty acid composition of the rat and human placenta. Secondly, to determine whether maternal obesity and the associated altered FA profile, impact on expression of key genes involved in the mechanism of parturition. It was hypothesized that dietary induced obesity would have a similar effect on the placenta as it did in the plasma and liver of our translational rat model and having an overweight or obese BMI prior to pregnancy would have a similar impact. The genes hypothesized to be altered with obesity were those particularly centred on prostaglandin production (COX1

and COX2, PTGES, PTGIS and AKR1B1 along with transport and activation enzymes), steroid synthesis and signalling (CYP17 and ER1 in rats and CRH and aromatase in human) and inflammation (PPARγ, NFκB, p65 and SIRT1) which could have negative implications on the birth outcomes.

3.2 Methods

3.2.1 Animal study tissue

All rat placental samples were taken from a previous study as detailed in Elmes et al (2011). All animal experiments were performed under The University of Nottingham Guidelines and approved by The University of Nottingham Animal Welfare Ethical Review. Within the animal facilities at the University of Nottingham, twenty virgin Wistar rat dams (Harlan Ltd, Belton, Leics, UK) weighing ∼60 g were pair-housed and randomly assigned to be fed either a CON (standard laboratory chow, B&K Universal Ltd, Hull, UK, n=10) or a HFHC (n=10) diet for 6 weeks. Rat dams were then mated naturally with Wistar stud males, and pregnancy was confirmed through the appearance of a semen plug on the cage floor. The pregnant rats were then housed individually and maintained on their CON or HFHC diets throughout gestation until birth at 22 days of gestation. Daily food intake and weight gains were recorded prior to and during pregnancy. At gestational day 20, hourly checks were made for signs of parturition, and following the birth of the first pup, each rat dam was immediately killed by CO2 asphyxiation and cervical dislocation. The breakdown of the ingredients to make HFHC diet is referred to in Elmes et al., (2011) as shown in Table 3.1 and the fatty acid profile of the diet is shown in Table 3.2 from Muir et al (2018). The placenta was dissected out, weighed and snap frozen in liquid nitrogen then stored at -80°C until further analysis.

Constituents	*g/kg*
Corn oil	100
Casein	200
Maize starch	218
Butter	295
Sucrose	100
Cellulose	50
Vitamin mix	5
Mineral mix	20
Methionine	10
Choline	2
Cholesterol	10

Table 3.1 Composition of the high fat high cholesterol diet used to induce obesity with components given in g/kg from Elmes et al (2011)

	CONTROL (%)	HFHC (%)
Total Saturates	20.2	46.3
14:00	0.4	7.7
15:00	0.1	0.8
16:00	14.8	26.8
17:00	0.2	0.5
18:00	3.8	9.9
20:00	0.3	0.3
22:00	0.3	0.1
24:00:00	0.2	0.1
Total Monos	20.9	29.9
16:1n-7	0.4	1.5
18:1n-9	19	27.6
18:1n-7	1.1	0.7
20:1n-9	0.4	0.2
22:1n-9	0	0
Total Omega 9	19.4	27.7
18:1n-9	19	27.6
20:1n-9	0.4	0.2
Total Omega 7	1.5	2.2
16:1n-7	0.4	1.5
18:1n-7	1.1	0.7
Total Omega 3	6	0.8
18:3n-3	6	0.7
Total Omega 6	52.8	19.8
18:2n-6	52.7	19.6
20:2n-6	0.1	0
20:3n-6	0	0.1
20:4n-6	0	0.1
n-6/n-3 ratio	8.8	24.75

Table 3.2 Fatty acid composition of the control and high fat high cholesterol diet (HFHC) from Muir et al (2018)

3.2.2 Human tissue collection

Ethical approval for the study was obtained from the Derbyshire Research Ethics Committee (Ref: 09/H0401/90). Placental samples were obtained from patients attending the Department of Obstetrics and Gynaecology, Royal Derby Hospital, Derby, UK. Patients provided informed, written consent prior to undergoing elective caesarean section at term gestation (>37 weeks), indications for which were maternal request, previous elective section or breech presentation while cases with diabetes, hypertension and pre-eclampsia were excluded. Placentae, once checked by the midwife and with the cord clamped, were transported to the lab within 20 minutes of delivery, where a section of placental trophoblast layer were dissected and frozen at −80 °C prior to extraction of RNA, protein or lipids. Participants were stratified based on a BMI measurement taken during an antenatal clinic appointment, resulting in three groups of women: BMI <25 kg/m2 (n = 13), BMI 25–30 kg/m2 (n = 7) and BMI >30 kg/m2 (n = 14). Subset of these women were used in fatty acid analysis BMI <25 kg/m2 (n = 9), BMI 25–30 kg/m2 (n = 7) and BMI >30 kg/m2 (n = 10).

3.2.3 Direct Fatty Acid Methyl Ester (FAME) synthesis

Frozen rat and human placental tissue was crushed into a fine powder under liquid nitrogen. 100 to 400mg of tissue was transferred into glass tubes for FAME synthesis. Direct FAME synthesis is based on the method developed by O’Fallon et al 2007 and is based on the concept that the base used in the procedure is strong enough to hydrolyze the fatty acids structures so they can be methylated without the need for organic extraction first (O’Fallon et al., 2007). 700µl of 10M KOH (560g potassium hydroxide pellets Sigma-aldrich, MO, USA to 1L distilled water) and 5.3ml of methanol (VWR BDH Chemicals, Briare, France) were added to the glass tubes containing the tissue, each sample was vortexed for 30 seconds. The samples were then incubated in a water bath (NE1-2.5 Unstirred Thermostatic water bath, Clifton, Nickel-Electro Ltd, Weston supermare, UK) at 55°C for 90 minutes with a 5 second vortex every 20 minutes for 3 rounds before being left for the final half an hour. The sample tubes were then cooled in an ice bath for 10 minutes. Next, 580µl of 12M H_2SO_4 was then added to each tube before vortexing again for 30 seconds. Samples were then returned to the water bath

again for another 90 minutes following the same vortex procedure and again cooled in an ice bath for 10 minutes. Once complete 1ml of Hexane (VWR BDH Chemical, Briare, France) was added to each sample and vortexed for 30 seconds, and then centrifuged at 2,000 rpm for 10 minutes (Fisherbrand GT4R Centrifuge, Thermo Fischer Scientific, Germany). The resulting top layer of hexane was removed and placed into solvent resistant tubes and stored at -20°C.

3.2.4 Gas chromatography Fatty acid analysis

FAMEs were removed form the -20 °C freezer, inverted a few times to homogenize the samples and 500µl was added to glass Gas chromatography (GC) vials (2 ml crimp top vials, Thermo Scientific) and sealed with screw cap lids (9mm screwcap orange silicon/ white PTFE septa, chromatographydirect.com, Northants, UK). FAME37 standards (Supleco37-Component FAME mix, Tracecert Sigma) were serially diluted from neat ½, ¼, 1/8, 1/16, 1/32, 1/64 and 500 µl placed in the glass vials. The 7 standards, hexane (as a negative control), all samples followed by another 7 standards were all loaded onto the GCFID machine carousel. 1µl was injected onto the GCMS (GC: Trace 1300, MS: Thermo Scientific ISQ 7000, Thermo Scientific, Milan, Italy). Conditions for the GC were as follows: column (CP-Sil 88, Agilent, 100 m, 0.25 mm, 0.20 µm), 260°C inlet temperature, split ratio of 1:10 for standards calibration, 1:50 for samples, oven held at 140°C for 5 minutes before increasing at 4°C/min until 240°C and then held for 10 minutes. Conditions for the MS were as follows: MAS transfer line temperature set to 250°C and the ion source temperature set to 200°C. Compounds were identified using GC retention time and MS scan data quantified using Chromeleon™ software (Thermo Scientific). The gas chromatography analysis was optimized and maintained by Nutritional Science technician Louise Williams and Dongfang Li. Chromeleon software calculates the amount of each of the 37 fatty acids measured and calculates them as a percentage of the total amount of fatty acids present (relative amount %). Total SFA, MUFA, PUFA, n 3 and n 6 were calculated by adding the relative amounts of the relevant fatty acids together for each sample.

3.2.5 RNA extraction

Samples were crushed under liquid nitrogen using a Biopulverizer.

Rat tissue: RNA was extracted from 20-25mg of crushed tissue using a Roche High Pure RNA Isolation tissue Kit (Roche Diagnostics Ltd., Burgess Hill, UK) according to manufacturer's instructions. Tissue was disrupted and homogenized in 300 µl Buffer RLT using pipetting. 400 µl Lysis-Binding buffer was added and vortexed for 15s, this homogenized and lysed tissue solution was added to upper reservoir of filter tube, inserted into a collection tube. The entire assembly was then centrifuged for 15 s at 8000 x g. Flow through liquid was then discarded and 90 µl DNase and 10 µl DNase I (Roche Diagnostics, GmBH, Manheim, Germany) solution per sample were combined, mixed and added onto the glass filter fleece of the filter tube. This was incubated for 15 min at room temperature. 500 µl of wash buffer **I** was then added to the upper reservoir of the filter tube assembly and centrifuged for 15 s at 8000 xg. Flow through was discarded and filter tube combined with the used collection tube. 500 µl wash buffer **II** added to the upper reservoir of the filter tube assembly and centrifuged for 15 s at 8000 x g. Flow through again was discarded and the filter tube combined with the used collection tube. 200 µl washer buffer **II** was then added to the upper reservoir of the filter tube assembly and centrifuged for 2 min at 13000 x g to remove any residual wash buffer. The filter tube was inserted into a clean 1.5ml microcentrifuge (Natural non-sterile polypropylene Graduated RNAse and DNAse free tubes, Alpha Labooratories, Hampshire, UK) and 50-100 µl of elution buffer was then added to the upper reservoir of the filter tube to elute the RNA. The tube assembly was centrifuged the tube assembly for 1 min at 8000 x g.

Human tissue: 1ml Qiazol lysis buffer (QIAGEN science, USA) was added to approximately 100mg of crushed placental tissue it was thenthoroughly mixed by pipetting up and down and left to incubate on the bench top for 5-10 minutes at room temperature. 200 µl chloroform (AnalR, NORMAPUR, VWR, BDH chemicals) was then added and vigorously shaken for 15 seconds, before being left for 2-3 minutes at room temperature. The lysed placental samples were then centrifuged at 12,000xg for 15 minutes at 4°C. The resulting upper aqueous solution was then transferred to a new tube and 500 µl isopropanol

(VWR International S.A.S, Rosny-sous-Bois-cedex, France) added and mixed by vortexing for 15s. The tubes were left on benchtop for 10 minutes before undergoing centrifugation at 12,000xg for 10 minutes at 4°C. The resulting supernatant was then aspirated and discarded and 1ml 75% ethanol added before further centrifugation at 7500xg for 5 mins at 4°C. The resulting supernatant was then removed completely, and the pellet left to air dry at room temperature for 2-5 mins. The dried RNA pellet was re-dissolved in 50 µl of Nuclease-free water (SIGMA-Aldrich, Saint Louis, USA) and added to filters from Roche High Pure RNA Isolation tissue Kit (Roche Diagnostics Ltd., Burgess Hill, UK). 10 µl of DNAse 1 recombinant was combined with 90 µl DNAse reaction buffer (Roche Diagnostics GmbH, Mannheim, Germany) per sample were combined and mixed by pipetting. 100 µl of this solution was added to each sample and incubated for 1 hour at room temperature. From this point, the procedure from the kit was followed from adding wash buffer 1 step as detailed in the rat RNA extraction.

3.2.6 RTqPCR

Primer design, cDNA synthesis and PCR method are detailed in section 2.1-2.3.

Target (°C)	95
Acquisition	Continuous
Ramp Rate (°C/S)	Auto set at 0.11
Acquisitions (per °C)	5

Table 3.3 Parameters for OliGreen cDNA quantification assay on LightCycler480

3.3 Results

3.3.1 Rat placenta

The pooled placenta tissues from rats used in this study was obtained from a previously published trial as detailed in Elmes et al (2011). From the original trial, rat dams exposed to the HFHC diet had a significantly higher weight at the end of the pregnancy, but similar gestational weight gain, gestational length, litter number, offspring weight and sex ratios. The gestation and birth information for these rats are shown in Table 3.4.

Information on Dams		*Control*		*HFHC*	
		Average	**SEM**	**Average**	**SEM**
End of pregnancy weight (g)		298.85	± 9.75	321.37	± 3.04
GWG (g)		94.27	± 5.93	110.33	± 4.16
Gestational length (days)		22.1	± 0.9	22.46	± 0.23
Litter number (n)		11.13	± 1.09	10.14	± 0.94
Litter weight (g)		61.23	± 5.7	54.46	± 4.54
Sex of pups	Female (n)	6		6	
	Male (n)	5		4	

Table 3.4 Information on rats used in the study for both control and HFHC groups detailing gestational and birth data

3.3.2 Fatty acid profile of the rat placenta

The FA profile of placentae from rats fed either a control or HFHC diet is shown in table 3.5. It is important to note that fatty acids are expressed as a percentage of total FAs and based on the 37 FAs we were able to detect. The 6 most abundant FAs in the rat placenta are palmitic (C16), stearic (C18), OA (C18:1n9c), linoleic acid (LA) (C18:2n6c), AA (C20:4n6) and DHA (C22:6n3) making up 95-97% of the total FAs measured. The relative amounts of these FAs for both dietary groups combined are shown in Figure 3.1. As expected, exposure to a HFHC diet had a significant impact on the fatty acid profiles of

the placenta, although some of these are found in very small amounts e.g making up less than 1% of total FAs.

Placentae from obese rat dams had significantly lower SFA ($p = 0.005$), PUFA ($p < 0.001$), N3 ($p = 0.001$) and N6 ($p = 0.007$) FAs than placentae from lean control rats (Table 3.5 and Figure 3.2 A, C, D, E). In contrast MUFAs were found to be 2-fold higher in the HFHC fed rats compared to CON ($p < 0.001$) (Table 3.5 and Figure 3.2 B), almost entirely due to an increase in the MUFA OA ($p < 0.001$) (Table 3.5 and Figure 3.3 C). Looking at individual SFAs, palmitic and stearic acid were both significantly lower in HFHC fed ($p = 0.017$ and $p = 0.001$ respectively) (Table 3.5 and Figure 3.3 A-B.). The n6 PUFA LA ($p = 0.003$) and n3 PUFA DHA ($p = 0.002$) were both significantly higher in control compared to HFHC fed rats (Figure 3.3 D-E.). The n6:n3 ratio was also calculated from total n6 and total n3 PUFAs, and exposure to the HFHC diet was found to significantly increase the n6:n3 ratio from 6.83 in CON to 14.15 in diet induced obese rats ($p = 0.001$). Estimates of desaturase enzyme activities were calculated as a ratio of product: precursor (see figure 3.4). SCD18 activity was estimated from OA: stearic acid ratios and was found to be significantly higher in HFHC fed rats ($p < 0.001$). Similarly, SCD16 activity was estimated through the ratio of palmitoleic: palmitic acids and activity was two-fold higher in HFHC fed rats ($p = 0.001$). The long chain n6 PUFA AA is produced from LA via a 3-step process. First LA is desaturated to γ-LA via Δ6D and then elongated to form DGLA that is subsequently desaturated by Δ5D to form AA. Δ6D activity, calculated by DGLA: LA ratio was found to be significantly increased in the HFHC rats ($p = 0.001$). AA: DGLA ratio was used as a measure of Δ5D and was found to be significantly reduced in HFHC fed rats ($p = 0.001$). Finally, OA: AA ratio was used to study the relationship between the two fatty acids and was found to be significantly higher in HFHC fed rats compared to control ($p = 0.001$), although this was not a linear relationship (figure 3.5).

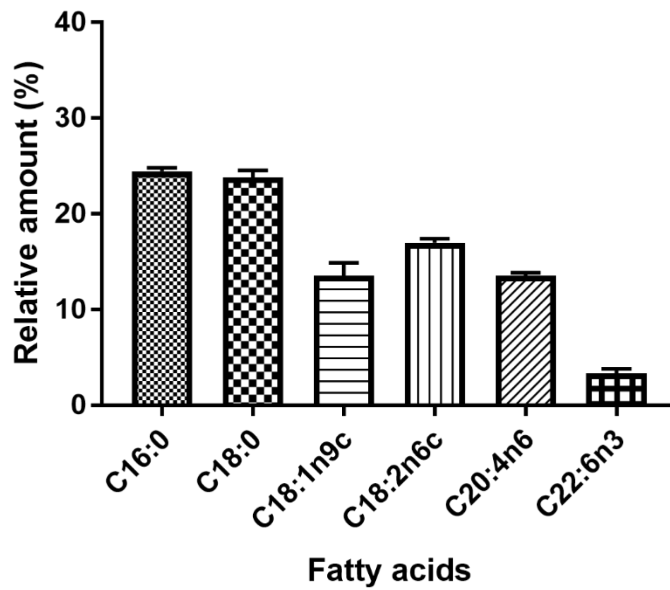

Figure 3.1 Fatty acid profile of the pooled rat placentae across both control n=6 and HFHC n=7 groups expressed as a percentage of total FAs (mean with SEM). Includes only those fatty acids that made up over 1% of total fatty acids. C16 = palmitic, C18 = stearic, C18:2n6c cis-linoleic, C20:4n6 = arachidonic, C18:19nc = cis oleic and C22:6n3 = docosahexanoic acid.

Fatty acid		*CONTROL*		*HFHC*		
		% total fatty acid	SEM	% total fatty acid	SEM	p-value
Caproic	C6:0	0.004	±0.001	0.004	±0.001	0.867
Caprylic	C8:0	0.003	±0.001	0.003	±0.001	0.363
Capric	C10:0	0.002	±0.001	**0.004**	±0.001	**0.078**
Undecanoic	C11:0	0.002	±0.001	0.002	±0.001	N/A
Lauric	C12:0	0.009	±0.002	**0.036**	±0.002	**<0.01**
Tridecanoic	C13:0	0.002	±0.001	0.003	±0.001	N/A
Myristic	C14:0	0.258	±0.006	**1.034**	±0.035	**<0.01**
Pentadecanoic	C15:0	0.120	±0.004	**0.284**	±0.007	**<0.01**
Palmitic	C16:0	**25.360**	±0.198	23.700	±0.358	**0.006**
Heptadecanoic	C17:0	0.307	±0.010	**0.428**	±0.009	**<0.01**
Stearic	C18:0	**26.000**	±0.592	21.971	±0.335	**0.001**
Arachidic	C20:0	0.156	±0.003	**0.202**	±0.006	**<0.001**
Heneicosanoic	C21:0	0.004	±0.001	**0.005**	±0.001	**0.03**
Behenic	C22:0	**0.077**	±0.003	0.062	±0.001	**0.018**
Tricosanoic	C23:0	0.007	±0.001	0.006	±0.002	0.486
Lignoceric	C24:0	**0.120**	±0.004	0.095	±0.005	**0.008**
Total SFA		**52.352**	±0.709	47.776	±0.593	**0.020**
Myristoleic	C14:1	0.011	±0.002	0.018	±0.003	0.17
Pentadecanoic	C15:1	N.D	N.D	0.012	±0.002	N/A
Palmitoleic	C16:1	0.520	±0.014	**0.985**	±0.048	**<0.01**
Heptadecanoic	C17:1	0.197	±0.020	0.212	±0.020	0.686
Oleic	C18:1n9c	8.523	±0.362	**17.791**	±0.279	**<0.001**
Eicosenoic	C20:1	0.101	±0.004	**0.154**	±0.010	**0.005**
Nervonic	C24:1	0.072	±0.014	0.042	±0.002	0.219
Total MUFA		9.475	±0.386	**19.956**	±0.314	**<0.001**
Linoleic	C18:2n6c	**18.215**	±0.337	15.933	±0.323	**0.002**
Linolenic	C18:3n6	0.045	±0.003	**0.058**	±0.002	**0.003**
DGLA	C20:3n6	0.431	±0.010	**0.663**	±0.023	**<0.001**
Arachidonic	C20:4n6	13.762	±0.295	13.337	±0.410	0.503
Total n6 PUFA		**32.493**	±0.379	30.035	±0.535	**0.010**
3n3 Linolenic	C18:3n3	**0.112**	±0.004	0.048	±0.003	**<0.001**
ETE	C20:3n3	0.214	±0.008	0.211	±0.006	0.896
EPA	C20:5n3	**0.043**	±0.002	0.031	±0.002	**0.006**
DHA	C22:6n3	**4.850**	±0.150	1.828	±0.077	**<0.001**
Total n3 PUFA		**5.219**	±0.156	2.119	±0.251	**<0.001**
Eicosadienoic	C20:2	**0.352**	±0.006	0.254	±0.009	**<0.001**
Docosadienoic	C22:2	0.042	±0.001	**0.047**	±0.002	**0.009**
Euricic	C22:3n9	0.014	±0.002	0.009	±0.002	0.175
Total PUFAs		**38.120**	±0.441	32.464	±0.463	**<0.001**
n6:n3		6.83		14.15		**<0.001**

Table 3.5 Fatty acid profile of the rat placenta. Mean relative amounts of each fatty acid calculated and identified on Chromeleon from FAME37 GCMS analysis. Bold indicates p<0.05, and highest mean relative amount, grey indicates all significant values with a greater than 1% relative abundance. N/A means the amounts were too low to be detectable.

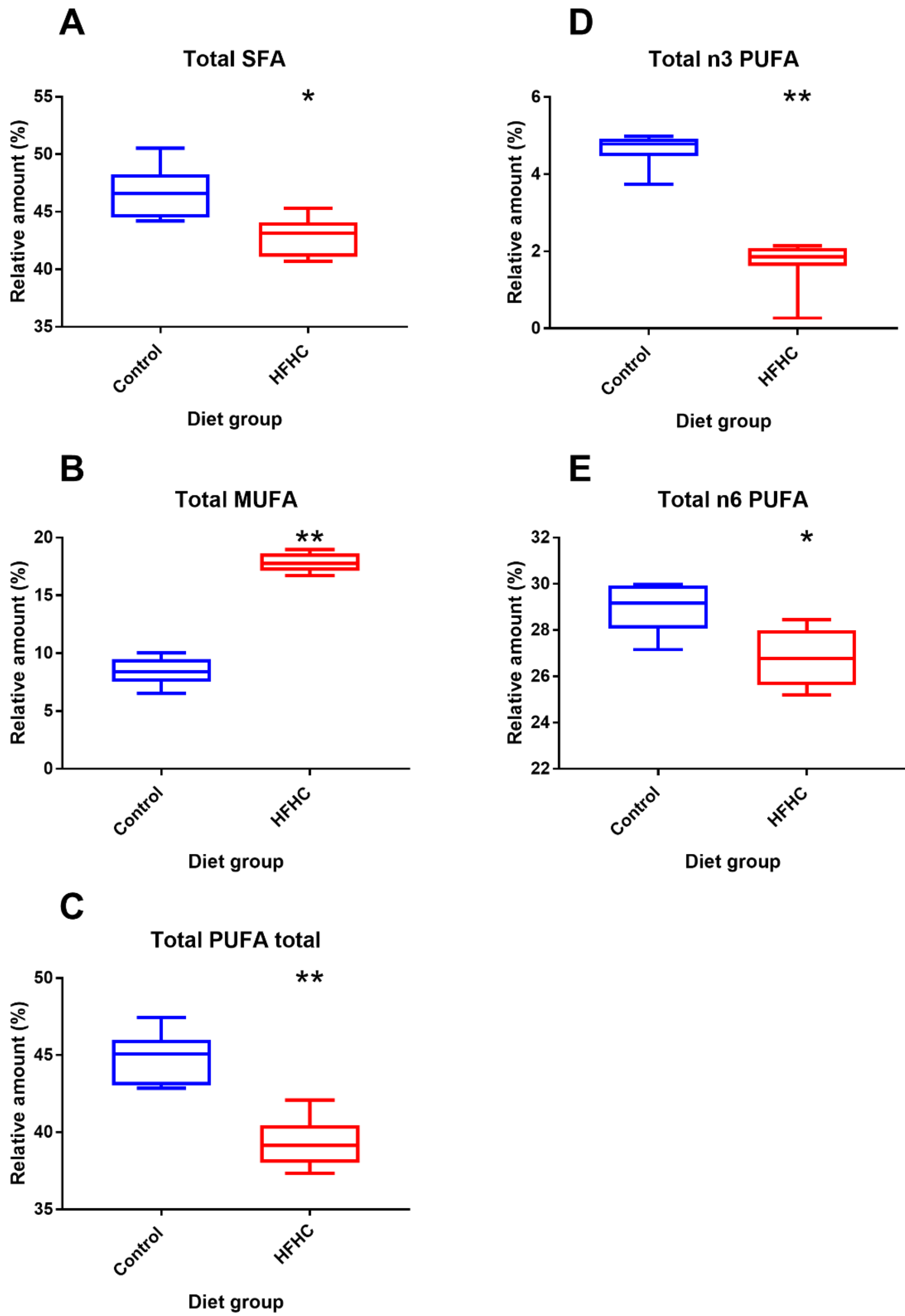

*Figure 3.2 Total Saturated, Monounsaturated, Polyunsaturated, n3 and n6 fatty acids displayed as median, interquartile range and upper/ lower range, averaged by diet group (control n=6, HFHC n=7). * indicated p < 0.05 **indicated p < 0.001*

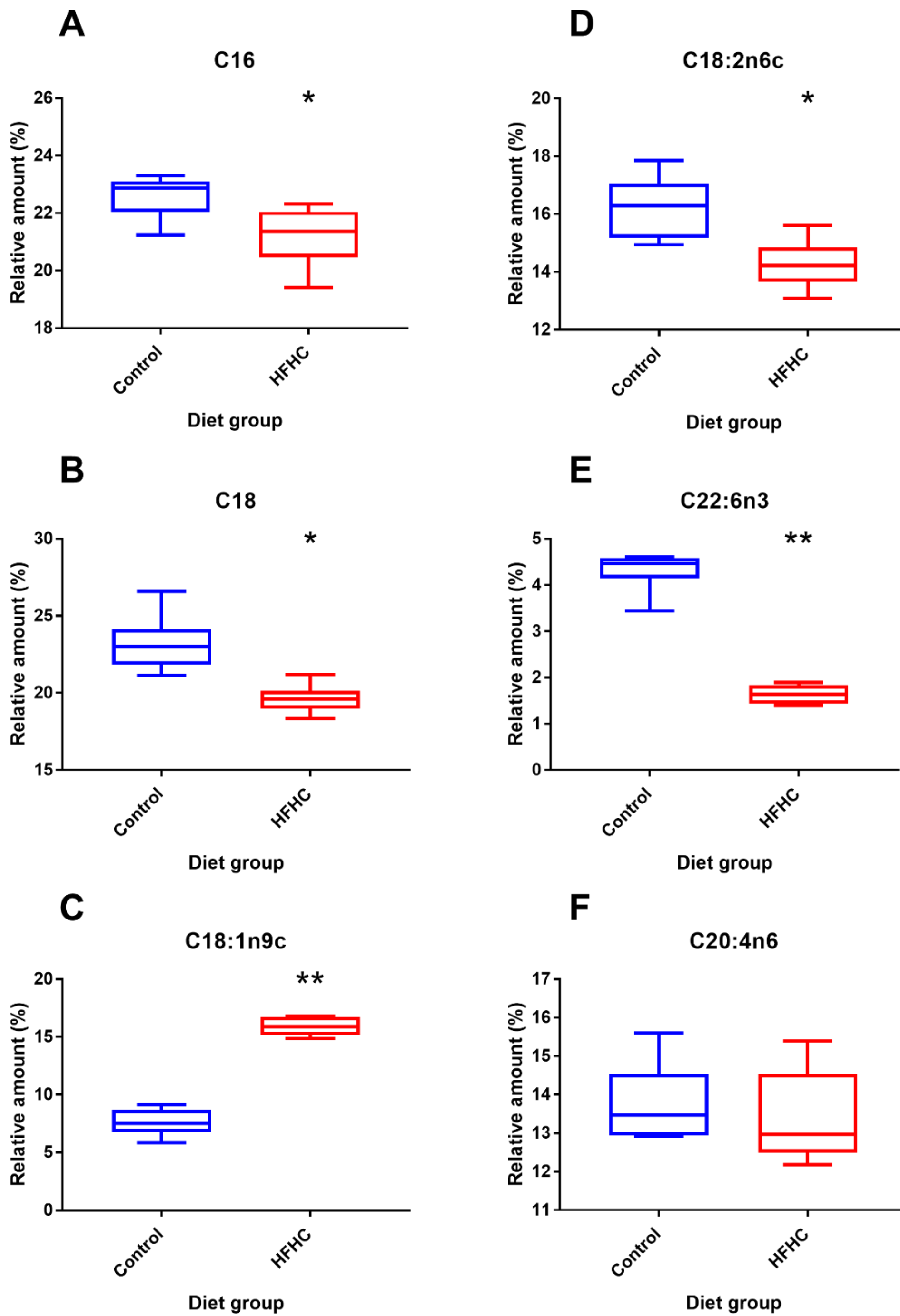

*Figure 3.3 The six most abundant fatty acids (that made up over 1% of total fatty acids) displayed as median, interquartile range and upper/ lower range, averaged by diet group (control n=6, HFHC n=7). * indicated p < 0.05 **indicated p < 0.001. C16 = palmitic, C18 = stearic, C18:2n6c cis-linoleic, C20:4n6 = arachidonic, C18:19nc = cis oleic and C22:6n3 = docosahexanoic acid.*

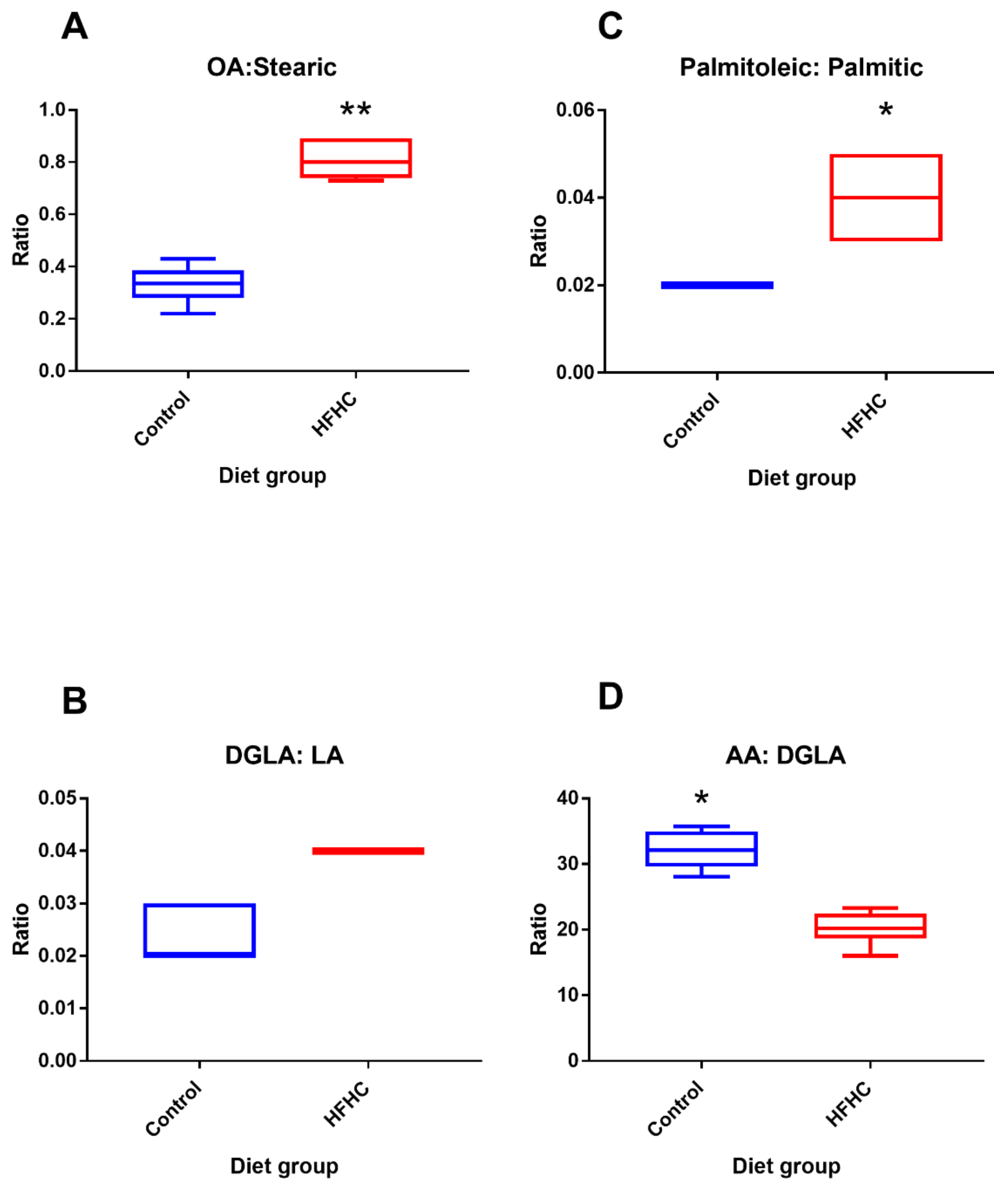

*Figure 3.4 Desaturase enzyme activity estimates calculated as ratios from product: precursor, displayed as median, interquartile range and upper/ lower range, averaged by diet group (control n=6, HFHC n=7). OA:stearic is SCD18, palmitoleic:palmitic is SCD16, AA:DGLA is Δ5D and DGLA:LA is Δ6D. * indicated $p < 0.05$ **indicated $p < 0.001$*

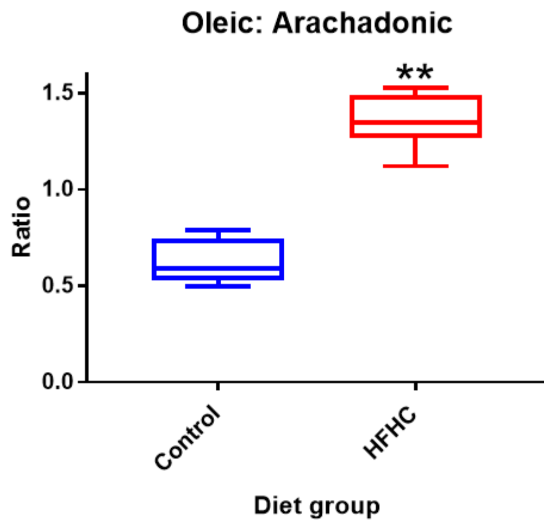

*Figure 3.5 OA to AA ratio displayed as median, interquartile range and upper/ lower range, averaged by diet group. * indicated p<0.05 **indicated p<0.001.*

3.3.3 Human placenta

Placentae were collected from women delivering their baby via elective CS at term from the Royal Derby Hospital (n=26). The demographics of these women including information on previous and current birth data is presented in Table 3.6. The average age of the women was 34 years old, with a mean BMI of 28.6. The mean gestational age of the offspring was 38.8 weeks, with no offspring born before 37 weeks gestation. 80.0% of offspring weighed between 2500 to 4000g, none were classified as low birth weight but 4 (15.4%) were classed as macrosomic. Birthweight grouped by maternal BMI (Figure 3.6) was not found to have any significant association. The most common reason for elective CS was previous CS for all BMI groups, followed by breech birth. Failed IOL was also reported but only within the obese BMI group.

Information on Mothers	Number of women (%)		
Age (mean =34)	37+	8	30.77%
	28-36	15	57.69%
	<27	3	11.54%
BMI (mean = 28.6)	Underweight	0	0.00%
	Normal	9	34.62%
	Overweight	7	26.92%
	Obese	10	38.46%
Previous births history			
Parity	0	5	19.23%
	1	17	65.38%
	2	4	15.38%
Gravida	1	5	19.23%
	2	12	46.15%
	3	4	15.38%
	4	2	7.69%
Current birth data			
Sex	Female	12	46.2%
	Male	13	50.0%
Gestational age at delivery	Less than 28	0	0.0%
(Mean =38.8)	28-31	0	0.0%
	32-36	0	0.0%
	37+	26	100.0%
Gestational weight	<2500g	0	0.0%
(Mean = 3487.6g)	2500g-4000g	21	80.8%
	>4000g	4	15.4%

Table 3.5 Demographic information, current and previous birth data from women used in the study, demonstrated as frequency and percentage.

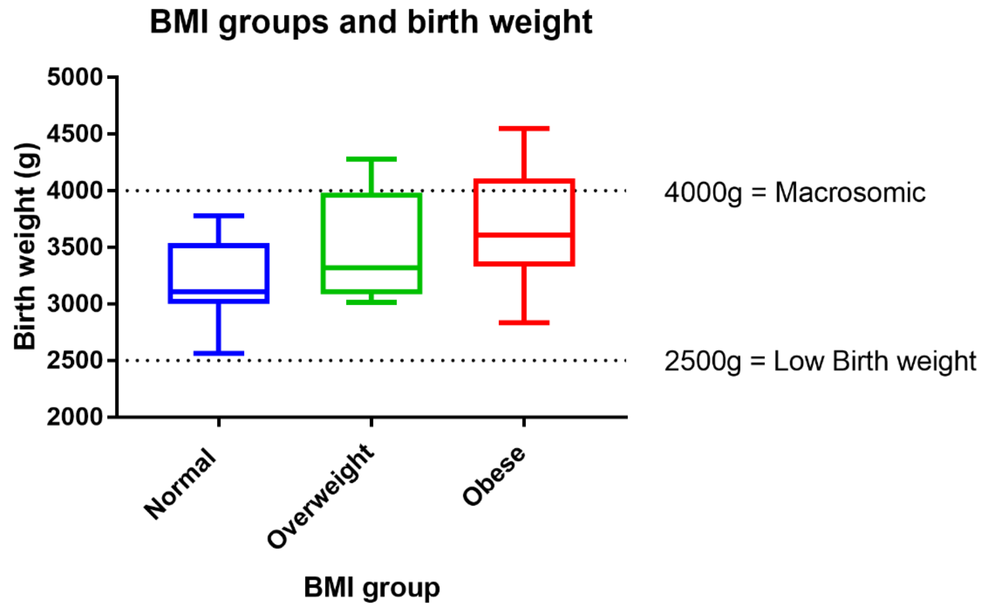

Figure 3.6 Birthweight median, interquartile range and upper/lower range for average birthweight per BMI group.Dashed line at 4000g indicates macrosomic classification.

3.3.4 Fatty acid profile of the human placenta

The fatty acid profile of the human placenta is shown in table 3.7. The 7 most abundant FAs in the human placenta are palmitic, stearic, OA, LA, DGLA, AA and DHA which make up around 97% of total fatty acids measured. The relative amounts for these are shown in figure 3.7. Palmitic is the most abundant FA in the placenta from women with similar levels in women with a normal, overweight and obese BMIs. The next most abundant FA is AA, followed closely by stearic in women with normal and overweight BMI. However, in obese mothers OA is higher and Stearic acid lower than in normal and overweight pregnant women. BMI was found to have a significant effect on the fatty acid profile, although to a small degree. There were no significant changes to the total SFA, MUFA, PUFA, n3 or n6 fatty acid levels with increasing BMI (Figure 3.8). Interestingly, BMI was however, seen to have a significant effect on Stearic acid, where being obese had significantly lower levels than placentae from overweight women (p = 0.011), but not dissimilar to women of normal weight (Figure 3.9). All the other significant changes are seen in FA with a relative abundance below 1%. Desaturase enzymes activities were estimated through ratios of product: precursor and plotted against BMI (Figure 3.10).

SCD18 activity was found to have a significant positive relationship with BMI oleic: stearic (p = 0.039) and palmitoleic: palmitic also had a positive relationship although this was not significant (p = 0.071). This suggests as BMI increases so does SCD18 activity and so oleic acid production. No effect of BMI was seen on either Δ6D activity or Δ5D activity. It was also evident there was no significant relationship between OA: AA and BMI either (Pearsons correlation r = -0.54, n=26, p = 0.792).

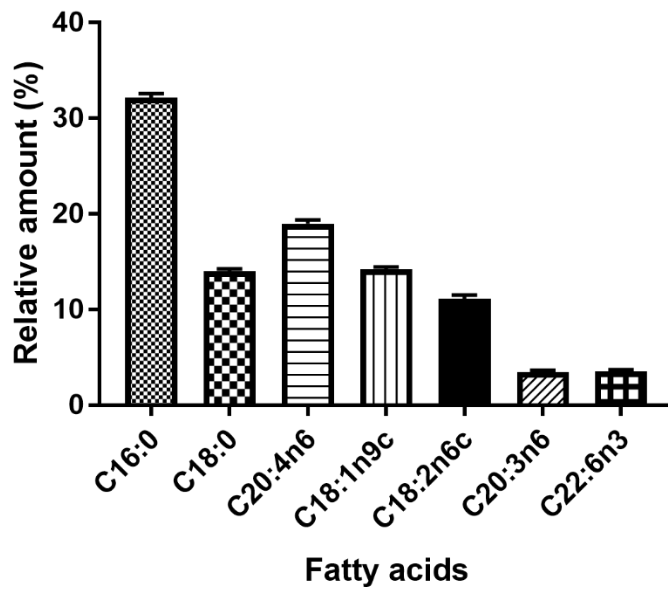

Figure 3.7 Fatty acid profile of the human placentae across all BMI groups (Normal n=9, Overweight n=7, Obese n=10) *expressed as a percentage of total FAs (mean with SEM). Includes only those fatty acids that made up over 1% of total fatty acids. C16 = palmitic, C18 = stearic, C20:4n6 = arachidonic, C18:19nc = oleic, C18:2n6c linoleic, C20:3n6 = DGLA, C22:6n3 = docosahexanoic acid.*

Fatty ACid		*Normal*		*Overweight*		*obese*		
		% total fatty acid	SEM	% total fatty acid	SEM	% total fatty acid	SEM	p-value
Caproic	C6:0	0.013	0.003	0.008	0.001	0.011	0.002	0.307
Caprylic	C8:0	0.021	0.006	0.008	0.002	0.010	0.002	0.093
Capric	C10:0	0.015	0.005	0.006	0.002	0.007	0.002	0.177
Undecanoic	C11:0	0.002	0.001	0.001	0.001	0.001	0.001	0.405
Lauric	C12:0	0.022	0.004	0.013	0.002	0.016	0.002	0.111
Tridecanoic	C13:0	0.002	0.002	0.002	0.001	0.001	0.001	0.173
Myristic	C14:0	0.443	0.035	0.346	0.028	0.402	0.022	0.143
Pentadecanoic	C15:0	0.114	0.009	0.090	0.005	0.104	0.005	0.160
Palmitic	C16:0	32.098	0.729	31.189	0.618	32.836	0.665	0.331
Heptadecanoic	C17:0	0.187	0.012	0.170	0.010	0.165	0.006	0.250
Stearic	C18:0	14.111	0.300	**14.835**	0.564	13.321	0.209	**0.034**
Arachidic	C20:0	0.055	0.002	0.051	0.003	0.050	0.002	0.330
Heneicosanoic	C21:0	0.001	0.001	0.001	0.001	0.002	0.001	N/A
Behenic	C22:0	0.049	0.002	0.043	0.002	0.039	0.002	0.013
Tricosanoic	C23:0	0.006	0.001	0.005	0.002	0.005	0.002	0.255
Lignoceric	C24:0	0.043	0.005	0.036	0.002	0.032	0.003	0.066
Total SFA		47.181	0.761	46.803	0.713	47.002	0.602	0.944
Myristoleic	C14:1	0.025	0.005	0.045	0.003	**0.048**	0.004	**0.009**
Pentadecanoic	C15:1	0.019	0.004	0.014	0.002	0.023	0.003	0.445
Palmitoleic	C16:1	0.598	0.031	0.537	0.051	**0.802**	0.065	**0.007**
Heptadecanoic	C17:1	0.027	0.003	**0.160**	0.001	0.064	0.009	**<0.001**
Oleic	C18:1n9c	14.076	0.227	13.932	0.255	14.555	0.529	0.553
Eicosenoic	C20:1	0.084	0.004	0.087	0.008	0.082	0.005	0.779
Nervonic	C24:1	0.188	0.084	0.117	0.072	0.238	0.068	0.606
Total MUFA		15.191	0.258	14.960	0.247	15.905	0.568	0.300
Linoleic	C18:2n6c	11.478	0.453	11.880	0.554	10.380	0.587	0.188
Linolenic	C18:3n6	0.045	0.002	0.045	0.003	0.046	0.004	0.995
DGLA	C20:3n6	3.505	0.190	3.559	0.287	3.431	0.278	0.950
Arachidonic	C20:4n6	18.613	0.751	18.930	0.373	19.353	0.637	0.739
Total n6 PUFA		33.731	0.680	34.457	0.606	33.324	0.443	0.474
3n3 Linolenic	C18:3n3	0.048	0.005	0.055	0.004	0.051	0.006	0.603
ETE	C20:3n3	0.020	0.003	0.015	0.002	0.020	0.001	0.280
EPA	C20:5n3	0.073	0.005	0.078	0.011	0.080	0.005	0.790
DHA	C22:6n3	3.607	0.316	3.546	0.224	3.514	0.208	0.970
Total n3 PUFA		3.748	0.314	3.694	0.236	3.665	0.203	0.975
Eicosadienoic	C20:2	0.176	0.007	0.188	0.014	0.157	0.007	0.073
Docosadienoic	C22:2	0.013	0.002	0.015	0.000	0.008	0.002	0.389

Euricic	C22:3n9	0.014	0.002	0.015	0.001	0.013	0.001	0.677
Total PUFAs		37.670	0.856	38.354	0.715	37.159	0.567	0.586
n6:n3		9.549		9.622		9.373		0.928

Table 3.7 Fatty acid profile of the human placenta for each BMI group (Normal n=9, Overweight n=7, Obese n=10). Mean relative amounts of each fatty acid calculated and identified on chromeleon from FAME37 GCMS analysis. Bold indicates p < 0.05, and highest mean relative amount, grey indicates all significant values with a greater than 1% relative abundance.

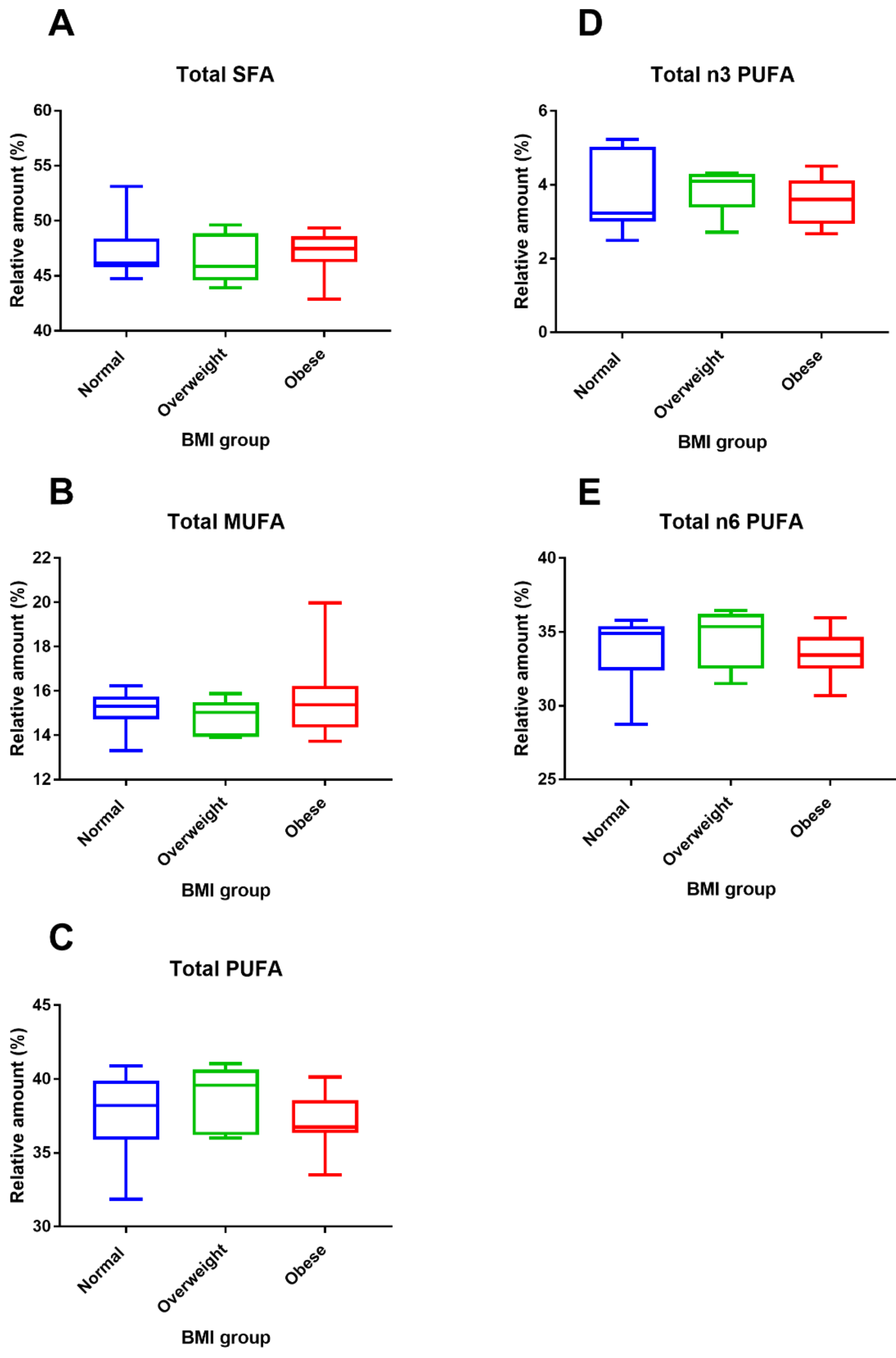

Figure 3.8 Total Saturated, Monounsaturated, Polyunsaturated, n3 and n6 fatty acids displayed as median interquartile range and upper/ lower range, averaged by BMI group (Normal n=9, Overweight n=7, Obese n=10). ** Indicates p<0.05 **indicates p<0.001*

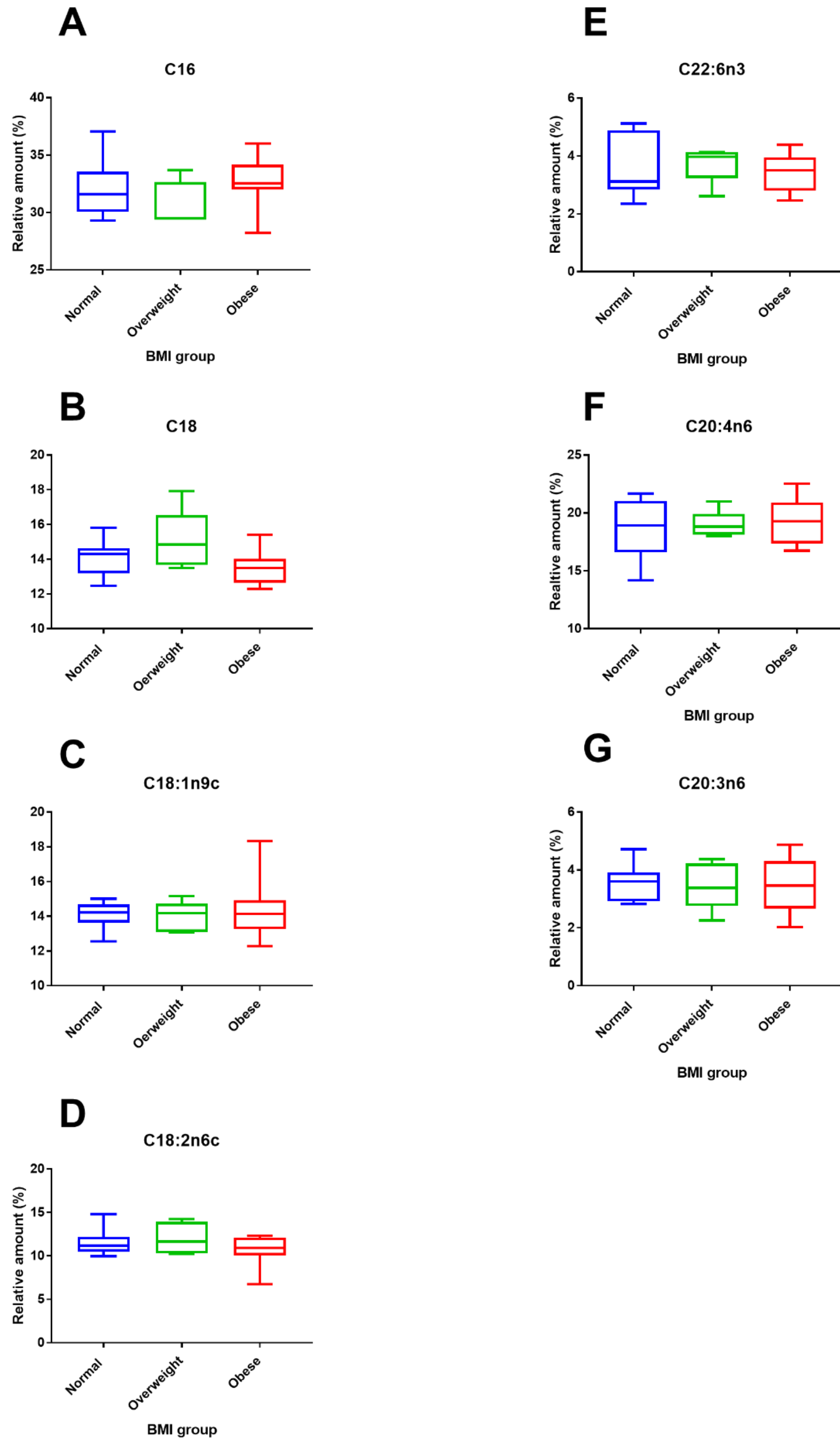

Figure 3.9 The 7 most abundant fatty acids in the human placenta (all those with relative abundance above 1%) displayed as median, interquartile range and upper/ lower range, averaged by BMI group (Normal n=9, Overweight n=7, Obese n=10). ** indicates p < 0.05 **indicates p < 0.001. C16 = palmitic, C18 = stearic, C20:4n6 = arachidonic, C18:19nc = oleic, C18:2n6c linoleic, C20:3n6 = DGLA, C22:6n3 = docosahexanoic acid.*

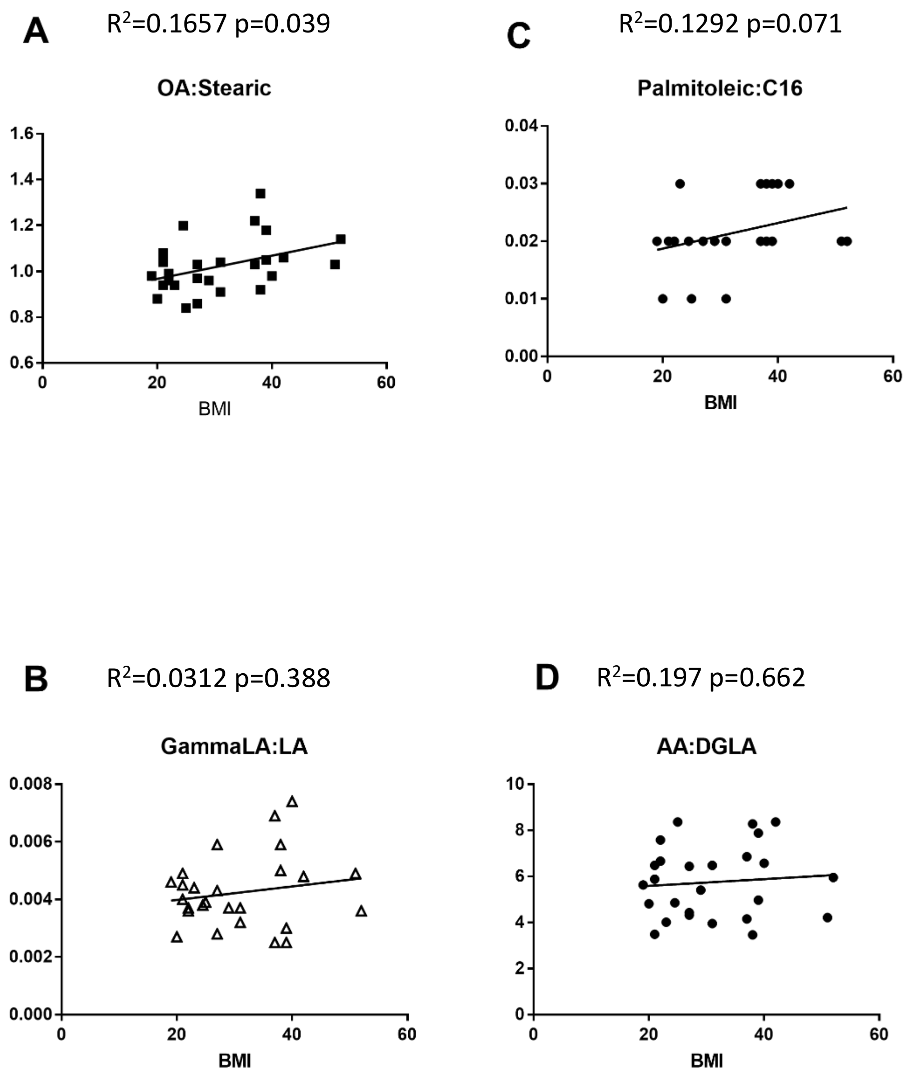

*Figure 3.10 Desaturase enzyme activity estimates calculated as ratios from product: precursor plotted with BMI. Oleic:stearic is SCD18, Palmitoleic:palmitic is SCD16, AA:DGLA is Δ5D and DGLA:LA is Δ6D. * indicated p<0.05 **indicated p<0.001*

3.3.5 Expression of genes in the rat placenta

RTqPCR was used to quantify and compare the relative abundance of mRNA in placentae from rats fed the control and HFHC diets. Cytochrome P450 17A2 (CYP17), the enzyme responsible for producing pregnenolone from cholesterol in the rat placenta as a precursor for oestrogen synthesis in the ovaries, and oestrogen receptor 1, one of the isoforms of receptor through which oestrogen acts (ER1) (Figure 3.11 A&E) were studied for changes in steroid hormone synthesis and signalling with, no significant difference observed. The three subtypes of peroxisome proliferator-activated receptors (PPARs) were measured as they are key regulators involved in lipid homeostasis and many lipids are ligands for them (Figure 3.11 B&F). Unexpectantly, PPARδ levels were too low to measure accurately. No significant difference was found with PPARα expression levels in placentae from rats fed Con or HFHC diet, however, PPARγ was found to be significantly elevated in placentae of rats exposed to HFHC diet ($p=0.004$). As PPARs are ligand activated transcription factors, we were interested in quantifying downstream targets that could have implications in labour. SIRT1 is an epigenetic regulator, negatively regulated by PPARγ but there was no significant effect of diet on mRNA expression (Figure 3.11 C). PPARγ activation has anti-inflammatory activity but no change was seen in NFkB or P65 mRNA expression, which are major transcription factors involved in the production of inflammatory cytokines and prostaglandins (Figure 3.11 G&D). Further inflammatory markers such as interleukins could not be measured due to low levels of expression within the placental tissue. PG production in the placenta is a key player in parturition. PPARγ and OA have both been down to have modulating effects on PG synthesis, so we looked at enzymes within the PG synthesis. The 2 COX enzymes PTGS1 and PTGS2 which are the first step in conversion of AA to PGs were not found to be altered by the obesity inducing diet (Figure 3.12 A&B). Other enzymes involved in PG synthesis (PTGES, PTGIS and AKR1B1), PG transport (ABCC4 and SLCO2A1) and activation (HPGD) were also found to be unaffected by the HFHC diet (Figure 3.12 C-H). Finally, SCD18 levels were measured to see if changes in mRNA occurred but again no significant differences in gene expression were identified (Figure 3.11 H).

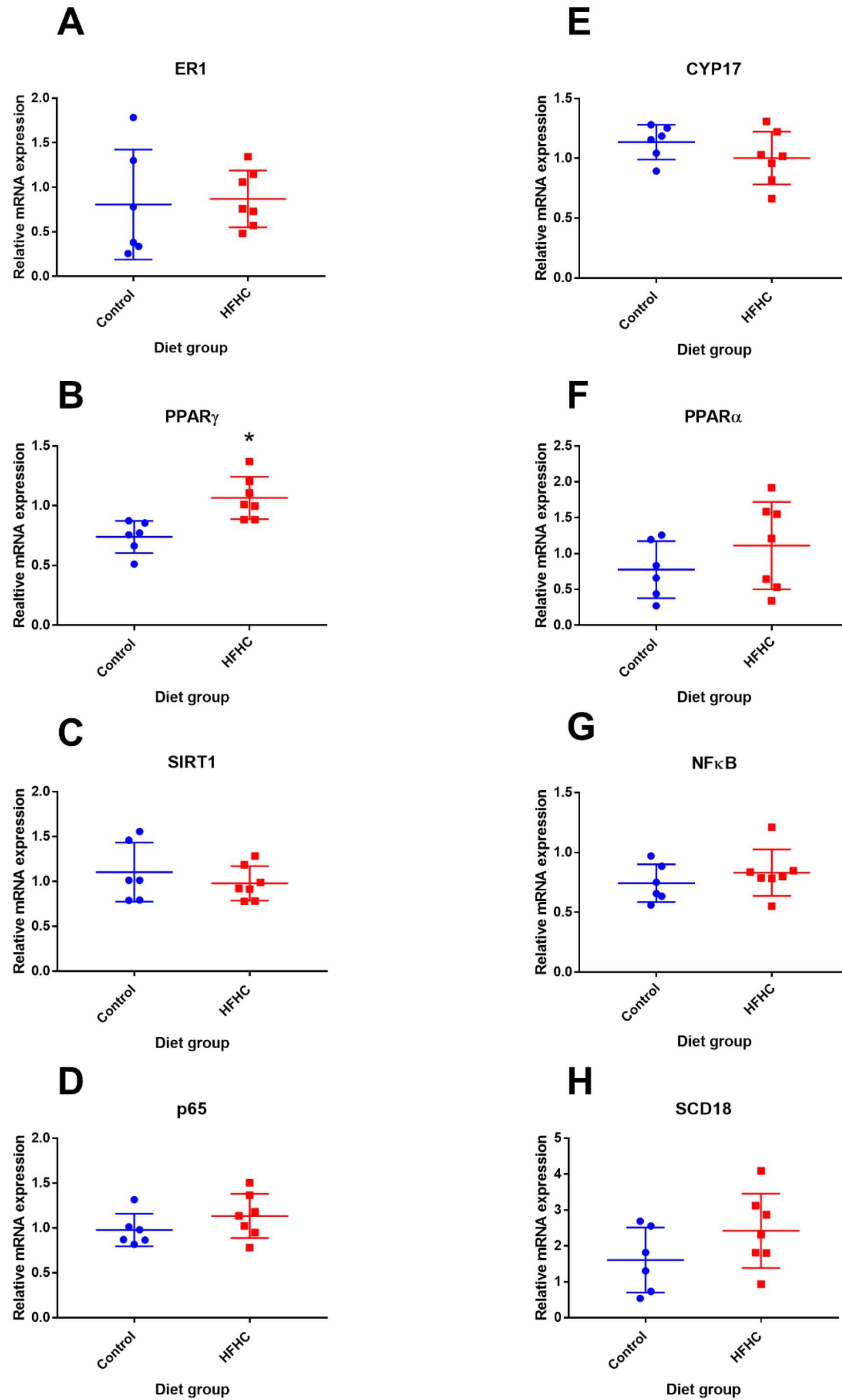

*Figure 3.11 Relative mRNA expression of A&E) steroid synthesis and signaling related genes CYP17 and ER1, B&F) Peroxisome proliferator-activated receptors (PPAR) γ and α C) SIRT1, D&G) NFkB family proteins NFkB and p65 and H) SCD18 enzyme, in rat placenta measured through RTqPCR and averaged by diet group (control n=6, HFHC n=7). Significant differences indicated by * = $p < 0.05$.*

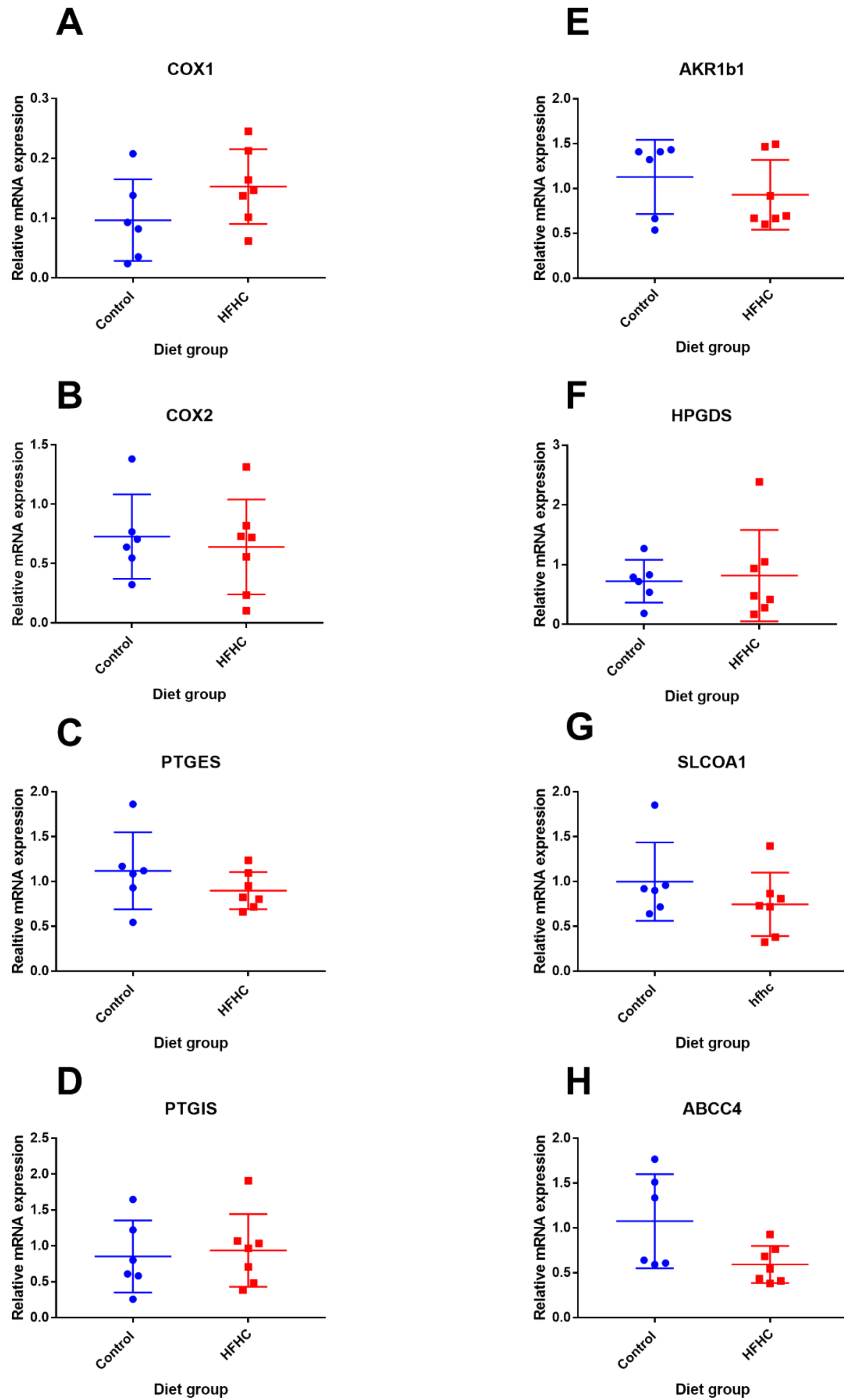

Figure 3.12 Relative mRNA expression of genes involved in: A-E) PG synthesis COX1, COX2, PTGES, PTGIS and AKR1B1 F) PG activation HPGDS and G-H) PG transport ABCC4 and SLCO2A1, in rat placenta measured through RTqPCR averaged by diet group (control n=6, HFHC n=7). No significant differences were found.

3.3.6 Expression of genes in the human placenta

To determine whether maternal obesity adversely affects steroid synthesis, gene expression of CRH and aromatase mRNA was quantified in placentae from women of different BMI, production but BMI was not shown to have significant effect on expression of either gene. In line with the gene expression findings in the rat placenta PPAR mRNA were also quantified in the human placenta. In parallel to the rat study, the PPARδ gene was again found to not be expressed highly enough in the near-term placenta and PPARα saw no significant change. In contract to the rat, PPARγ mRNA levels in the human placenta were not found to be significantly altered by BMI (Figure 3.13).

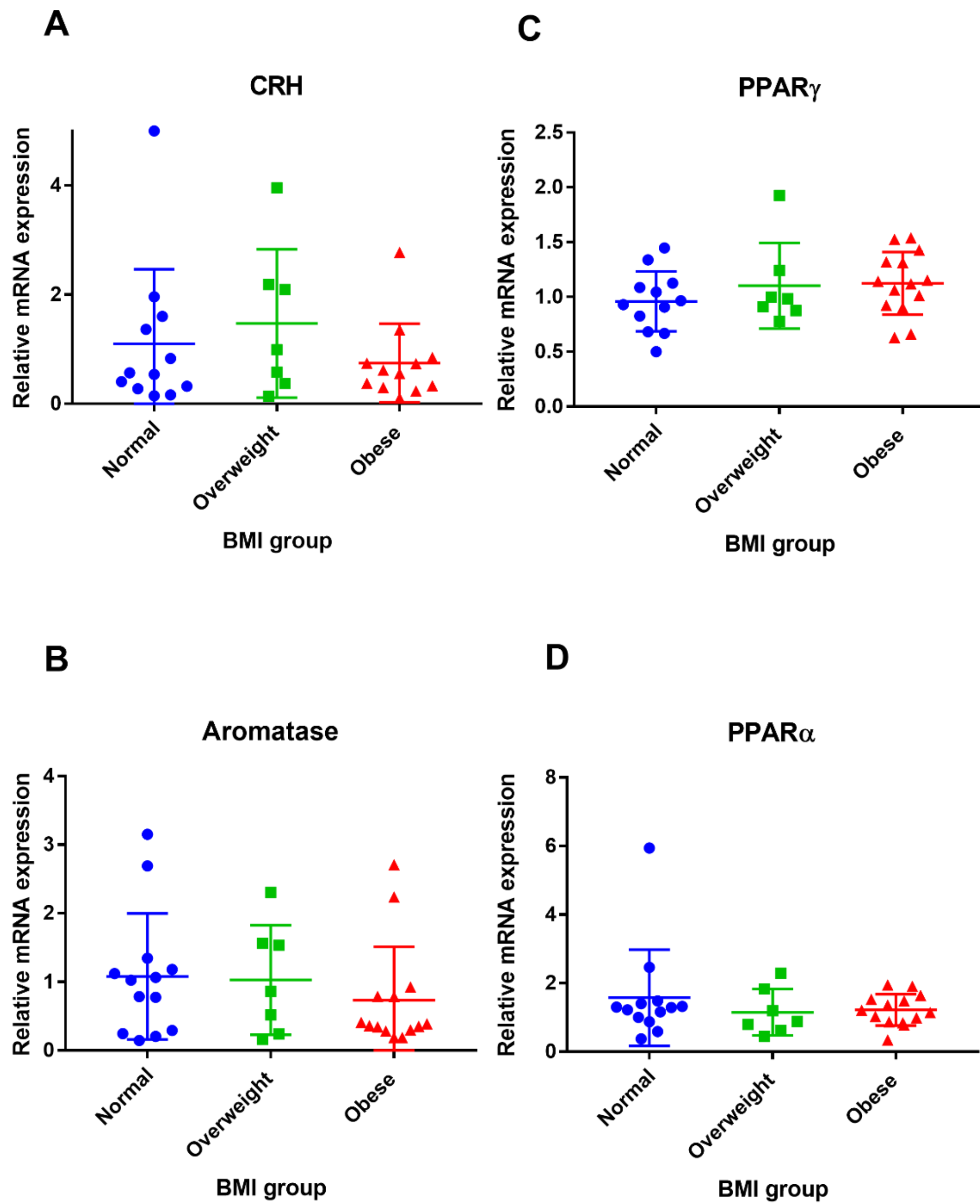

Figure 3.13 Relative mRNA expression of A) CRH, B) aromatase, C)PPARg and D) PPARa in human placenta, measured through RTqPCR and averaged by BMI group (normal n=13, overweight n=7, obese n=14).

3.4 Discussion

Overall, the translational rat model of HFHC diet to induce maternal obesity significantly altered the fatty acid profile of the placenta and expression of a few key genes. There was a 2-fold increase in MUFAs, specifically made up almost entirely of an increase in OA along with a parallel decrease in SFAs and both n3 and n6 PUFAs. Estimates of desaturase enzyme activity were all found to increase in the placenta of HFHC fed rats, and an increase OA: AA was observed, indicating decreased precursors for prostaglandin synthesis. Analysis of the human placenta identified that increasing BMI also has a significant effect on fatty acid profile but, to a much a lesser degree. Increasing BMI had no effect on total SFAs, MUFAs or PUFAs in the placenta but obesity did significantly decrease stearic acid. Only SCD18 activity was found to increase with BMI, similar to the rat model but the OA: AA relationship was not observed in the human placenta. The placental gene expression work identified that only PPARγ mRNA increased in the obese HFHC fed rats, but this occurred with no significant change in expression of any other potential downstream effector genes measured or genes involved in hormone synthesis. In contrast to the rat model of maternal obesity, there was no significant effect of BMI on the same genes quantified in the human placenta.

General composition of the FA profile of the placenta from rats and humans show they are reasonably similar. If we focus on the fatty acids that were quantified at physiological levels making up >1% of total fatty acids, palmitic was the most abundant (24.47%), followed by stearic (23.83%), LA (16.99%), AA (13.53%), OA (13.51%) and finally DHA (3.34%). In the human placenta palmitic acid was similarly the most abundant fatty acid (32.14%), however AA was second (18.98%), followed by stearic or OA depending on BMI group. Interestingly the human placenta was found to contain DGLA at high levels too at 3.49%, which was not evident in rat placentae. The differences here particularly for AA can be explained by the fact the rat placentae were taken during active labour and human pre-labour as taken from elective CS. AA is a precursor for 2 series prostaglandins which are important for successful labour (Smith, 2001). Increased PG synthesis has been demonstrated in term laboring tissues (Brennand *et al.*, 1995). Increased COX and LOX enzymes are observed at term and are associated with increased AA metabolism

(Bennett *et al.*, 1993). AA was found to increase leading up to term labour, with increased LA and AA in cord artery and vein of spontaneous labouring women compared to elective CS, meaning an increased delivery of AA and LA to the fetus and to the placenta (Birchenall, Welsh and López Bernal, 2022). Further to this LA metabolites were found to be increased in spontaneous labouring womens cord vein blood (Birchenall, Welsh and López Bernal, 2022). The increased levels of delivery and metabolism within labour could explain the discrepancies between the relative amount of these FAs.

The FA profile of the placenta was found to be very susceptible to dietary changes in the rat. This has been demonstrated in other studies, for example, dietary fat intake has been demonstrated to significantly alter both maternal plasma and placental levels of FAs in rats (Amusquivar and Herrera, 2003). Furthermore, increasing the intake of just one FA specifically, found alterations in the entire fatty acid composition of the placenta including increases in SFAs and decreases in MUFAs, n3, n6 and n7 (Shrestha *et al.*, 2020). A human study found BMI to alter maternal blood lipid concentrations but not foetal blood lipid concentrations, suggesting the placenta may have a mediating effect (Geraghty *et al.*, 2017).

Within the rat placenta there was a marked change in reduction of SFAs and PUFAs and an increase in MUFAs. In a previous study, using the same diet, similar patterns within the plasma and liver were observed. Although there was no change in total SFA in the plasma, an increase in total MUFAs, specifically OA and decreased total PUFAs and SFA were evident but only in the liver (Muir *et al.*, 2018). The FA profile changes can be partially described by the FA composition of the HFHC diet. The FA composition of the HFHC diet used in Muir et al (2018) study was analysed and compared to control chow. The HFHC diet comprised of 35% total fat with increased saturated fat, increased MUFAs and decreased n3 and n6 PUFAs with an increased n6:n3 ratio. Not surprisingly exposure to the same diet increased MUFAs, including OA and decreased n3 and n6 in the placenta. However, the HFHC diet contains double the relative amount of SFA but the placentae of rats fed this diet have significantly less SFA compared to the control fed animals. Furthermore, the difference in OA between HFHC and control is much more

pronounced in the placental FA profile than in the diet. SFAs, particularly palmitic and stearic acid have been shown to create a lipotoxic environment and increase inflammation in the placenta (Ricchi et al., 2009; Myatt and Maloyan, 2016; Shirasuna et al., 2016; Rogers et al., 2020). The decreased SFA and increased MUFA in parallel to the increased desaturase enzyme ratios, that estimate an increased conversion of saturated stearic acid to OA, could be a response from the placenta to ameliorate the environment caused by excess SFA. Evidence of this is seen in HEPG2 (liver cancer cells) cells treated with palmitic acid, is associated with an increase in palmitoleic acid which is suggested to be an attempt to detoxify via SCD16 (Ricchi *et al.*, 2009). Furthermore, in a human dietary study estimated SCD16 and Δ6D activity has been shown to be increased in individuals consuming a diet higher in SFA, and although those in the high SFA group were consuming over double SFA amount, plasma levels of SFA remained the same. This suggests SCD activity may increase as a consequence of SFA intake as an attempt to decrease SFAs in circulation and in tissues (Warensjö *et al.*, 2008). Dietary cholesterol is also known to induce SCD PUFAs can negatively regulate SCD (Nakamura and Nara, 2004), the dietary intake of cholesterol and PUFAs in the HFHC diet are increased and decreased respectively so could also contribute the alterations seen here. In adipose and skeletal muscle, increased SCD1 may help sequester lipids in triglycerides, thereby decreasing lipotoxicity (Sampath and Ntambi, 2008). Demonstration of OA detoxification in response to SFA has been observed in trophoblast cells specifically, when treated with palmitic and stearic acid to cause lipoapoptosis but the addition of MUFAs including OA mitigated this response (Natarajan *et al.*, 2021). Although the human placentae in this study did not show an overall decrease in SFAs and increase in MUFAs like in the rat model, there was significantly less stearic acid (one of the main SFA in the placenta) in obese mothers and SCD18 ratio was found to increase with increasing BMI suggesting this response could also be occurring. Although, it is important to note this is impossible to tell without any information on the levels of SFA and MUFA consumed within the diet, especially as SCD16 activity ratio could be used as biomarker for high SFA intake but SCD18 is not so reliable as is often skewed by OA intake (Warensjö *et al.*, 2008). It is also worth noting there are other dietary regulators of SCD including carbohydrates as well as

hormones such as insulin so this cannot be solely attributed to higher dietary SFAs (Nakamura and Nara, 2004).

Interestingly, AA was found to halve in the plasma and liver of HFHC fed rats compared to controls in the Muir et al (2018) study but in the current study no significant differences in placental AA were found between HFHC and control fed rats. FA profile of the diets showed there was very little AA in either diet (0.0% in CON and 0.1% in HFHC) but over 2.5 fold less LA, in the HFHC diet compared to lean controls (52.7% CON and 19.6% in HFHC). The decreased amount of AA in the plasma of HFHC fed rats is easily explained by the decreased amount of precursor LA available. However, as there was no significant change within the placenta, despite a reduced amount of LA, it demonstrates the placentas ability to manipulate lipids available to suit it's demands. Human placental membranes have demonstrated a preferential uptake of long chain PUFAs (LCPUFAs) with binding sites having preference for fatty acids as follows: AA $>$ LA $>$ a-LA$>$ OA (Campbell, Gordon and Dutta-Roy, 1996). As the placenta is taken from the rat during active labour, where AA is being used up rapidly for PG production this could lead to the similar levels seen in the placenta between the two dietary groups. There is also a decreased magnitude in the difference between LA in the diet and the placenta, despite both being significantly lower in HFHC group. Within the placenta there is only a 3% relative difference. The two desaturase enzyme ratios involved in the production of AA from LA have differing results, Δ6D activity is increased in HFHC but Δ5D is decreased. This could suggest increase AA production although these results are unclear. Although AA levels are similar between diet groups during labour, replacement levels of AA are also essential for continued PG production (Lamming, 1993) and reduced amounts of LA could lead to decreased replacement particularly with maximal production occurring during labour. This could become a rate limiting step in PG production, reducing overall levels and therefore causing dysfunctional contractions. Furthermore, DGLA significantly increased in the rat placenta and DGLA is precursor to PGE_1 which is functionally opposite to $PGF_{2\alpha}$ muscle contracting and vascular constrictor properties (Bitsanis *et al.*, 2005).

In the rat both n3 and n6 fatty acids are significantly lower in the HFHC group. Relative availability of n3 and n6 fatty acids may affect both the quality and quantity of eioscanoids produced by the placenta (Lewis, Childs and Calder, 2018). In particular, DHA is found to be lower in the HFHC fed rats. Studies have found links with DHA and an increase in gestational length (Mattos, Staples and Thatcher, 2000), and thought to impact the inflammatory state of the placenta through transcription factors such as NFκB and PPARγ (Lewis, Childs and Calder, 2018). N3s have been demonstrated to compete with AA for COX enzymes reducing the amount of 2 series PGs ($PGF_{2\alpha}$ and PGE_2) produced, as well as, HETEs and LTCs which are essential for labour (Allen and Harris, 2001). The n6:n3 is greater in the HFHC fed rat. This trend of greater n6 compared to n3 is seen among studies of obese women (Eastman *et al.*, 2021). Whilst n3 promote an anti-inflammatory state, n6 are pro-inflammatory and these n6 FAs are also reduced in this rat model of obesity. It could be that competition between the n3 and n6 FAs is not the main issue in this model but rather a general lack of pro-inflammatory mediators to stimulate contractions for successful and continued labour.

The MUFA OA was found to be significantly increased in the HFHC fed rat placentas with over a 2-fold increase in relative abundance. There is published evidence OA reduces inflammation and prostaglandin production (Yamaki et al., 2002; Oh et al., 2009; Bhattacharjee et al., 2020; Müller et al., 2021; Zhang et al., 2022), however this varies between cell types and conditions (Garnacho-Montero et al., 2002; Cheng et al., 2015; Hang et al., 2019; Müller et al., 2021). There is evidence of OA significantly reducing prostaglandin production in allanochorion cells from ewes (Cheng *et al.*, 2015), but very little research in trophoblasts specifically. A potential suggested mechanism of OA is an inverse relationship with AA, either through displacement or through disruption of AA production (Hostmark and Haug, 2013). Hostmark et al (2013) describe this relationship through ratios of relative amounts of AA to OA, and other desaturase enzyme ratios for example SCD18 (OA:stearic), with negative relationships between these being found in rat serum. Our study found a significant difference between the OA: AA ratio based on diet, with HFHC fed rats having much more OA compared to AA, however, no further correlations were seen with AA, OA and desaturase ratios. This could be important in the

production of PGs and other inflammatory markers in the initiation of labour. A more detailed look at this is needed before any conclusions can be drawn. Interestingly, a rat study found differences in phospholipid compared to neutral lipid fractions, where AA was found to be the most abundant PUFA in phospholipid fractions, whereas LA and OA were the most abundant fatty acids in neutral lipids (Amusquivar and Herrera, 2003). Our study looked at total lipids of the placenta and did not differentiate between the fatty acids in the membrane (phospholipids) and stored lipids within lipid droplets (neutral lipids) which could be an important factor in the OA and AA relationship in terms of PG production as liberation of AA from the membrane is the initial step in PG production.

The very limited FA changes seen within the human placenta are somewhat surprising as other studies have found BMI to have significant effects on FA profiles. A BMI >25 was found to be a significant contributor to serum levels of FAs and saw increased MUFAs compared to lean counterparts (X. Chen *et al.*, 2010). BMI > 30 had higher levels of total SFAs, MUFAs and AA in serum in both trimester 1 and 3 (Martín-Grau *et al.*, 2021). Obesity has been shown to alter the way fats are processed in the placenta, with a particular increase in esterification and storage of oleic acid in the triglyceride fraction of obese women (Ferchaud-Roucher *et al.*, 2019). There are limited studies looking at the fatty acid profile of the placenta and even fewer looking at the impact of obesity. In studies looking at other factors, the control groups from these found the most abundant class of lipid in placenta to be PUFAs, mostly consisting of n6 FAs, followed by SFA and then MUFAs equating to around 11% (Prieto-Sánchez *et al.*, 2019). Both the rat and human placenta in our study find SFA to make up the greatest proportion of FA class, followed by PUFA but do find n6 to be the vast majority of this class. A control group from another study found palmitic, stearic, OA and AA as the most abundant FA within the placenta (Hulme *et al.*, 2019), so very similar to our findings. Although the main focus of Hulme et (2019) was on the impact of glucose and maternal diabetes, part of their analysis split the control group consisting of uncomplicated pregnancies into BMI< 30 and BMI>30. They also found no significant changes in the FA composition of the placenta according to BMI. They found no difference in stearic acid, however, the difference seen within our data set was between overweight and obese women (so BMI

25-30 and BMI >30) but no significance between normal BMI and obese, and the authors have not distinguished between normal and overweight women which could explain the discrepancy.

Placental TG levels have been correlated with pregestational BMI (Garcia-Santillan *et al.*, 2022). Segura et al (2017) compared the placenta of normal, overweight and obese women alongside those with gestational diabetes mellitus (GDM). When looking at total lipids they had similar findings – no change in MUFAs, OA, short chain PUFAs or LA. Similar to our findings in the rat, they identified a reduction in SFA (Segura *et al.*, 2017). However, an increase in LCPUFAS, including AA was found in overweight and obese women. In the TG fraction specifically, very similar changes were observed, although no significant increase in long chain PUFAs. Further investigation suggested these changes could be attributed to FATPs. Reduced FATP1 and 4 and increased FATP6 and FAT/CD36 found in obese placentas may account for the changes in SFA and LCPUFAs respectively (Segura *et al.*, 2017). A further study in agreement with ours also found no association of BMI with LA, AA nor DHA but there was no further information on other FAs or total lipid classes (Bitsanis *et al.*, 2005). Many of these studies described the placentas as 'term' but give little information on delivery type, with some of them sampling from both spontaneous vaginal births and CS. This can have a vast impact on the structure, metabolome, signalling and gene transcription (Burton *et al.*, 2014) which could explain some discrepancies seen.

Despite no significant change in OA of the human placenta with obesity, a positive association with SD18 activity and BMI was observed. OA also became the third most abundant FA in the placenta in obese women, as opposed to fourth in normal and overweight. The lack of dietary information from the women who donated the placental biopsies makes it very difficult to compare with the rat placenta, where diet was tightly controlled with exact FA profiles calculated. Although obesity is generally associated with an increase in saturated fat (Rifas-Shiman *et al.*, 2009), the dietary differences between 2 women of the same BMI could be vast. With diet having a direct effect on serum levels of FAs from both current diet and past diet through adipose tissue (Amusquivar and Herrera, 2003) trends can be much harder to identify. Regardless, the increase in the

SCD18 activity does suggest a greater increase in OA production. Although limited studies compare the placental FA profile among obese women there is some evidence plasma FA changes in obesity. Two studies found increased total MUFA with increased BMI but no specific comment on OA (Al-Otaibi, 2020; Martín-Grau *et al.*, 2021) but this is not seen across all studies (Gulecoglu Onem *et al.*, 2021). This could be due to other factors including gestational weight gain with excessive gestational weight gain associated with increased MUFAs including OA (Vidakovic *et al.*, 2015).

There is a large body of evidence showing that obesity induces gene changes in the placenta. In rats, high fat diets have been found to alter the transcriptome of rat placenta (Nitert et al., 2013; Lin et al., 2019; Wang et al., 2021). A human study comparing the placenta of lean vs obese women found 288 genes to be differentially expressed (Saben *et al.*, 2014). Genes found to be significantly altered by dietary fats are involved in pathways including lipid metabolism, PPAR and NFκB signalling pathway. FAs can alter transcription through nuclear receptor activity, impacting the nuclear content of transcription factors indirectly or through alteration of lipid rafts and signalling (Jump, 2004). To understand if maternal obesity could affect placental function in terms of labour initiation and progression, RTqPCR was used to look at mRNA changes in genes involved in steroid hormone production and signalling, and inflammation.

CRH has been described as a major driver of parturition with production predominantly taking place in the placenta (Lamming, 1993). Previous studies have identified maternal BMI to have a negative inverse relationship with maternal plasma CRH in mid gestation (Kramer et al., 2010; Y. Chen et al., 2010). Plasma CRH levels were found to rise in obese women but when compared to lean women were found to be significantly lower. Furthermore, CRH levels at 36 weeks correlated negatively with gestation at deliver in obese women (Stirrat *et al.*, 2014). One of the key processes described in the initiation of labour is the switch from progesterone dominance to oestrogen. In humans this is not clear in serum levels of oestrogen and progesterone but there is evidence of localized shift to an oestrogenic environment (Birchenall, Welsh and López Bernal, 2022) and theories of functional changes through a reduction in progesterone receptors (Zakar and

Hertelendy, 2007; Arruda et al., 2013). CRH drives oestrogen synthesis through stimulating DHEA-S release from the foetus (Lamming, 1993) and is converted to oestrogen via the aromatase enzyme, in the human (Rainey, Rehman and Carr, 2004). Circulating levels of both progesterone and oestrogen have also been found to be lower in obese women (Lassance *et al.*, 2015). Neither CRH nor aromatase were found to be altered by BMI at the mRNA level in humans. CRH has been found previously to be decreased in placenta from obese women (Saben *et al.*, 2014), but most studies have focused on plasma levels as detailed above.

In the rat, oestrogen synthesis occurs in the ovaries from androgens produced in the placenta, involving CYP17 (a cytochrome P450 enzyme). Alterations in this enzyme could result in changes to oestrogen synthesis. Earlier work on the HFHC rat model for maternal obesity identified progesterone levels to be higher in the HFHC fed rats at term (Muir *et al.*, 2016) , so genes involving oestrogen synthesis and signalling were quantified to see if this could further contribute to a skewed progesterone to oestrogen ratio. Oestrogen receptor 1 (ER1) and2 (ER2) are responsible for the oestrogen signalling within the placenta so alterations could modulate the power of oestrogen signalling. None of these were found to be affected by a HFHC diet. Enzymes involved in the synthesis pathways (17b HSD, 3bHSD and cytochrome P450) for these steroid hormones have not been found to be significantly altered by obesity, but instead a cholesterol transport gene was found to be 2-fold lower in the obese placenta which could account for the change (Lassance *et al.*, 2015). A proteomic study in pigs identified DAPs involved in steroid biosynthesis were down regulated and again found down regulation of a cholesterol transporter genes (J. W. Li et al., 2019). High fat and high LA feeding in rats lead to an upregulation in genes involved in cholesterol transport to the foetus (Draycott *et al.*, 2020). There was some evidence of this in humans with higher BMI but a much more limited impact was seen on cholesterol transport (Draycott *et al.*, 2020). Cholesterol transport genes have also been identified to be reduced in HF fed rat placentas (Ye et al., 2017; Louwagie et al., 2018). Together, these could suggest alterations seen with steroid hormone biosynthesis could be a consequence of reduced cholesterol transport as opposed to downstream enzyme changes.

Lipid metabolism pathways were implicated in multiple studies looking at the transcriptomics (Saben et al., 2014; Wang et al., 2021). PPARs are key nutrient sensors and involved in lipid homeostasis and metabolism (Hihi, Michalik and Wahli, 2002). All 3 PPAR isoforms were quantified in rat placenta, however, only PPARγ was found to be impacted by diet. All 3 PPAR isoforms have been identified trophoblasts but PPARδ and PPARα decrease after differentiation (Kadam, Kohan-Ghadr and Drewlo, 2015). PPARs are described as promiscuous in interactions with ligands and one of the major ways FAs elicit transcription changes through nuclear receptor signalling (Kliewer *et al.*, 1997). Both PPARγ and α are identified as related targets of OA (Xue *et al.*, 2013). PPARγ was found to be significantly increased in HFHC fed rats. This agrees with several other studies finding high fat diets to increase PPARγ in the placenta of rats (Hsu *et al.*, 2020), mice (Qiao *et al.*, 2015) and placental cells treated with fatty acids (Sundrani, Karkhanis and Joshi, 2021). This is reasonably unsurprising finding as obesity of high fat diets have been found to increase the lipid content in placentas (Hirschmugl et al., 2017; Lewis & Desoye, 2017; L. Song et al., 2017) and PPARγ primarily promotes lipid storage and is a key driver of lipid droplet formation (Kadam, Kohan-Ghadr and Drewlo, 2015). No change was seen with any of the PPARs in human placenta based on BMI. This is surprising as other studies have found this (Calabuig-Navarro *et al.*, 2017). Although not in all (Dubé *et al.*, 2012).

PPARγ has been shown to be reduced in foetal membranes (Dunn-Albanese *et al.*, 2004) and DNA binding activity in labouring tissues (Lager *et al.*, 2014). Increased levels in term placenta as a result of a high fat diet could have negative implications on parturition. PPARγ is important in the development of the placenta particularly in vascularization but is also known to have anti-inflammatory effects in trophoblasts through NFκB (Bo et al., 2016; Sundrani, Karkhanis and Joshi, 2021). We attempted to measure inflammatory cytokines including IL-1b and IL-6 in excised placental tissue through RTqPCR however this was not possible due to seemingly low expression. Other studies have been able to measure cytokine mRNA expression in the placenta through PCR (D. W. Kim et al., 2014; M. Wang et al., 2022; X. Yang et al., 2016). Alternative methods for cytokine expression could be through ELISAs or multiplex assays. Both NFκB and P65 (transcription factors

involved in NFκB signaling pathway) were not found to be affected by diet in the rat placenta. Previous studies have seen increases in P65 and IkB-a (another element in the pathway) with a high fat diet (Zhang et al., 2023). It would be more beneficial to have performed a DNA binding activity assay here as although mRNA has not changed, activity may still be regulated. Other activity assays involving NFκB saw an upregulation in both placenta and liver in rats fed lard aimed to induce maternal dyslipidaemia (Breetha and TR, 2018). However, in human no association with NFκB activity was identified with BMI (Aye *et al.*, 2014).

SIRT1 mRNA drops significantly at labour and expression is generally reduced by pro-labour mediators (Lappas *et al.*, 2011). SIRT1 is reduced by pro-inflammatory mediators and PPARγ is anti-inflammatory so higher levels of PPARγ could lead to increased SIRT1 levels indirectly. However, the relationship between the two suggests some downregulation between each other but the relationship is complex and often depends on activators of both proteins (Wątroba, Szewczyk and Szukiewicz, 2023). No change was seen in response to diet in this study. Several other studies have found high fat diets to decrease levels of SIRT1 in rat placentas (Hsu et al., 2020; Zhang et al., 2023), however, these tissues were taken pre-labour (gestational day 18.5 and 21 respectively). The tissue in this rat study was taken during active labour therefore this could explain why there was no significant differences in SIRT1 observed between the CON and HFHC as SIRT1 decreases in active labour. On the other hand, in humans, vistafin (an adipokine increased in obesity) was found to have an inverse relationship with SIRT1, suggesting that these higher vistafin levels prevents labour associated decrease in SIRT1 which could lead to post term deliveries (Tsai *et al.*, 2015). As SIRT1 activation decreases LPS mediated PGE_2 and $PGF_{2\alpha}$ release (Lappas *et al.*, 2011), sustained levels could prevent this initial influx. A SIRT1/ PPARγ relationship with obesity-related inflammation is unknown. A mouse model feeding a high fat diet observed decreased SIRT1 and increased PPARγ expression and correlated this with LPL activity (Wątroba, Szewczyk and Szukiewicz, 2023).

Prostaglandin production is one of the placentas key roles in the initiation and success of labour. The HFHC model has previously demonstrated decreased plasma levels of $PGF_{2\alpha}$

(M. J. Elmes et al., 2011). PPARγ has been demonstrated multiple times to decrease PG production in cells from the feto-placental-uterine unit (Dunn-Albanese *et al.*, 2004; Froment *et al.*, 2006; MacLaren *et al.*, 2006). Pathcards is an integrated database of biological pathways which identifies genes which are clustered into paths. Prostaglandin synthesis and metabolism has 45 genes listed within it. These genes were cross referenced with OA related targets obtained from the Chinese medicinal database (Xue *et al.*, 2013). PPARγ, COX1 and COX2 were all identified as OA targets within the prostaglandin pathway. These genes alongside other enzymes responsible for prostaglandin synthesis, metabolism and activation were investigated. No enzymes involved in prostaglandin synthesis, metabolism or activation were found to be impacted by diet. COX2 has been shown to be increased by high fat diet in other rat studies (Nitert et al., 2013; Zhang et al., 2023). COX1, COX2 and HPGD have also shown increases but only in female offspring, along with phospholipase2 which could create more substrate for COX2 (Nitert *et al.*, 2013). This would suggest increased PG production which is not seen in our study.

3.4.1 Limitations

It is important to note there are some limitations to the study. Firstly, direct comparison of the rat and human placenta cannot be made as whole rat placenta were taken during the active expulsive stage of labour and pooled whereas human placental biopsies were obtained from women undergoing elective CS at term so before labour had been initiated or established. This has huge implications on the FA profiles due to the increased metabolism of the placenta during labour to produce all of the necessary pro-labour mediators as previously discussed. A metabolomics study comparing pre and post labour placentas saw increased LA pathways and varying AA levels dependent on delivery type (Birchenall, Welsh and López Bernal, 2022). It would be useful to compare non-labouring and labouring placental FA profiles in both rat and from human to identify any trends between labouring and non-labouring tissue which may identify potential impactful changes. There is also no dietary information collected from the women in the human tissue study. Whilst the rat diet is very tightly controlled, 2 women could ingest entirely different energy intakes, macro and micronutrients which can influence the FA

profile. Further information on this and matching of erythrocyte, plasma and placental fatty acid profiles would provide a clearer picture of how current and previous diet could influence placental FA profiles and the effects this may have.

3.5 Conclusions

In conclusion, the HFHC diet in the rat elicits some key changes in the fatty acid profile and mRNA expression of PPARγ within the placenta. These fatty acid changes could have anti –inflammatory effects and a reduction in the production of PGs. Reduced LA could mean reduced synthesis and replacement of AA and decreased ability to produce the 2 series PGs consistently throughout labour, that are key for stimulating contractions and cervical dilation. OA has been demonstrated to have anti-inflammatory effects and negative effects on PG production, although this does vary based on cell type. Our finding in the rat model that maternal obesity is associated with a significant increase in PPARγ that has anti-inflammatory effects again supports this theory. However, no pathway in which it may do this has been identified. Combining all these findings suggests a decrease in the pro-labour mediators produced by the placenta, leading to a general reduction in inflammatory cytokines and PGs which could prevent the initiation and continued success of labour, which is often associated with maternal obesity. Although findings in the human placental tissue did not quite match what was observed in the rat, this is unsurprising due to the varied diets between humans. There is evidence of an increased production of OA and a decrease in SFAs.

4 Effects of oleic acid on the production of pro-labour mediators PGE_2, $PGF_{2\alpha}$ and IL-6

4.1 Introduction

In the Chapter 3 we identified a significant alteration in the fatty acid profile of the placenta in the diet induced maternal obesity model. A significant reduction in SFAs, increase in MUFAs and desaturase enzyme activity suggests a potential mechanism for reducing lipotoxicity induced by a diet high in SFAs (Ricchi et al., 2009; Natarajan et al., 2021). In particular, this increase in MUFAs was driven by OA being over 2-fold higher and becoming the third most abundant FA in the placental tissue. Although this clear-cut trend was not observed in obese humans, a few characteristics within the FA profile suggested a similar response.

The impact OA may have on placental trophoblasts cells is unclear in terms of labour initiation and success. OA has been investigated in trophoblast cells showing impacts on migration (C. Yang et al., 2017), and increase in lipid droplets (Colvin *et al.*, 2017). There is some evidence from a study investigating the effects of OA in foetal membranes, but this showed varying results based on dose of OA, exposure and responsiveness to other treatments such as LPS (Cheng *et al.*, 2015). Results from other studies investigating effects of OA on different tissues and cell types, including adipocytes, lung cancer and kidney injury have demonstrated reduced PGs, inflammatory markers and toll-like receptor signalling (Shaw et al., 2013; Yamaki et al., 2002; Zhang et al., 2022). Along with this there is evidence to suggest OA increases markers associated with anti-inflammatory effects in a trophoblast cell line (Easton *et al.*, 2023). However, this decrease in inflammation and PG synthesis is not consistent across all studies. Others have reported increases in PGs and inflammatory markers (Haastrup *et al.*, 2001; Kwon *et al.*, 2018; Lai *et al.*, 2022). Further work is therefore needed in trophoblast cells to determine the specific impact increased OA may have in these cells on pro-labour mediators and how this might occur.

The second significant finding in the previous chapter is the increased PPARγ expression found in rats fed a HFHC diet but no clear mechanism on how it may affect labour processes. There is evidence of PPARγ exerting anti-inflammatory and PG inhibitory effects (Mendez and LaPointe, 2003; Ackerman IV et al., 2005) and a demonstrated relationship with FAs, specifically OA (Shaw et al., 2013; Zhang et al., 2022). However, so far little has been done to investigate the relationship of OA, PPARγ and PG in trophoblast cells.

4.1.1 Aims

With inconsistencies in the literature, this experimental chapter aims to investigate the effect high physiological concentrations of OA have on a trophoblast cell line in terms of growth, viability and synthesis of pro-labour mediators including PGs and pro-inflammatory cytokines. In addition, we will also attempt to unravel how OA may elicit these effects and whether PPARγ is involved. The hypothesis to be tested is that OA at high physiological concentrations relevant will reduce the production and secretion of PGE_2, $PGF_{2\alpha}$, IL-6 and TNF.

4.2 Methods

4.2.1 Cell culture

4.2.1.1 Cells

The HTR8-SVneo cell line(Graham et al., 1993) was obtained from American Type Culture Collection through an official distributor in the UK (LGC, Middlesex, UK). Cells were stored in liquid nitrogen at passage 21 and all experiments were performed at passage 22. Cells were maintained in RPMI ATCC specification media (2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4500 mg/L glucose, and 1500 mg/L sodium bicarbonate) supplemented with 5% Fetal Bovine Serum (heat inactivated, Gibco, Brazil) and 1% penicillin/streptomycin (10,000 units penicillin and 10mg streptomycin/ml, sterile filtered, Bioreagent, Merck Life Science, UK) maintained at 37°C and 5% CO2 in T75 flasks (Corning 75cm2 Cell Culture Flask, canted neck, nonpyrogenic, polystyrene, Corning, China). The culture media changed every 2-3 days.

4.2.1.2 Treatments

HTR8-SVneo cells were seeded in 6-well plates (Flat bottom, tissue culture treated, non-pyrogenic, polystyrene, Costar, Corning, ME, USA) at a density of $3.5x10^4$ in 2ml media. After incubation for 24 hours media was replaced with Serum Free media (same RPMI but with no FBS added). Oleic acid solutions were made up in Serum free RPMI media from Oleic acid-Albumin from bovine serum (Sigma-Aldrich, Saint Louis, USA) to a final concentrations of 50uM, 100uM, 200uM and 400uM. BSA (Bovine Serum Albumin, heat shock fraction, Sigma-Aldrich Chemie GmbH, Steinhem, Germany) was dissolved in sterile PBS (Phosphate buffer saline tablet, Sigma-Aldrich, USA) to a concentration of 108mg/ml to match protein concentration in oleic acid. All treatments were carried out in the same volume of culture media and concentration of BSA in PBS solution. Vehicle control wells contained no oleic acid just BSA, PBS solution and control wells had no addition of anything just serum free media. Media was collected at 0h, 2h, 24h and 48h post treatment into eppendorfs, and these were immediately centrifuged at 1,500rpm for 10 minutes at 4°C, The resulting supernatant was then aliquotted and stored at -80°C. The remaining cells had the media removed, and washed with 2ml of PBS before 400 µl

Milli-U water was added. Cells were then removed with a cell scraper (Starstedt, NC, USA) and removed into Eppendorf tube and stored at -80°C until futher analysis.

4.2.1.3 DNA Assay

To determine the effect of treatments on cell proliferation and cell number, HTR8-SVneo cells were seeded at $1.35x10^4$ per well in 200 µl of ATCC specification media media in a 96 well plate. The same treatment, time and concentrations were carried out as described above but with an extra control of media containing serum. The media was removed from cells, then washed with PBS before addition of 100 µl Milli-U water added to all wells. The 96 well plate was then frozen at -20°C and freeze thawed 3 times before the DNA assay was run. A DNA standard curve was produced from stock calf thymus DNA in distilled water (Medchem express). 10x TNE stock (100mM Tris [pH 7.4], 10mM EDTA, 1M NaCl) was diluted to 2xTNE and Hoescht Dye solution diluted to 2ug/ml. To each well 100 µl of dye solution was added and fluorescence plate read on the omega plate reader set at (350nm excitation and 460nm emission).

4.2.1.4 CCK8 Assay

To determine the effect of treatment on cell viability HTR8-SVneo ells were seeded at $1.35x10^4$ per well in 100 µl media in a 96 well plate (Flat bottom, tissue culture treated, non-pyrogenic, polystyrene, Costar, Corning, ME, USA). The same treatments and timelines were used as already described above (See section 4.2.1). 10 µl of CCK-8 solution (Vazyme, Nanjing, PRC) was added to each well and the plate was shaken gently or 30 seconds. The 96 well plate was then incubated for 2 hours at 37°C with 5% CO_2 and then read at 450nm on the omega plate reader. The viability of the cells were calculated as follows: Cell viability % = [(A-C)/(B-C)] × 100% (A: Experimental group OD (contain medium, cells, drugs and CCK-8 Solution) B: Control OD (contain medium, cells, CCK-8 Solution) C: Blank OD (contain medium, CCK-8 Solution).

4.2.1.5 Nile Red

Nile Red is a fluorescent stain used to detect intracellular lipid droplets, with better specificity at yellow gold fluorescence than red fluorescence (Greenspan et al., 1985). A

stock solution of 1mM Nile Red was prepared by dissolving 15.8mg Nile Red (Sigma) and 500mg Pluronic F-127 (Sigma) in 50ml dimethyl sulfoxide (DMSO). The stock solution was aliquoted and stored at –20°C. 1mM Nile Red was diluted in Hank's buffered saline solution (HBSS, Sigma) to 30µM immediately before use and protected from light using foil. The cells were washed twice with HBSS before adding an appropriate volume of Nile Red to each well (100µl for a 96- well plate). The plate was left to incubate in the dark for 15 minutes. After this time, the waste liquid was removed and the cells washed with HBSS. The cells were covered with HBSS for measuring fluorescence on a FLUOstar Optima or FLUOstar Omega microplate reader (BMG Labtech). Wavelengths were for yellow gold fluorescence (excitation 485nm, emission 590nm). Orbital scanning was used at a diameter of 3mm to take an average of ten points around the well. If images were taken, they were taken at this point using a Leica DM IL fluorescence microscope.

4.2.2 Enzyme linked immunosorbent assays- ELISAs

4.2.2.1 PGE2 (cat: EHPGE2, Invitrogen, ThermoFisher Scientific, Vienna, Austria): The methods were followed according to the manufacturer's instructions. All solutions and plates were defrosted for 30 minutes at room temperature. PGE2 standards were then diluted in RPMI cell culture media and 7 serial dilutions made (standard 1 = 2500pg/ml through to standard 7 = 39.1pg/ml and standard 8 was 0pg/ml). 100 µL of RPMI culture media was added into the non specific binding (NSB) and the B0 (0 pg/mL Standard) wells. Next, 100 µl of standards and media samples were added into appropriate wells. 50 µL of diluent reagent was next added into the NSB wells. 50 µL of the blue PGE2-AP conjugate was then added into each well, except the Total Activity (TA) and blank wells, followed by 50 µL of the yellow PGE2 antibody into each well, except the Blank, TA and NSB wells. Each standard, sample and control was run as duplicate. The ELISA plate was then sealed and incubated at room temperature on a microplate shaker set at 500rpm for 2 hours. After incubation, all wells were washed with 400 µl of wash buffer 3 times and the plate firmly tapped over absorbent tissue on a bench to remove any excess buffer. 5 µl of PGE2-AP conjugate was then added to the TA wells and 200 µl of substrate solution placed in every well. The ELISA plate was sealed again and incubated at room temperature for 45 minutes without shaking. 50 µl stop solution (sodium phosphate

tribasic dodecahydrate) was then added to every well and the plate was read immediately at 405nm OD with correction at 580nm. For analysis a four parameter logistic regression curve was generated using MARS data analysis software and from this curve the unknown sample concentrations were calculated using blank corrected and 580nm minus data. Intra-assay precision for PGE_2 concentrations were (~490pg/ml) with 5.8% CV, and the Inter-assay 5.1% CV.

4.2.2.2 PGF2a (Cat: EU0204, Fine Test, Wuhan, China): All equipment and kit reagents were brought to room temperature before use. Standards were made up by 1ml sample dilution buffer added to standard tube and left to stand for 2mins before inverting the tube several times. 7 serial dilutions were prepared in sample dilution buffer (standard 1= 500pg/ml, standard 7 = 7.812pg/ml and standard 8 = 0pg/ml). The ELISA plate was washed twice before 50 µl of standard, samples and sample dilution buffer (as blank) were placed into the wells of the pre-coated plate. All samples and standards were run in duplicate. 50 µl of Biotin-labelled antibody was immediately added to each well and mixed gently by the tapping the plate for 1 minute. The ELISA plate was then incubated at 37°C in Envirogenie (Scientific Industries, New York, USA) for 45 minutes. Liquid from each well was then aspirated and washed 3 times with 350 µl wash buffer. Next 100 µl of HRP-streptavidin conjugate (SABC) was added to each well and the plate was sealed before incubating at 37°C in the dark for 10-20 minutes. The microplate reader was preheated to 37°C for 15 minutes before reading the plate. 50 µl of stop solution was then added to each well and the OD absorption read immediately at 450nm. A four parameter logistic regression curve was calculated using MARs software on blank corrected data and sample concentrations calculated from this. Intra and inter-assay precision was 5.35%CV and 5.24%CV respectively.

4.2.2.3 IL-6 (Cat:88-7066, Invitrogen, ThermoFisher Scientific, Vienna, Austria) and TNFa (Cat:88-73466, Invitrogen, ThermoFisher, Vienna, Austria): Corning™ Costar™ 9018 The ELISA plate was coated with 100 µL/well of capture antibody in Coating Buffer, sealed and incubated overnight at 4°C. Each wells was then aspirated and washed 3 times with >250 µL/well with Wash Buffer, allowing approximately 1 minute for soaking before

blotting on absorbent paper. Wells were next blocked with 200 µL of ELISA/ELISPOT Diluent (1X) at room temperature for 1 hour. Again the liquid from each well was aspirated and all wells washed once again with Wash Buffer. Standards were prepared by 2-fold serial dilutions of the top standards to make the standard curve for a total of 8 points (standard 1 = 200pg/ml, standard 7 = 1.56pg/ml and standard 8= 0pg/ml) and 100 µl of each standard and sample (neat cell culture media) was added to each well and 100 µL of ELISA/ELISPOT Diluent (1X) to the blank well. Each sample and standard was run in duplicate. The ELISA plate was sealed and incubated overnight at 4°C. Wells were aspirated and washed 5 times before 100 µl of detection antibody was added to all wells. The ELISA plate was once again sealed and incubated at RT for 1 hour and wells washed a further 5 times . Next 100 µl of Streptavidin-HRP was added to each well and incubated at room temperature for 30 minutes and washed 7 times. 100 µl MB solution added and incubated for 15 minutes before the addition of 100 µl stop solution to each well. The ELISA plate was then read at 450nm with 570nm subtraction. A linear standard curve was created using MARS data analysis software and from this sample concentrations were calculated.

4.2.3 RNA extraction and RTqPCR

Same process as Rat tissue but Roche High Pure RNA Isolation Kit (Roche Diagnostics Ltd., Burgess Hill, UK) used (See section 3.2.5). cDNA synthesis, primer design and RTqPCR protocol are described in sections 2.1-2.3.

4.2.3.1 Oligreen assay (Quant-iT OliGreen ssDNA reagent, Molecular Probes Life Technologies, Sigma-Aldrich, USA): Make a 1:200 working dilution of OliGreen in 1x TE buffer (diluted from 20X in nuclease free water) and 5ul added to 5ul cDNA in a 96 PCR plate. Serial dilution of stock cDNA to make a standard curve. This is then incubated in the dark for 5 minutes before reading on the LightCycler 480 machine. Settings were as described in table 3.3. Data is analysed using reactions at 80°C to increase specificity by reducing fluorescence given off by OliGreen binding to RNA (Rhinn *et al.*, 2008). Standard Curve is calculated and cDNA quantity is calculated form standard curve and relative fluorescence.

4.3 Results

4.3.1 Cell proliferation and viability

The Hoescht dye DNA assay was used as a measure of cell number and a marker of proliferation of trophoblast cell line treated with increasing concentrations of OA (0-400µM) (Figure 4.1 A). There was no significant effect of OA concentration on cell number when treated for 48 hours. This was important as it confirms OA and BSA have no effect on cell proliferation in comparison to control media with no serum. Cell viability was measured via a CCK-8 assay and expressed as % viability of control at 100% (Figure 4.1 B). OA was found to have a significant effect on cell viability ($p < 0.001$). The viability of the trophoblast cells significantly increased with exposure to higher OA concentrations, with the highest concentration 400µM having the same viability as control (100.22% +/- 7.28). Both control and 400µM were significantly greater than vehicle (73.29% +/- 3.26) and 50µM (78.73% +/- 4.21) of OA respectively, but no significant difference was observed between 100µM (83.3% +/- 4.88) and 200µM (95.26% +/- 4.7) concentrations. Although cell proliferation was unchanged, cell viability was impacted by BSA and rescued by increasing levels of OA.

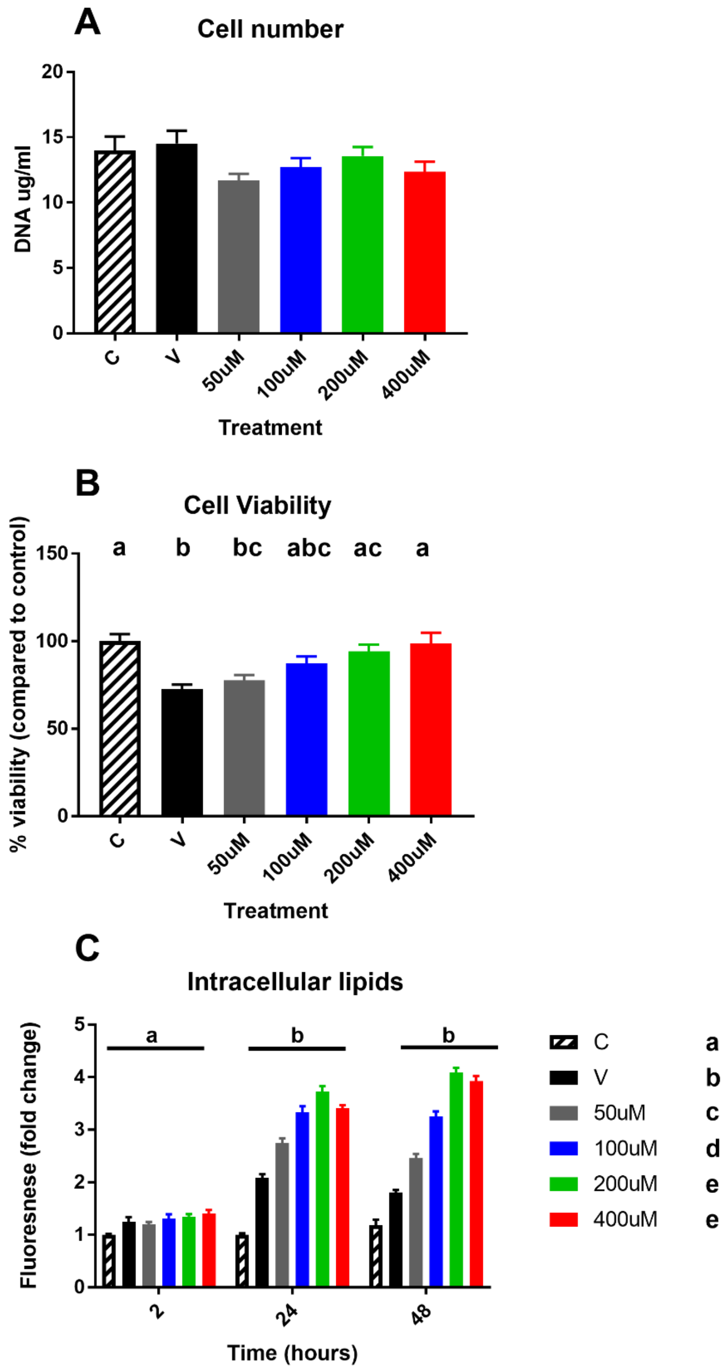

Figure 4.1 Cell based assays for proliferation, viability adnd instracellular lipids in response to OA. A) measure of cell number through quantifying amount of DNA using the Hoescht dye, as a marker of proliferation B) measure of cell viability expressed as % of control using CCK8 assay and C) measure of intracellular lipids using Nile red assay. All comparing control (serum free media), vehicle (BSA) and OA concentrations. Letters show significant differences as per Bonferoni post-hoc testing in GENSTAT for time (over the top of bars) and treatment (annotated next to treatment key) those with the same letter notations are not significantly different from one another.

4.3.2 Fatty acid uptake

Nile red dye (9-diethylamino-5H-benzo-α-phenoxazine-5-one) stains intracellular lipids of cells and fluorescence intensity of the dye is directly proportional to amount of lipids present. This is expressed as fold change from the control cells which had no treatment applied (Figure 4.1 C). A significant effect of both time ($p < 0.001$) and treatment ($p < 0.001$) was seen, with a significant time*treatment interaction ($p < 0.001$). Both 24 and 48 hours had significantly more lipids present than 2 hours of treatment. The intracellular lipid increased dose dependently with OA concentration from 0-200 µM, but there was no additional increase at the 400 µM OA concentration. Overall, the data highlights that higher levels of OA are absorbed when exposed to the fatty acid for longer or exposed to a higher OA concentration, however intracellular lipid capacity is reached at 200 µM. Images showing lipid droplets within cells are shown in Figure 4.2.

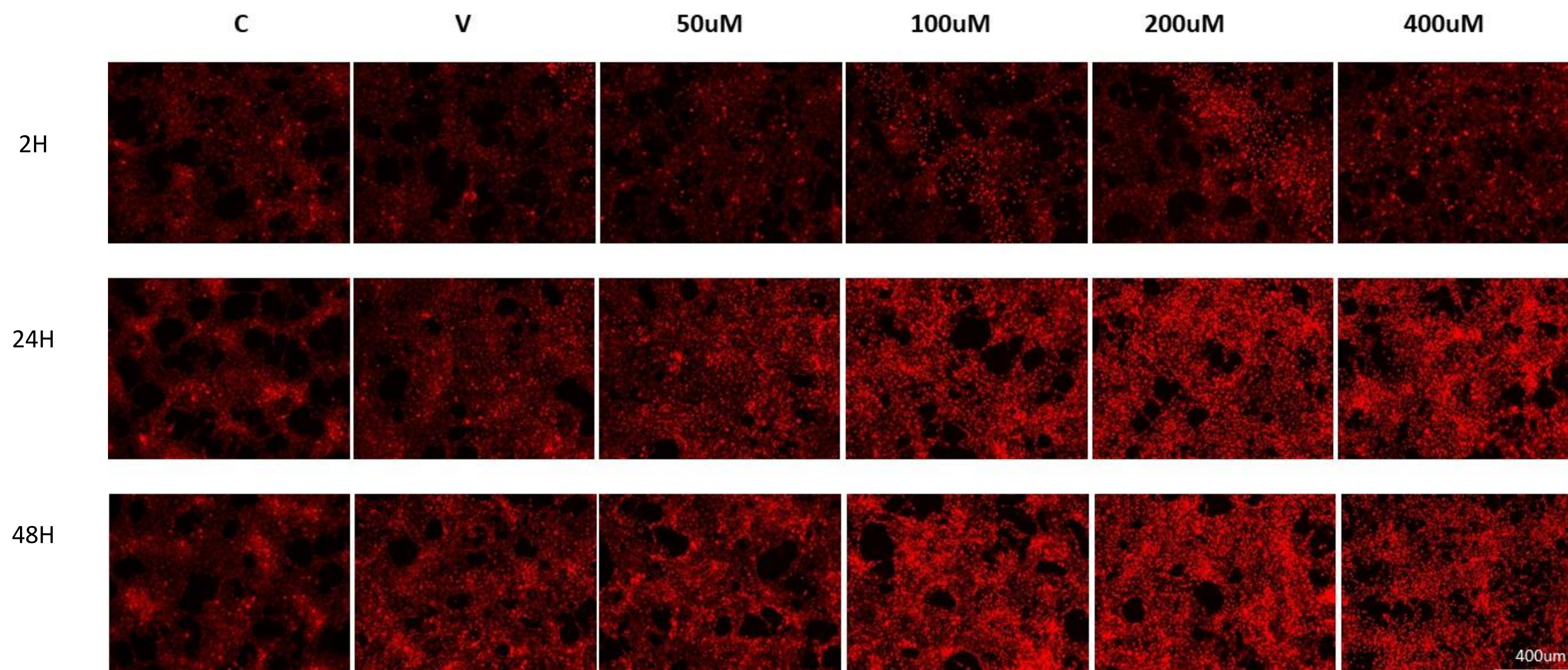

Figure 4.2 Images taken of HTR8-SVneo cell svisualising intracellular lipids. Taken on EVOS microscope at 10x magnification, after treatment with increasing concentrations of OA, after 2, 24 or 48h. Visualized using Nile red dye.

4.3.3 Prostaglandin and inflammation expression

ELISAs were used to measure the amount of PGE_2, $PGF_{2\alpha}$, IL-6 and TNF secreted by the trophoblast cells into the culture media over 2, 24 and 48 hours in response to OA (Figure 4.3). PGE_2 was significantly affected by both time ($p = 0.007$) and treatment ($p < 0.001$) but there was no significant interaction between the two ($p = 0.898$) (Figure 4.3A). Control treated cells with no exposure to BSA, or OA synthesized very little PGE_2 with a mean of 97pg/ml (+/- 67.05) across all time points. Vehicle BSA treatment led to an increase in PGE_2 production and release (660pg/ml +/- 63.68) that was further increased by 50 µM OA (747pg/ml +/- 180.9). Subsequent exposure to 100 µM and 200 µM of OA decreased synthesis and secretion of PGE_2 production at 532pg/ml (+/- 57.79) and 406pg/ml (+/- 64.43) respectively, with the highest 400u µM concentration of OA reducing PGE_2 synthesis to levels lower than cells exposed to BSA alone (41pg/ml +/- 27.32). This response to increasing concentrations of OA was seen across 2-, 24- and 48-hour time points. 400 µM was significantly lower than all other treatment groups, aside from control which was also significantly lower than all other treatments, excluding 200 µM (Figure 4.3 A). The maximum amount of PGE_2 synthesized was seen after 24 hours exposure with a mean of 566.7pg/ml (+/- 120.8) and was significantly higher than PGE_2 synthesised at both 2 and 48 hours at 326pg/ml (+/- 60.32) and 348.4pg/ml (+/-61.86) respectively. $PGF_{2\alpha}$ was also significantly impacted by OA treatment ($p < 0.001$) and time ($p < 0.001$), again with no significant interaction between treatment and time ($p = 0.228$). Similar to PGE_2, control cells synthesized and secreted very little expression of $PGF_{2\alpha}$ at 2.6pg/ml (+/- 0.42) and again the same trend was observed, with the vehicle control (BSA) increasing production to 7.37pg/ml (+/-1.47) and 50 µM OA further increasing PGF2α to 8.166pg/ml (+/-1.47). However, 100 µM OA slightly increased $PGF_{2\alpha}$ production compared to Vehicle control at 7.649pg/ml (+/- 1.2) but did not reach significance. Exposure to 200 µM and 400 µM OA decreased $PGF_{2\alpha}$ in comparison to Vehicle control with mean values of 7.51pg/ml (+/- 1.09) and 5.31pg/ml (+/-0.42). However, $PGF_{2\alpha}$ synthesis in response to 400 µM OA was significantly lower than all other treatments except the controls where $PGF_{2\alpha}$ was not dissimilar (Figure 4.3 B). Secretion of $PGF_{2\alpha}$ was highest in culture media after 2 hours at a mean concentration of 8.77pg/ml (+/- 1.12) and was significantly higher than both 24 and 48-hours incubation where mean concentrations were 5.687pg/ml (+/-0.73) and 4.355pg/ml

(+/-0.48) respectively. Time ($p < 0.001$), treatment ($p < 0.001$) and a time*treatment interaction ($p < 0.001$) were all found with trophoblast cell production of IL-6. Control treated cells didn't secrete IL-6, whereas BSA induced synthesis of IL-6 to 100.5pg/ml (+/- 0.81), which subsequently decreased with increasing concentrations of OA. 50 µM (97.45pg/ml +/- 1.19) and 100 µM (96.87pg/ml +/- 1.54) did not significantly decrease IL-6 concentrations compared to Vehicle. However, exposure to 200 µM OA significantly decreased IL-6 concentrations to 64.69pg/ml (+/- 0.85), which is significantly lower than Vehicle control, 50 µM and 100 µM. In contrast, IL-6 concentrations when exposed to 400 µM OA were significantly lower to all other treatments except control where the IL-6 levels were similar with a mean concentration of 13.87pg/ml (+/- 1.09). , Increasing the incubation time significantly increased IL-6 levels in the culture media increasing from a mean of 4.59pg/ml (+/- 0.69) after 2 hours, to 52.04pg/ml (+/- 1.12) after 24 and 129.93pg/ml (+/- 0.94) after 48 hours where all were significantly higher. The time and treatment interaction indicates that the effect of OA on IL-6 production increases with time (Figure 4.3 C). Interestingly, there was no TNF detected in the culture media, suggesting that the HTR8-SVneo cells may require extra stimulation to induce production and excretion of this cytokine.

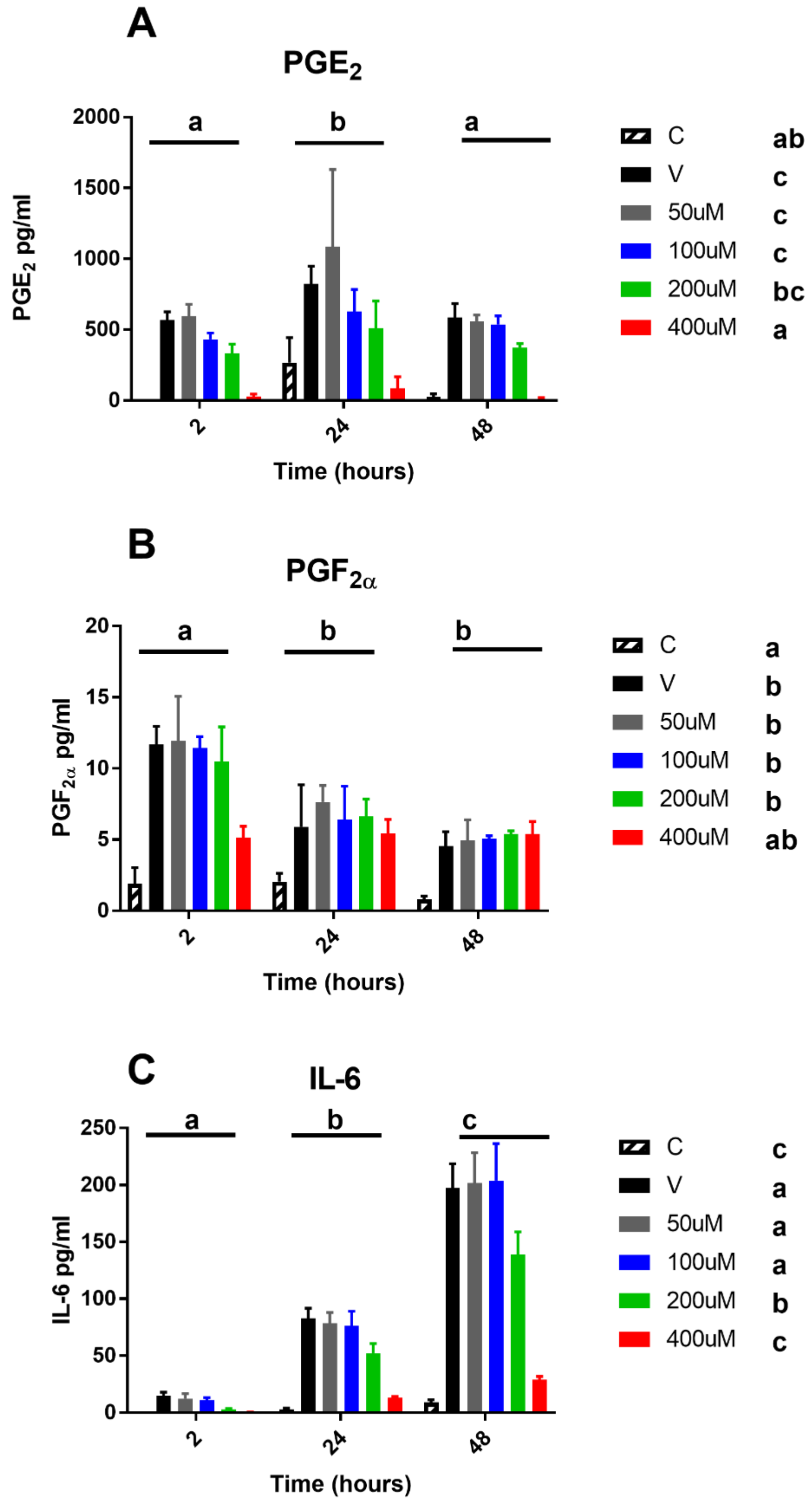

Figure 4.3 Secreted A) PGE_2 B) $PGF_{2\alpha}$ and C) IL-6 from HTR8-SVneo trophoblast cell line in response to Oleic acid (50uM, 100uM, 200uM and 400uM) after 2, 24 and 48 hours exposure. Measured from culture media using ELISA. Letters represent significant differences as per Bonferoni post-hoc test in Genstat for time (over the top of bars) and treatment (annotated next to treatment key), those with the same letter notations are not significantly different from one another.

4.3.4 Expression of genes associated with prostaglandin production and inflammation

RTqPCR was used to measure the mRNA expression of genes after both 24 and 48 hour timepoints. COX1 and COX2 are the first 2 genes in the production of prostaglandins from AA. COX1 levels were too low to accurately measure. COX2 saw no significant effect of time or treatment despite a visual trend of decreasing amounts of PTGS2 with increasing OA, across both time points but more prominent at 48h. The anomaly to this is at 400 µM at 48h where a large increase in PTGS2 is seen (Figure 4.4 A). PTGES is the enzyme that produces PGE_2 and was found to be significantly decreased with increasing amounts of OA ($p = 0.002$), with 400uM being significantly lower than C, V and 0uM but not 100 or 200uM (Figure 4.4 B). AKR1C3 and AKR1B1 are involved in the production of $PGF_{2\alpha}$ from PGE_2. Both were found at levels too low to be measured as the average CP values fall outside of the desired range (indicating very low levels low confidence in product due to large number of amplifications). EDN1 is implicated in PG synthesis (Belinky *et al.*, 2015) and has been demonstrated to be a target of OA (Xue *et al.*, 2013). OA treatment was found to have a significant effect on EDN1 mRNA expression in trophoblast cells ($p < 0.001$). EDN1 was found to be highest in control treated cells but this was significantly decreased by all other treatments. There were no other significant differences between treatment groups and the same pattern was observed after both 24 and 48 hours (Figure 4.4 C). PPARγ has anti-inflammatory signalling properties. No effect of treatment was seen on PPARγ mRNA levels but time was found to have a significant effect ($p < 0.001$) with 24 hours incubation having significantly higher mRNA expression levels of PPARγ (Figure 4.4 D).

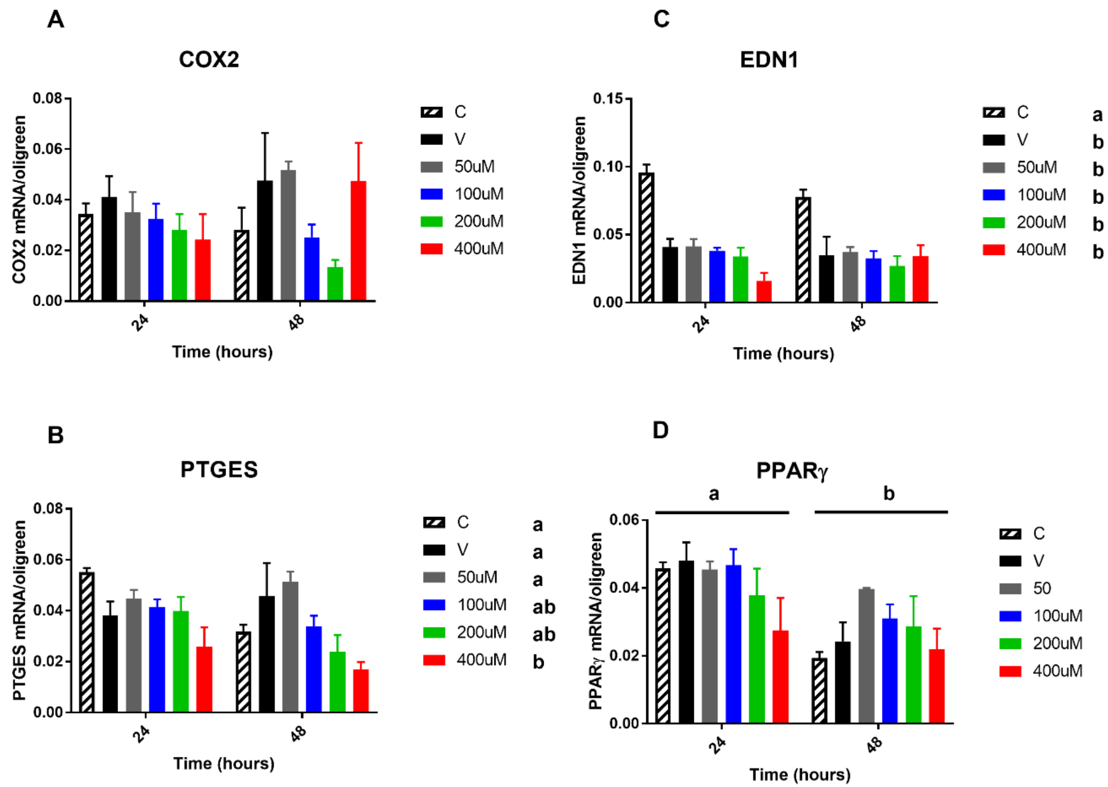

Figure 4.4 mRNA expression of genes involved in PG synthesis (A-C) and inflammation (D) measured through RTqPCR in response to in response to Oleic acid (50uM, 100uM, 200uM and 400uM) after 2, 24 and 48 hours exposure. Normalised to cDNA amount measured using oligreen. Letters represent significant differences as per Bonferoni post-hoc test in Genstat for overall time and treatment - time (over the top of bars) and treatment (annotated next to treatment key), those with the same letter notations are not significantly different from one another.

4.4 Discussion

Overall, OA was not found to have a significant effect on cell number but was seen to have a positive impact on cell viability. Doses of OA found at a physiological level (50-400 µM) were found to reduce the secretion of PGE_2, $PGF_{2\alpha}$ and IL-6 in HTR8-SVneo trophoblast cell line. A limited effect was found on the expression of genes involved in PG production and inflammation. No effect of OA was found on mRNA expression of PPARγ, PTGS2 nor AKR1B1. However, there was a significant decrease in PTGES expression with increasing concentrations of OA and time of exposure.

No treatment was found to have an impact on cell proliferation as determined by measuring cell number, however, OA was found to have a positive impact and improved cell viability. BSA is commonly used as a carrier for small molecules including FAs as we have used here. As varying concentrations of OA were used in these experiments a BSA solution was made up to keep the amount of BSA within the culture media consistent between treatment groups. BSA alone was used as a vehicle control, however, the CCK8 assay used to determine cell viability, saw a decrease in viability of around 25% via the addition of BSA compared to the control (serum-free media). This was subsequently rescued with increasing amounts of OA up to the maximum concentration of 400 µM taking cell viability back up to 100%. This was unusual and unexpected as BSA is often used as a supplement to help cells grow, particularly in serum free media as used here. There are 2 potential explanations for this. Firstly, the BSA used as the supplement to top up the other solutions, so they all contained consistent amounts of BSA was contaminated with something that decreased cell viability. We conclude that this is very unlikely as we would have been expected to also see a reduction in cell number through the DNA assays but there is no sign of this. The second potential explanation is it could be due to the colour of the BSA solution. The BSA solution was much darker and more yellow in colour in comparison to the media OA was diluted in and this was visible to the eye when solutions were made up, especially compared to the serum free media. As the CCK8 assay is measured through optical density and the media is not removed this could have altered readings for the plate reader. The colour of the BSA solution was also diluted when combined with OA solutions which was much paler and so again could explain the increasing cell viability with increasing OA concentration. In hindsight, an

alternative method of cell viability could have been performed to confirm the effects of OA on cell viability. However, it is still clear from both the DNA assay and CCK8 assay that OA does not have a detrimental effect on cell growth and survival and so any decreases in products seen from these treatments cannot be due to a decrease in number of cells or cell death. In the published literature the effect of OA on cell proliferation and viability is found to be inconsistent and probably due to differences in cell types used. Caco2 cells show an increase in the rate of proliferation (Storniolo *et al.*, 2020), however, in T-lymphocyte cells and HEPG2 OA was found to reduce cell viability (Fernanda Cury-Boaventura *et al.*, 2006; Kwon *et al.*, 2018). In the T-lymphocytes this was described to be due to excess triglycerides although they used 200 µM OA but in HEPG2 500 µM was used, it could be that OA has anti-proliferative effects when in excess and these upper limits can vary based on cell type. This is demonstrated in Stornolio et al (2020) as 1 µM OA had much greater mitogenic effects than 10 µM and this was further decreased with 50 µM in both cell number and DNA synthesis.

Absorption and incorporation of OA was measured through the use of Nile red assays which allowed us to measure total lipid content of the cells and also visualize lipid droplets. Intracellular lipids were seen to increase with time, as expected, as increased exposure to OA would allow a greater amount to enter the cells, however, no significant difference was seen between 24 and 48 hours and 200 µM and 400 µM, suggesting a saturation point. Several other studies have also seen an increase in lipids upon treatment with OA in multiple different cell types (Y. S. Liang et al., 2018; Malodobra-Mazur et al., 2019; Moreira et al., 2009). In a different trophoblasts cell line (BeWo) specifically, OA treatment lead to an increase of the triglyceride abundance in neutral lipids (Easton *et al.*, 2023).

Inflammatory overload within the uterine cavity is essential for the transition of myometrium from a quiescent to active state. A synergistic increase in pro-inflammatory mediators, IL-6, IL-8, IL-1B, TNF, COX2, PGE_2 and hCAP18 from amnion, chorion, placenta, decidua and myometrium is seen in labouring tissues compared to term non-labouring (Hadley *et al.*, 2018). Therefore, we looked at PGE_2, IL-6 and TNF secretion from placental cells to determine how OA may impact this, along with $PGF_{2\alpha}$

and expression of genes involved in prostaglandin synthesis pathway, including COX2 and anti-inflammatory pathways.

OA had a negative effect, decreasing PGE_2 and $PGF_{2\alpha}$ production. Control cells, who only had serum free media, had very little PGE_2 production with minimal amounts being detected in their media. The addition of BSA significantly increased the amount of PGE_2 produced, and this was then dose dependently decreased by OA supplementation with the highest concentration reduced to control levels, seeing very little to no production of PGE_2. $PGF_{2\alpha}$ had similar pattern, although much less production was seen from these cells and levels were closer to/overlapping with the limit of detection from the kit so caution should be taken when interpreting. The effect of OA in lower doses was much less pronounced, however, similar to PGE_2 the levels of $PGF_{2\alpha}$ with 400 µM treatment was reduced to levels similar to control cells. Several studies have seen extracts from medicinal plants decrease PG expression where the main constituents of these extracts are OA but also contain other fatty acids and substances such as polyphenols (Hosseinzadeh et al., 2017; Ko et al., 2016; G. Li et al., 2013). In a feeding study when OA was fed alongside high amounts of the short chain fatty acid precursor for prostaglandins LA, reduced levels of PGE_2 in multiple tissues compared to a diet high in LA alone (Barzanti *et al.*, 1999). Several other in vitro studies in multiple cell types have found OA treatment to reduce PGE_2 levels induced by pro-inflammatory mediators including LPS and TNF (Oh et al., 2009; Aryaeian, Shahram and Djalali, 2016; Lamy et al., 2016). Furthermore, in prostatic tissue treatment with OA reduced PGE_2 levels from malignant tissue back to ranges in benign tissue (Chaudry *et al.*, 1994). Prostaglandin synthesis from maternal endometrial cells isolated from ewes, stimulated by LPS or dexamethasone was significantly inhibited by OA, however this was not seen in fetal chorion cells (Cheng *et al.*, 2015).

IL-6 is part of the pro-inflammatory surge and is found in greater levels in labouring tissues and TNF is released from placental tissues and induces the formation of prostaglandins (R. Smith, 2001). No extra-cellular TNF was demonstrated within this study. It could be because this is only produced at term or when these cells are stimulated which in this study were not. IL-6 has been shown to be secreted from human trophoblast cells (Kameda et al., 1990; Stephanou et al., 1995). In this study

IL-6 was not produced in control cells. Previous studies have found similar with very little IL-6 production in control treated cells and much less than the BeWo cell line (Oliveira *et al.*, 2021). BSA has been shown in macrophages to increase IL-6 secretion suggesting an inflammatory activity (Shacter et al., 1993). Our results show a dose dependent decrease in baseline IL-6 production and an increasing effect of time on the potency of OA. This has been observed in many other studies. Macrophages treated with OA have been demonstrated to show a reduction in inflammatory cytokines including IL-6 (Müller *et al.*, 2021; Santamarina *et al.*, 2021) and others including TNF and IL-1b (Karasawa *et al.*, 2018). In other cell types, OA has also demonstrated anti-inflammatory effects (Matesanz *et al.*, 2012) again specifically looking at IL-6 and TNF (Pupe et al., 2001; Beaulieu et al., 2021). Particularly BeWo cells, another trophoblastic cell line saw, increased markers associated with anti-inflammatory pathways (Easton *et al.*, 2023).

There are several proposed mechanisms for these changes observed. Fatty acids can elicit changes through a variety of mechanisms including changes in transcription through nuclear receptor activity, indirectly impacting transcription factors or alteration of lipid rafts and signalling (Jump, 2004). There are very few studies looking at the impacts of OA in trophoblasts, particularly regarding prostaglandins and inflammation so information is pulled from many other cell types, alongside our PCR work to try and conceptualize a mechanism.

The first proposed route through which OA can reduce PG expression is through a reduction in COX enzymes. Control treated cells had similar levels of COX2 to other treatments yet very little PGE_2 and $PGF_{2\alpha}$ production. This could be explained by the reduction in EDN1. Control treated cells had significantly higher END1 mRNA compared to any of the OA treated or vehicle cells. In amnion cells EDN-1 inhibited PGE_2 production (Mitchell *et al.*, 1990). This study found no significant effect of OA on COX2 mRNA expression at any concentration or time point. At 24h a pattern of decreasing COX2 with increasing OA was observed, although subtle. This is more pronounced at the 48h time point, however, treatment with 400 µM OA, increased COX2 mRNA expression dramatically. 5 studies have demonstrated a reduced protein and/or mRNA expression of COX2 enzyme (Oh et al., 2009; Bastiaansen-Jenniskens et al., 2013; Ko et al., 2016; Lamy et al., 2016; Song et al., 2019). All of these studies

were in different cell types, however, all had stimulation with either LPS or TNF that induced the expression of COX2 and little or no response was seen without this stimulation. Our cells had no stimulation from LPS or TNF and control cells expressed similar levels of COX2 despite secreting very little PGE_2 or $PGF_{2\alpha}$. As no significant change in COX2 was observed, we decided to look at mRNA expression of other enzymes involved in PG synthesis. COX1 was at levels too low for detection. OA and time were seen to have a significant negative effect on PTGES mRNA expression, which decreased as OA increased. 400 µM of OA was found to be significantly lower than vehicle and 50 µM but not the other concentrations of OA. PTGES is responsible for the production of PGE_2 so this could explain the reduction seen here. A study in rabbit kidney microsomes also found PGE_2 inhibition and suggested this be due to a reduction in PTGES, although this was using the fatty acyl coA derivative of OA (Fujimoto, Yonemura and Sakuma, 2008). The enzymes involved in $PGF_{2\alpha}$ production were also measured. AKR1C3 and AKR1B1 had very low expression which could explain the much lower levels of $PGF_{2\alpha}$ seen.

Although we saw no significant change in COX2 mRNA expression the differences seen with OA could be due to inhibition of the enzyme. Several enzyme activity assays have demonstrated COX2 inhibition by OA, although to varying degrees (Momin, De Witt and Nair, 2003; Sato, Kofujita and Tsuda, 2007; Hosseinzadeh et al., 2017; Termer et al., 2021). This has also been demonstrated in tissue (S. H. Kim et al., 2021). However, this is not always the case, other have found no COX2 inhibition (Ringbom et al., 2001; Fujimoto, Yonemura and Sakuma, 2008; VanHorn et al., 2012) or OA action being entirely independent of COX2 (Bozza *et al.*, 1996). It has also been shown that OA can bind to the COX1 active site despite not being a substrate but instead could act as a competitive inhibitor to AA which could then reduce PG production (Termer *et al.*, 2021). Alternative enzymes in prostaglandin synthesis have been found to be impacted, in rat neurons OA has been shown to inhibit PGDH (Hiroyoshi *et al.*, 1983).

A third mechanism of action is suggested to be displacement of AA, leading to less substrate for COX enzymes and so reduced PG production. Feeding rats a diet high in OA was found to decrease the amount of AA in the spleen and lung but did not result in altered PG synthesis (Lokesh, LiCari and Kinsella, 1992). Within macrophages this

was dependent on amount of OA used, at 100 µM concentration a decrease in relative amounts of AA was seen but at 200 µM free AA levels were found to be increased (Müller *et al.*, 2021). In breast cancer OA has been found to stimulate AA release via a phospholipase C dependent pathway (Soto-Guzman *et al.*, 2013). We did not measure FA profiles of these cells but future work would be useful to understand how OA could affect relative amounts, as well as membrane and free FA profiles to understand how OA may affect AA. Within our rat study we found an altered OA:AA ratio based on the HFHC diet which indicated greater OA levels in the placenta compared to AA, however AA levels remain consistent despite dietary changes.

OA could also be acting through PPARs to elicit it's anti-inflammatory effects. OA has found to induce expression of PPARγ in hepatocytes (Edvardsson et al., 2006; Medeiros-De-Moraes et al., 2018), intestinal epithelial cells (Y. S. Liang et al., 2018) and adipocytes (Kokta et al., 2008; Cheguru et al., 2012; Luo et al., 2013; Chung et al., 2016; Turner et al., 2017). One suggested mechanism for OAs control of PPARγ expression is altering the methylation status of the PPARγ promoter site (Malodobra-Mazur, Cierzniak and Dobosz, 2019). In our rat study, an increase in PPARγ mRNA was observed and coupled to an increase in OA. When we looked at trophoblast cells treated with OA, no PPARγ mRNA increase was seen at any concentrations. Again, this may not solely to be associated with expression but alterations in signalling could explain the responses seen. Increased binding of PPAR response elements has been seen with OA treatment in pancreatic cells (Vassiliou *et al.*, 2009) and T-lymphocytes (Fernanda Cury-Boaventura *et al.*, 2006). Other demonstrations of OA signalling through PPARγ have shown the reduction or reversal of the effects of OA treatment from the use of PPARγ inhibitors (Luo *et al.*, 2013). OA may act directly as a ligand to PPARγ as competitive binding assays have identified OA as a strong activator of PPARγ (Kliewer *et al.*, 1997) in both rat and human assays (Evans *et al.*, 2022). Alternatively, OA has been shown to influence the deacetylation of PGC1a (a PPARγ co-activator) via SIRT1 deacetylation activity through AMPK signaling (Palomer *et al.*, 2018). PPARγ could also be involved with OA signaling as OA has been demonstrated to be a stronger activator of PPARα as opposed to gamma (Kliewer *et al.*, 1997) and has been

shown to stimulate activity of PPARα (Hanley et al., 1998; Coll et al., 2008; Evans et al., 2022).

NFκB signaling has been implicated in many studies looking at OA. PPARγ has been shown to antagonize NFκB and inflammatory gene activation (Ackerman IV *et al.*, 2005). NFκB is a family of major inflammatory regulators. Acting through the canonical or non-canonical pathway as an inducible transcription factor, regulating a large array of genes involved in inflammatory responses such as cytokines and inflammasomes (T. Liu et al., 2017). The canonical pathway is triggered in response to a diverse array of stimuli including LPS and TNF and involves IKK complex (IKKa and IKKb catalytic subunits and NEMO a regulatory subunit) phosphorylating IkBa leading to it's degradation and so allowing the canonical NFκB members (p50/RelA (also known as p65), p50/c-Rel) to translocate to the nucleus and bind DNA (T. Liu et al., 2017). In terms of labour, NFκB is central to labour associated pathways. Activation through stretch and many other cytokines up regulated during labour such as IL-1β and TNF, as well as, being directly observed to increase pro-inflammatory cytokines itself. NFκB is also implicated in PG synthesis through COX2 and PLA2 expression, along with MMP expression (T. Liu et al., 2017). Furthermore, DNA binding activity has been demonstrated to be upregulated in trophoblasts in primary cultures from term tissue (Gómez-Chávez *et al.*, 2021). OA has been shown to reduce LPS induced NFκB activation in HUVEC cells (Massaro *et al.*, 2002). This has been shown to occur by blocking the phosphorylation of IkB in murine microglia (Oh *et al.*, 2009) or decreasing p65 phosphorylation in macrophages (Santamarina *et al.*, 2021). An alternative mechanism of reducing NFκB signalling has been proposed within diabetes and insulin resistance as OA has been shown to inhibit the action of palmitic acid. Addition of palmitic acid to cells increases DAG content within the cell through the reduction in DGAT2 gene, which converts DAG into TAG. OA has been shown to prevent this down regulation of DGAT2 and so increase TAG levels and consequently reduce DAG levels. DAG stimulates PKC0 which in turn activates IKKb resulting in the phosphorylation and degradation of IκBa thus allowing NFκB complex to initiate transcription of cytokines. Reduction of DAG reduces this signaling pathway therefore reducing NFκB signaling (Palomer *et al.*, 2018). OA has been shown to increase TAG levels within trophoblast cells (Easton *et al.*, 2023) so this could be a viable

mechanism of action. This is not definitive as 2 studies have demonstrated OA can activate PKC (Egan, Lu and Greene, 1999; Haastrup et al., 2001). NFκB signalling can be elicited through direct stimulation from inflammatory factors present during labour such LPS and TNF and has been demonstrated a potential role of OA for reducing this.

There are other signalling pathways that OA has been demonstrated to influence which can act independently or interact with NFκB aswell. In primary trophoblast cells OA has been found to act through mTOR (Silva *et al.*, 2023). In the same cell line used here, OA has signalled through PI3/Akt pathway through increasing levels of p-S6 (C. Yang et al., 2017). In addition to this, most MAPK signalling proteins including ERK1/2, P90RSk, JNK and p38 were phosphorylated in response to OA treatment in trophoblasts with activation of PI3/AKT and ERK 1/ 2 crosstalk demonstrated (C. Yang et al., 2017). But when being used as a therapeutic agent JNK and ERK phosphorylation is suppressed (Perdomo et al., 2015; Ko et al., 2016; Lamy et al., 2016) and no evidence of change in p38. OA has also demonstrated to affect AMPK signalling. When utilized alongside palmitic acid, OA was found to restore AMPK activity (Palomer *et al.*, 2018) and sustained AMPK signaling was shown to disrupt NLRP3 inflammasome in adipose tissue (Palomer *et al.*, 2018).

Further extracellular signalling pathways may also be involved. From our data there is no significant increase in intracellular lipids within the cells for 200 µM and 400 µM treatment of OA but for nearly all markers measured 400 µM is significantly more effective. Furthermore, there is little change in intracellular lipid levels between the treatment groups after 2 hours incubation, but OA is impacting all markers from the 2h time point. This would suggest OA is not solely acting via transport into the cell but also binding receptors on the cell surface. OA lipid body biogenesis is mediated by a Gai-coupled receptor on the cell surface in epithelial cells which can lead to rapid and specific intracellular signaling (Moreira *et al.*, 2009). MUFAs have been demonstrated to bind GPCR40 and elicits cytoprotective effects possibly through PI3 and PKB signaling pathways (Nolan and Larter, 2009). Furthermore, OA has been shown to bind GPCR120, however, this resulted in an increase in ERK1/2 and COX2 mRNA, which lead to increased PG synthesis (Widmayer *et al.*, 2019).

There are huge discrepancies on the impacts OA is seen to have on inflammation processes and PG synthesis, and how these effects are elicited. The majority of evidence discussed above are in cells with extra stimulation such as LPS, TNF or SFAs such as palmitic acid that induce inflammatory responses. In these studies, OA is being used as a therapeutic agent, neutralising the inflammatory effects of these challenges. However, there are a range of studies which find opposing effects, and these are often when OA is being used alone to induce inflammation, injury or toxicity. One common example of this is the study of non-alcoholic fatty liver disease (NAFLD). High concentrations of OA are used to induce steatosis in liver cells (Rogue et al., 2014; Kwon et al., 2018; Song et al., 2023). Kwon et al (2018) treated HEPG2 cells with increasing amounts of OA until a negative effect on cell viability was seen and this wasn't until levels exceeding what we used here at 500 µM. This was described to be due to lipid accumulation within cells and hypothesized that reduced AMPK signalling through LKB1 as the cause along with an increase in TNF (Kwon *et al.*, 2018). In contrast, when OA is treated alongside palmitic acid it's found to reverse the down regulation of AMPK signalling caused by palmitic acid (Palomer *et al.*, 2018). In these models OA is found to decrease PPARγ protein levels (Song et al., 2023) and activation of PPARγ signaling was found to reduce the effect of OA induced steatosis (Rogue *et al.*, 2014). A different liver cell line, LO2, when treated with OA to induce steatosis was not found to increase PPARγ protein expression but 4 genes in the PPARγ signalling pathway were found to be significantly increased, although at much lower OA doses of approximately 177 µM compared to 500 µM (Zhang et al., 2021). In this instance it is hard to tell whether the discrepancies are due to the fact it different cell lines or lower doses are used. In ovarian granulosa cells 500 µM of OA was also found to be inflammatory, upregulating cytokine production including IL-6 (Lai *et al.*, 2022). This could mean OA becomes lipotoxic after a certain threshold and this could also vary between cells. The doses used in our study could be below the lipotoxic range, however, these were chosen to be within physiological range of pregnant women in late gestation (Villa *et al.*, 2009). Differing effects of OA have been found within the same cell type under different conditions. Llor et al (2003) treated a cancerous colorectal cell line and a normal colorectal cell line with OA and saw differing responses. OA had strong apoptotic effects within the cancerous cells, but minimal apoptosis was seen within the normal cell line (Llor *et al.*, 2003). Method of administration of OA seems to alter results. Focusing on lung studies and OA

within animal trials, it is established that an injection of OA causes lung injury in mice (Hang *et al.*, 2019), however OA given to mice orally has been shown to reduce symptoms of asthma and inflammation severity (S. H. Kim et al., 2021) and reduced tumorigenesis in lung tissue when fed to mice (Yamaki *et al.*, 2002). This is suggested to be due to the method of OA administration. A study comparing intravenous and intragastric administration of OA found intravenous to cause acute lung injury but intragastric not toxic at any doses tested, suggested to due to esterification during the absorption within the intestines and a slower release of OA (Gonçalves De Albuquerque *et al.*, 2012). These studies provide further evidence that OA actions vary based on the context of the experiment, including variables such as dosage, other treatments or challenges and how OA is given.

From our rat model and some of our human data it suggests that an increase in OA is maybe a response to higher dietary consumption SFA often seen in obesogenic diets and, as demonstrated in multiple studies, OA reduces the inflammation caused by SFA (Colvin *et al.*, 2017; Natarajan *et al.*, 2021). In this scenario it could mean that OA, levels often seen in obese women, are anti-inflammatory within placental cells and when coupled with excess SFAs could reduce their lipotoxic effects. Furthermore, as labour is an inflammatory state OA in greater amounts could again reduce inflammatory response form TNF. This could lead to a net anti-inflammatory placenta with reduced cytokine and PG synthesis thus disrupted labour. Further work needs to be done to confirm this, particularly in trophoblast cells to clarify the effect of OA on PG and inflammatory cytokine release, especially under more labour like condition.

4.4.1 Limitations

One of the key limitations of this experimental study is the fact that the cell line is derived from first trimester trophoblasts. This was due to lack of availability of term trophoblast cells and HTR8-SVneo being a commonly used trophoblast cell line capable of producing prostaglandins as BeWo and JEG-3 (the alternatives) have been shown to not possess the COX2 enzyme (Johansen *et al.*, 2000). To improve this primary trophoblast cells from term placenta would be the best option, particularly if these could be sampled from women with different BMI. These cells were also not under 'labour like' conditions, treatment under an oestrogenic environment could give a clearer picture of how this would work at term. Finally, this study only looks at

OA in isolation and FAs modulate each other extensively. Fatty acids have been described as having unpredictable effects when used in combination (Motaung E and al, 1999) and particularly concerning PPARs the degree of activation of these is suggested to be due to a function of the sum of FAs and metabolites that can interact with the receptor (Kliewer *et al.*, 1997). Cell lines are useful models but are not fully reflective of the complex environment in which the human placenta is maintained in vivo and the combined effects of additional dietary nutrients (Easton *et al.*, 2023).

To gain a better understanding of how OA acts in trophoblast cells I would repeat the experiment but with dexamethasone, a synthetic glucocorticoid, which can mimic the endogenous cortisol rise and induce parturition as used in Cheng et al (2015). It would be expected that this would increase the effects on PGs and inflammatory cytokines of OA seen here based on other studies finding OA neutralizes the effects of other agents. Following this, treatment with PPARγ inhibitors would be used at maximal concentration to see if PPARγ signalling is involved in the effects of OA. As NFκB, PI3/AKT and mTOR signalling have all been demonstrated to be associated with OA actions in trophoblast cells, modulators of these signalling pathways would be used alongside OA treatments to try and elucidate a clearer mechanism of OA action.

4.5 Conclusions

In conclusion, this chapter has shown compelling evidence that the increased OA, found in the placenta from the maternal obesity rat model, can have negative impacts on pro-labour mediators. OA at concentrations within the physiological ranges found in obese women, significantly reduce the production of both PGE_2 and $PGF_{2\alpha}$, as well as IL-6. These are key mediators in the inflammatory cascade seen within the initiation of labour and form part of positive feedback loops which further perpetuate expression of PGs and pro-inflammatory mediators within the intra-uterine cavity. Although, a complete mechanism of this has not been proven, a potential signalling pathway and experimental design has been proposed.

5 Retrospective and prospective cohort study in pregnant Pakistani women

5.1 Introduction

Poor birth outcomes, including PTB and LBW are a major public health problem across the world, particularly in LMICs such as Pakistan (World Health Organisation, 2012). Central and southern Asia account for 17% of all maternal deaths worldwide with maternal mortality rates for southern Asia estimated to be 134 per 100,000 live births (Fazel et al., 2018). Complications of pregnancy and childbirth remains the leading cause of death and disability for childbearing women in Pakistan (Z. Mumtaz et al., 2014). PTBs disproportionately affect LMICs with more than 60% occurring in Africa and South Asia (World Health Organisation, 2012). Rates in Pakistan exceed 15%, making them the top 10 highest globally for PTB (World Health Organisation, 2012). LBW (< 2500g) rates are found to be 12.6% in Pakistan and again also seen as one of the highest LBW rates across the world (Pusdekar et al., 2020). Adverse pregnancy outcomes such as PTB, CS and LBW all have negative impacts on the mother, her offspring and the wider population. Despite CS improving maternal and foetal outcomes where necessary, WHO has stated that there are no additional benefits when CS rates increase above 10–15% (Maciej Serda et al., 2010). A recent study estimated CS rates in Southern Asia to be 19% (Amjad et al., 2020) with a reported 19.6% CS rate in 2018 for Pakistan, far exceeding the WHO recommendations (Betran et al., 2021).

Unfortunately, Pakistan is not on track to meet their recommended improvements in antenatal care and to reduce maternal and infant morbidity and mortality (Waqas et al., 2020). Due to population growth and limited resources the situation is only worsening (Waqas et al., 2020). A recent scientometric analysis of births in Pakistan identified a number of priorities to address the poor maternal and infant health outcomes. A key priority was to focus on identifying factors affecting maternal and infant morbidity but also the early life causes and predictors of NCDs to understand the impact and to identify new ways to address the problem (Waqas et al., 2020). It's important to fully understand the drivers of adverse pregnancy outcomes to help break these intergenerational cycles and help improve the health of the Pakistan

population. Maternal health is key to improving wellbeing today and for the future (Hussein, 2017).

Investigating the impact of NCDs on adverse pregnancy outcomes is important but NCDs can be even more exasperated in lower-middle income countries (LMICs), where often joint burdens of obesity, NCDs and poor dietary health is evident linking both poor maternal and neonatal health. The rising rates of obesity in every region of the world is thought to be increasing at greater rates in LMICs where women appear to bear the larger burden (Ford et al., 2017). The Pakistan demographic health survey 2017-18 found 22% of women aged 15-49 to be obese (BMI over 30) and a further 30% to be overweight (BMI 25-29.9). This survey identifies a 12% increase in the level of overweight and obese women since the 2012-13 survey (National Institute of Population Studies (NIPS) [Pakistan], 2019). NCDs are responsible for 71% of all global deaths, disproportionately affecting the LMIC's. Some of the world's highest rates of NCDs are in South Asian countries and often described as an epidemic (Waqas et al., 2020). This widespread occurrence of NCDs not only affects older generations but spans the entire population including women of reproductive age (Hussein, 2017). Studies have found a high burden of NCDs in urban (F. S. Khan et al., 2013) and semi-urban settings (Naseem et al., 2016) within Pakistan. In the South Asian mega city, Karachi, 8% of the population are diabetic and 18% hypertensive. Furthermore 39% of the population have been determined to be pre-hypertensive and 40% pre-diabetic, emphasising that the burden will only increase further (F. S. Khan et al., 2013). A survey of 12000 households identified that almost 39% exhibit high blood pressure and approximately 15% were diabetic (Naseem et al., 2016). It is well established that obesity and risk of developing NCD's are related (Selassie and Sinha, 2011), but any research linking NCDs and adverse pregnancy outcomes is currently lacking. As was observed in chapter 3 and research published by our laboratory (Muir *et al.*, 2018), habitual diet often associated with maternal obesity significantly alters the fatty acid status and composition of the placenta and plasma and can have detrimental effects on pregnancy outcomes. Nutrition, obesity and NCDs are intertwined, with the increase of NCDs and obesity being led by a nutritional and lifestyle transition (Government of Pakistan Planning Commission Nutrition Section, 2019). Healthy dietary patterns decrease the risk of all-cause mortality and cardiovascular disease (Kant, 2004) and adherence to a lacto-vegetarian diet (high in

fruit, vegetables and unprocessed food) has been shown to be inversely associated with BMI (Koutras et al., 2022). Similarly, adherence to the healthy eating index or low inflammatory diet have been linked to reduction in risk of type 2 diabetes (Brelk & Gregoric, 2022; Micha et al., 2020). Pakistan faces a rise in NCDs coupled with over and under nutrition that puts a huge strain on the healthcare system (Iqbal et al., 2017).

5.1.1 Aims

The aim of the current chapter was to evaluate the impact of obesity, NCDs and dietary patterns on pregnancy outcomes. The retrospective study obtained data from medical records of women who had given birth at Aga Khan University hospital with existing NCDs. It is hypothesised that obesity and or habitual diet will increase the risk of negative pregnancy outcomes and the inclusion of NCDs within the model will help identify whether they increase the risk of developing adverse pregnancy outcomes. The prospective study consisted of a food frequency questionnaire given to a subset of pregnant women, to gain a better understanding of their dietary habits. This was used to try and understand the diet quality and consumption patterns of Pakistani women and the impact this has on pregnancy and birth outcomes. We hypothesised that consumption of foods high in saturated fat to have an adverse impact on labour outcomes, and a healthier diet to be associated with positive pregnancy outcomes. This research could help us understand whether obesity, in combination with diet and NCDS are key drivers behind adverse pregnancy outcomes.

5.2 Methods

5.2.1 Study design, participants and data

Non-communicable diseases: The data used in the analysis is from a retrospective cohort study that took place at Aga Khan University Hospital, Pakistan. As the study involved human subjects not involved directly, and with no intervention the Institutional Ethics Review Committee at Aga Khan University (AKU) issued and approved an exemption letter for full ethics review prior to the study commencing. The cohort consists of pregnant women, admitted to the obstetrics department that gave birth between 2015 and 2016, with pre-existing NCDs. The data collected comprised of maternal age, marital status and socioeconomic status. Participants BMI was calculated from their height and weight, recorded prior to pregnancy. A detailed medical history including details of their NCDs, the duration of these diseases and obstetric information from previous pregnancies were retrieved (live births, miscarriages, ectopic pregnancies, induced abortions, caesarean sections, stillbirths). For the current pregnancy, detailed obstetrical information was also recorded (gestational age at delivery, labour and delivery types, delivery outcome, medical and obstetric complications, infections, ICU/critical care admissions, duration of hospital stays, birthweight, APGAR scores, sex, birth injuries and any other anomalies). 857 women with existing NCDs were randomly selected from patient records. After excluding all those with missing information, 817 subjects were included in the final analysis. Pre-pregnancy BMI was calculated from recorded height and weight at first visit. Obesity was classified as those with a BMI greater than 23 as according to the adjusted South Asian BMI classifications published from the WHO. Pre-gestational type 2 diabetes and hypertension were the NCDs chosen as the exposure variables (yes/no). Non-communicable disease burden was calculated by summing the number of diseases an individual had.

Diet study: The data in the prospective cohort study also took place at Aga Khan University hospital, Pakistan that consisted of women who gave birth in 2018. Data was collected from 74 women and comprised of demographic information – age, marital status, area, education and occupation of mother and husband. BMI was calculated from weight and height at first visit. Screening for nutritional and lifestyle habits included information on activity levels at work and leisure time; smoking and

addiction status; special diets and food frequency questionnaire from CARRS surveillance study version 1.1 2009 (Nair et al., 2012), with 18 foods listed and ranked from 1 never consumed/less than once, 2 monthly, 3 weekly and 4 daily. NCD, medical and family history was also obtained. Detailed obstetrical information from current labour including gestational age at delivery, outcome, mode of delivery and any complications as well as neonatal outcomes including birth weight, APGAR scores, sex, injuries and anomalies. AKU has its own local ethics research committee (ERC). Professor Qureshi (PI, AKUH) steered the study through this Ethics Committee as well as the National Bioethics Committee of Pakistan. The patient information sheet and consent form were available in Urdu as the native language, as well as in English. All patient data has been anonymised.

5.2.2 Variables

Non-communicable diseases: The primary aim of this study was to look at the impact of obesity on pregnancy outcomes, so premature birth (before 37 weeks/ 37+ weeks gestational age at delivery), mode of delivery (spontaneous vaginal delivery, elective CS and emergency CS) and birth weight (g) were chosen as the outcome variables. Secondary to this the impact of NCDs on these outcomes, along with the combined impact of obesity and NCDs. The following confounding variables were chosen based on existing well-established interactions with our outcome variables, these included maternal age (years); previous number of live births, CS and preterm births; pre-eclampsia (yes/no); sex of offspring (male/female); birth weight (g) and gestational age at delivery (weeks - not used in preterm).

Diet study: The primary aim of this study was to look at the influence diet may have on pregnancy outcomes, so premature birth (before 37 weeks/ 37+ weeks gestational age at delivery) and mode of delivery (spontaneous vaginal delivery, elective CS and emergency CS) were chosen as outcome variables. The following confounding variables were again chosen based on existing knowledge of interactions with our outcome variables, these included maternal age (years); BMI (kg/m^2); previous number of live births, CS and preterm births; pre-eclampsia (yes/no) ; sex of offspring (male/female); birth weight (g) and gestational age at delivery (weeks - not used in preterm). To calculate diet scores individual foods were grouped based on commonalities as follows: Dairy (Milk, eggs and milk products); high saturated fat (deep-fried western, deep fried desi and western desserts); Grains (cereals and whole

grains); protein (meat, fish, shellfish and legumes) and healthy fats (fish, shellfish and nuts). These had the scores totalled up for how often they were consumed (max of 4 per item), the higher the score the more frequent the consumption. These were then grouped as high or low consumption – high dairy score of over 8 (max score 12), low saturated fat equal to 6 and below (max score 12), high protein greater than 10 (max score 16) and high grains equal to 6 and greater (max score 8). A healthy fat to saturated fat ratio was calculated by combining the frequency scores of each item per group (max of 12 in each) and converting to a decimal, the higher the decimal the higher the consumption of healthy fats vs saturated fat. This was split into two 50% groups of high healthy fat consumption and low healthy fat consumption relative to saturated fat consumption. The PDGN diet adherence score was calculated by using the PDGN dietary recommendations for pregnant women (Milk and milk products 2-3 servings/day; Cereals and grains 5-6 servings/day; Vegetables 2-3 servings/day; Fruits 2-3 servings/day; Meat (including fish) pulses and eggs 2-3 servings/day). The foods for each category were grouped accordingly and if consumed daily (the highest frequency we could obtain) a score of 1 was awarded per group and 0 if not. These were then totalled to form a total adherence score with a maximum of 5, being most adherent to guidelines. This was then split into high (scores of 3 and 4) and low adherence (scores of 1 and 2) groups.

5.2.3 Statistical analysis

All statistics were done in IBM SPSS Statistics program V.27 (IBM Armonk, NY: IBM Corp) and figures produced in GraphPad Prism version 8.0.0 (GraphPad Software, San Diego, California USA, www.graphpad.com). Frequency tables were used to produce demographic information.

Non-communicable diseases: Crosstabs with column percentages were produced to show the proportions of each disease with the outcomes and chi-squared to tests for associations with categorical variables. For premature birth, crude odds ratios (cOR) were calculated. For mode of delivery cORs were calculated for each category with spontaneous birth used as the reference category. Multinomial logistic regression models were run to produce the adjusted ORs (aOR) including all NCDs and confounding variables for preterm birth and mode of delivery outcomes. Means and standard deviations were calculated, and t-tests were run for birth weight and each NCD group.

Diet study: Crosstabs with column percentages were produced to show the proportions of each food or diet score group with the outcomes and chi-squared to tests for associations with categorical variables. For premature birth crude odds ratios (cOR) were calculated using binary logistic regression model. Multinomial logistic regression models were used for each diet group (as a continuous variable) and mode of delivery to calculate cORs with spontaneous birth used as the reference category.

5.3 Results

5.3.1 BMI:

5.3.1.1 Demographics

817 women delivered singleton babies during the study time period. Key demographic and previous birth data are shown in Table 5.1. BMI ranged from 14 to 52 kg/m^2, with a mean BMI of 29kg/m^2. Information on NCDs and current birth data is presented in Table 5.2. The BMI categories for the study were based on WHO adjusted values for south Asian population, so a BMI over 27 is considered obese (Nishida et al., 2004). 445 women (54.5%) were classed as obese and a further 248 (30.4%) considered overweight.

Information on Mothers		*Number of women (%)*
Age (mean =30.8)	37+	100 (12.2%)
	28-36	505 (61.9%)
	<27	212 (25.9%)
Marital status	Divorced	5 (0.6%)
	Married	812 (99.4%)
Occupation	Employed	64 (7.8%)
	Housewife	753 (92.2%)
BMI (mean = 28.6)	Underweight (BMI<18.5)	22 (2.7%)
	Normal (<18.5 BMI≤)	102 (12.5%)
	Overweight (BMI 23-27)	248 (30.4%)
	Obese (BMI>27)	445 (54.5%)
Previous live births history		
Live Births	0	279 (34.1%)
	1	251 (30.7%)
	2	153 (18.7%)
	3	91 (11.1%)
	4+	44 (5.3%)
Miscarriages	0	558 (68.3%)
	1	156 (19.1%)
	2	61 (7.5%)
	3+	42 (5.2%)
Ectopic pregnancies	0	804 (98.4%)
	1+	13 (1.6%)
Stillbirths	0	784 (96%)
	1	27 (3.3%)
	2+	6 (0.7%)
Induced abortions	0	796 (97.4%)
	1+	21 (2.5%)
Caesarean Sections	0	493 (60.3%)
	1	176 (21.5%)
	2	92 (11.3%)
	3+	56 (6.8%)
Preterm	0	737 (90.2%)
	1	68 (7.1%)
	2+	22 (2.7%)

Table 5.1 Demographic information and previous birth history for all 817 mothers included in the final analysis. Count and percentage shown for each category and mean for maternal age and BMI.

NCDs		*Number of women (%)*
Diabetes Type 1	Yes	20 (2.4%)
	No	797 (97.6%)
Diabetes Type 2	Yes	95 (11.6%)
	No	722 (88.4%)
Hypertension	Yes	153 (18.7%)
	No	664 (81.3%)
Asthma	Yes	273 (33.4%)
	No	544 (66.6%)
Thyroid disorder	Yes	376 (46%)
	No	441 (54%)
Burden	1	722 (88.4%)
	2	83 (10.2%)
	3	12 (1.5%)
Current birth data		
Sex	Female	397 (48.6%)
	Male	420 (51.4%)
Gestational age at delivery	Less than 28	4 (0.6%)
(Mean =37)	28-31	13 (1.6%)
	32-36	183 (22.5%)
	37+	616 (75.3%)
Pre-eclampsia	Yes	36 (4.4%)
	No	78 (95.6%)

Table 5.2 Non-communicable disease prevalence and current birth data for all 817 women included in the analysis. NCD burden was calculated by how many NCDs each woman had. Count and percentage of all women shown for each category and mean weeks calculated for gestational age at delivery.

5.3.2 Preterm birth

The earliest recorded birth was at 25 weeks gestation, with the average being 37.25 weeks. Premature delivery was defined as earlier than 37 weeks gestation, 200 women (24.7%) delivered preterm. Proportion of each BMI group who delivered Term or Preterm are shown in Figure 5.1 A. Binary logistic regression found no BMI categories to be significantly associated with the risk of preterm birth (Normal BMI cOR = 0.48, p = 0.15; Overweight cOR = 0.42, p = 0.07; Obese cOR = 0.67, p = 0.38).

5.3.1.3 Mode of delivery

Elective CS was the most common type of delivery with 281 women (34.4%) delivering this way, followed by emergency CS with 269 women (32.9%). A further 267 women (32.7%) had a spontaneous vaginal delivery (SVD). The proportion of each mode of delivery per BMI group are shown in Figure 5.1 B. Obesity was found to significantly increase the risk of elective CS (cOR = 2.35 p = 0.02), and emergency CS (cOR = 1.86 p = 0.017). Being overweight also increased the elective CS risk (cOR = 1.86, p = 0.033). After adjusting for confounding variables no associataions remained significant (Table 5.4).

5.3.1.4 NCDs

Pre-gestational Type 2 diabetes and hypertension, were added to the multinomial logistic regression model as often highly correlated with obesity and placental disorders. Following this correction, no associations were significant (table 5.5).

5.3.1.5 Birthweight

Median, interquartile ranges, minimum and maximum birthweights are shown for each BMI group in Figure 5.1 C. ANOVA found no significant differences between the groups, suggesting that BMI had no effect on birthweight.

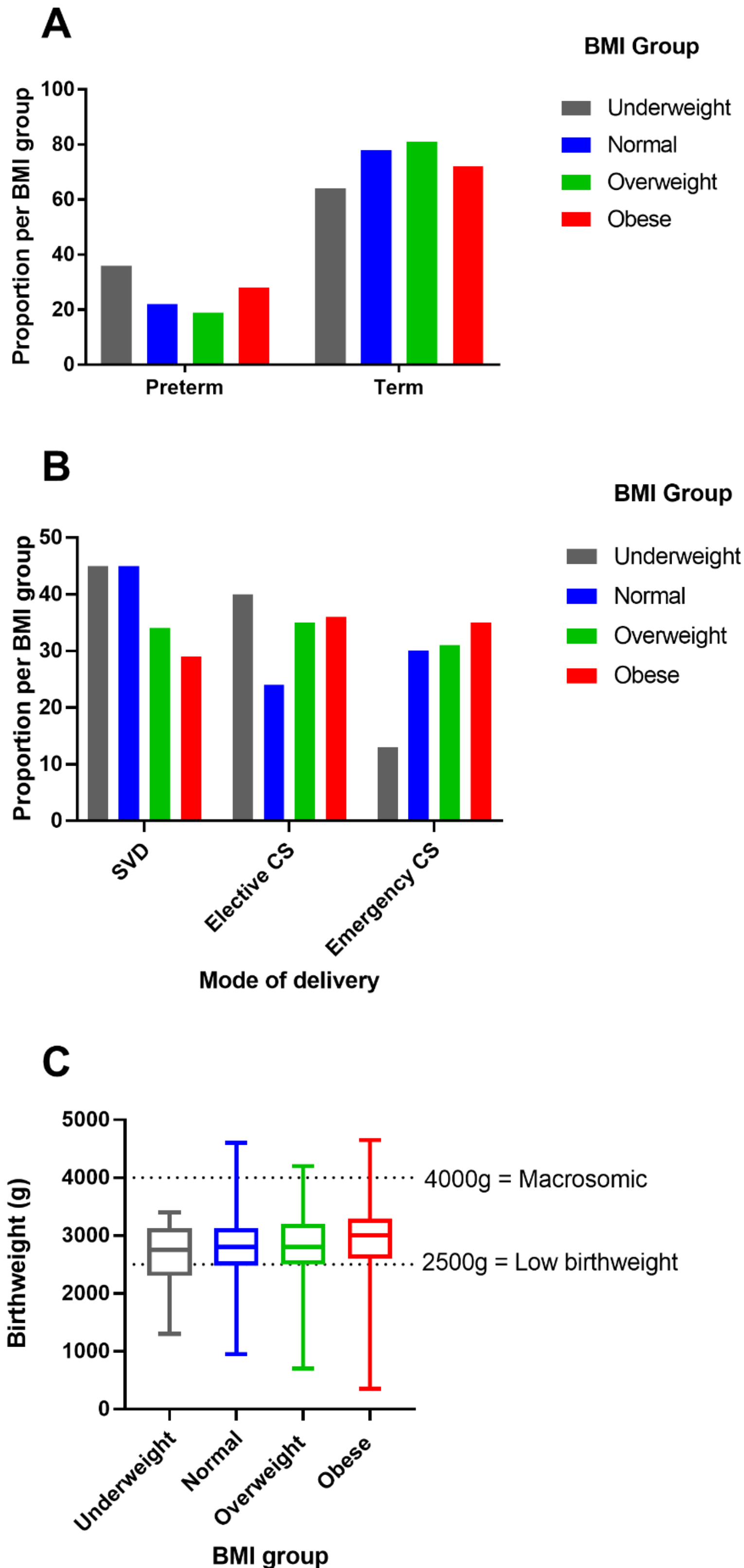

Figure 5.1 Proportions of A) preterm or term birth and B) mode of delivery for each BMI group and C) birthweight. Displayed as median, interquartile ranges and upper and lower limits of birthweight split by BMI group C). Dashed lines indicate cut offs for low birthweight and macrosomia.

BMI	*Elective*			*Emergency*		
	aOR	**95% CI**	**p-value**	**aOR**	**95% CI**	**p-value**
Underweight	1.34	(0.369-4.84)	0.659	0.435	(0.100-1.89)	0.266
Normal	Ref					
Overweight	1.41	(0.578-2.25)	0.703	1.25	(0.675-2.29)	0.438
Obese	1.27	(0.658-2.44)	0.479	**1.78**	(0.99-3.22)	**0.053**

Table 5.3 Adjusted odds ratios, 95% CI and p-values from multinomial logistic regression model for mode of delivery after adjusting for confounding variables. Spontaneous vaginal delivery is the refernece category. Those in bold represent those that remained significant. The following confounding variables were chosen based on existing well established interactions with our outcome variables, these included maternal age (years); previous number of live births, CS and preterm births; pre-eclampsia (yes/no); sex of offspring (male/female) and birth weight (g).

Exposure variable	*Elective*			*Emergency*		
BMI	aOR	95% CI	p-value	aOR	95% CI	p-value
Underweight	1.35	(0.372-4.88)	0.650	0.436	(0.101-1.89)	0.267
Normal	*Reference*					
Overweight	1.13	(0.571-2.23)	0.728	1.22	(0.661-2.26)	0.524
Obese	1.17	(0.600-2.27)	2.27	1.62	(0.890-2.94)	0.115
Type 2	1.51	(0.721-3.14)	0.277	1.34	(0.666-2.69)	0.412
Hypertension	1.45	(0.786-2.67)	0.235	1.65	(0.938-2.89)	0.083

Table 5.4 Adjusted odds ratios, 95% CI and p-values for each mode of delivery with both BMI and all NCDs included, along with confounding variables. Spontaneous vaginal delivery is the reference category. All those in bold are those that remained significant. The following confounding variables were chosen based on existing well established interactions with our outcome variables, these included maternal age (years); previous number of live births, CS and preterm births; pre-eclampsia (yes/no); sex of offspring (male/female); birth weight (g) and gestational age at delivery.

5.3.2 Diet study:

5.3.2.1 Demographics

74 women delivered singleton babies within the study time period. Demographics and previous birth data are shown in Table 5.5. 40.5% of the women had no recorded NCDs, 51.4% had 1 and a further 8.1% had 2 NCDs. Data on the subsequent birth are presented in table 5.6.

5.3.2.2 Dietary Groupings

The foods included in each food group, max scores and high and low group cut offs are found on table 5.7. The frequency and percentage of those in each high and low divisions for each food group are shown in table 5.8. The PDGN adherence score comprises of the consumption of 5 food groups each with a score of 0 or 1 dependent on if the individual foods in that group were consumed daily or not. The frequency and percentage of women meeting (adherence score = 1) or not meeting (adherence score = 2) the recommended amounts are shown in table 5.9. The total score for adherence to the PDGN guidelines (max = 5) are shown in table 5.10. None of the women in this study were totally adherent to the dietary guidelines.

5.3.2.3 Preterm birth

The average gestational age at delivery was 37.4. 21 women (28.4%) gave birth prematurely. The crude odds ratios and p-values for each food grouping and adherence scores are shown in table 5.11. No food groups or adherence scores were found to be significant. But high adherence to the dietary guidelines tended towards significance of reducing the risk of preterm birth (cOR = 0.383 p = 0.076).

5.3.2.4 Mode of delivery

Of the 74 women 14 (18.9%) delivered through spontaneous vaginal delivery (SVD) and 60 (81.1%) had a CS delivery. No food groups were found to significantly affect the mode of delivery table 5.12.

5.3.2.5 Birthweight

10 women (13.5%) delivered a low birthweight (defined as <2500g). No food groups were found to significantly affect birthweight, however, a high PDGN adherence score

was found to significantly decrease the risk of a low birthweight baby (Table 5.13). After adjusting for confounding variables this was no longer significant.

Information on Mothers		*Number of women (%)*	
Age (mean =32)	37+	12	16.22%
	28-36	46	62.16%
	<27	16	21.62%
Marital status	Divorced	0	0.00%
	Married	74	100.00%
Occupation	Employed	19	25.68%
	Housewife	55	74.32%
BMI (mean = 28.6)	Underweight	1	1.35%
	Normal	5	6.76%
	Overweight	15	20.27%
	Obese	53	71.62%
NCD Burden	0	30	40.54%
	1	38	51.35%
	2	6	8.11%
Previous live births history			
Live Births	0	13	17.57%
	1	25	33.78%
	2	26	35.14%
	3	7	9.46%
	4+	3	4.05%
Miscarriages	0	47	63.51%
	1	12	16.22%
	2	10	13.51%
	3+	4	5.41%
Ectopic pregnancies	0	73	98.65%
	1+	1	1.35%
Stillbirths	0	68	91.89%
	1	5	6.76%
	2+	1	1.35%
Induced abortions	0	74	100.00%
	1+	0	0.00%
Caesarean Sections	0	23	31.08%
	1	20	27.03%
	2	21	28.38%
	3+	10	13.51%
Preterm	0	66	89.19%
	1	7	9.46%
	2+	1	1.35%

Table 5.5Demographic information and previous birth history for all 74 mothers included in the final analysis. Count and percentage shown for each category and mean for maternal age and BMI.

Current birth data		Number of women (%)	
Sex	Female	38	51.4%
	Male	36	48.6%
Gestational age at delivery	Less than 28	0	0.0%
(Mean =37)	28-31	0	0.0%
	32-36	21	28.4%
	37+	53	71.6%
Mode of delivery	Spontaneous vaginal	14	18.9%
	Caesarean section	60	81.1%

Table 5.6 Current birth data showing count and percentage. Mean weeks calculated for gestational age at delivery.

Food group	*Food items*	*Max score*	*High category*	*Low category*
Dairy	Milk, eggs and milk products	12	> 8	≤ 8
Saturated fat	deep-fried western, deep fried desi and western desserts	12	> 6	≤ 6
Grains	cereals and whole grains	8	≥ 6	< 4
Protein	meat, fish, shellfish and legumes	16	> 10	≤ 10
Healthy fats	fish, shellfish and nuts	12	Ratio = Total score Healthy fats: Total score Saturated fats	

Table 5.7 Each food contained in each food group, the maximum frequency score and the definitions of the high and low categories for each grouping.

Food group	*Consumption*	*Number*	*Percentage*
Dairy	High	43	58.11%
	Low	31	41.89%
Saturated fat	High	22	29.73%
	Low	52	70.27%
Protein	High	41	55.41%
	Low	33	44.59%
Grain	High	53	71.62%
	Low	21	28.38%
Healthy:saturated fat	High	37	50.00%
	Low	37	50.00%

Table 5.8 Each food group with the frequency and percentage of women in each division.

PDGN category	Adherence score	Frequency	Percentage
Fruit	0	29	39.2%
	1	45	60.8%
Veg	0	47	63.5%
	1	27	36.5%
Grains	0	74	100.00%
	1	0	0.00%
Meat and pulses	0	34	45.9%
	1	40	54.1%
Dairy	0	5	6.76%
	1	69	93.24%

Table 5.9 The frequency and percentage of women meeting (adherence score =1) or not meeting (adherence score =2) the recommended amounts Scores were calculated by whether or not the individual consumed the foods within those groups to meet the recommended consumption by the Pakistani dietary guidelines for pregnant women.

PDGN adherence score	Frequency	Percentage
0	1	1.35%
1	11	14.86%
2	25	33.78%
3	28	37.84%
4	9	12.16%

Table 5.10 The total score for adherence to the PDGN guidelines (max = 5). These are calculated by totalling the scores from the 5 food groups listed in table

Food	*Consumption*	*Preterm*		*Term*		*cOR*	*Risk ratio*	*Chi-squared p*
		Frequency	**(%)**	**Frequency**	**(%)**			
Dairy	High	12	27.90	31	29.00	0.95	0.96	0.92
	Low	9	29.00	22	71.00			
Deep Fried	High	5	22.70	17	71.60	0.66	0.74	0.48
	Low	16	30.80	36	69.20			
Protein	High	11	26.80	30	73.20	0.84	0.89	0.74
	Low	10	30.30	23	69.70			
Grain	High	17	32.10	36	67.90	2.01	1.68	0.26
	Low	4	19.00	17	81.00			
Healthy fat:saturated fat	High	12	32.40	25	67.60	1.49	1.33	0.44
	Low	9	24.30	28	75.70			
PDGN adherence	High	7	18.90	30	81.10	0.38	0.50	0.07
	Low	14	37.80	23	62.20			

Table 5.11 Count, percentage, crude OR, relative risk and p-value for term and preterm delivery for each food group based on consumption.

Food		SVD		CS		
		Count	**(%)**	**Count**	**(%)**	**cOR (p-value)**
Dairy	High	6	14.0%	37	86.0%	2.15 (p=0.205)
	Low	8	25.8%	23	74.2%	
Deep Fried	High	4	18.2%	18	81.8%	0.933 (p=0.916)
	Low	10	19.2%	42	80.8%	
Protein	High	8	19.5%	33	80.5%	0.917 (p=0.885)
	Low	6	18.2%	27	81.8%	
Grain	High	10	18.9%	43	81.1%	1.01 (p=0.986)
	Low	4	19%	17	81.0%	
Healthy fat: saturated fat	High	7	18.9%	30	81.1%	1.00 (p=1.00)
	Low	7	18.9%	30	81.1%	
PDGN adherence	High	2	5.40%	27	73.00%	1.43 (p=0.554)
	Low	5	13.50%	20	54.10%	

Table 5.12 Frequency, percentage, crude odds ratios and p-values for mode of delivery per food group consumption.

Food	*Consumption*	*Low birth weight*		*Normal birthweight*		*cOR*	*Chi-squared p*
		Frequency	**(%)**	**Frequency**	**(%)**		
Dairy	High	4	9.3%	39	90.7%	0.43	0.21
	Low	6	19.4%	25	80.6%		
Deep Fried	High	1	4.5%	21	95.5%	4.4	0.14
	Low	9	17.3%	43	82.7%		
Protein	High	6	14.6%	35	85.4%	1.24	0.75
	Low	4	12.2%	29	87.9%		
Grain	High	9	17%	44	83%	4.09	0.17
	Low	1	4.8%	20	95.2%		
Healthy fat: saturated fat	High	8	21.6%	29	78.4%	4.828	0.09
	Low	2	5.4%	35	94.6%		
PDGN adherence	High	1	2.7%	36	97.3%	0.086	**0.007**
	Low	9	24.3%	28	75.7%		

Table 5.13 Frequency, percentage, crude odd ratios and p-values for mode of delivery per food group consumption.

5.4 Discussion

5.4.1 Obesity and Non-communicable diseases study:

5.4.1.1 Obesity and pregnancy outcomes

Nearly a quarter (24.7%) of the births in this study were premature (delivered before 37 weeks gestation) which fits the current landscape, with Pakistan being identified as one of the top 10 countries in the world with high PTB rates (Blencowe et al., 2012). Recent research identified PTB rates of Pakistan to be approximately 22% for the whole country and when broken down by province found rates of 22.5% for the Sindh province, which is where the hospital providing data for the current study is located (Hanif et al., 2020). Interestingly, in the current study obesity was found to have no significant impact on preterm birth, which is in contrast to some published studies. Studies across the globe have found obesity to increase the risk of preterm birth, especially extreme preterm birth (before 32 weeks gestation) as reviewed in (Vats et al., 2021). This is not the case across all studies as a systematic review of 84 studies from both developed and developing countries, provided evidence that there is no significant association between obesity and overall PTB. However, the study did indicate that PTB risk did increase with BMI as did labour induction before 37 weeks (Mcdonald et al., 2010). A further study found no association, apart from induced PTB in cases of excessive gestational weight again (above recommended guidelines) and was suggested to be due to an increased underlying inflammatory state (Faucher et al., 2016). Further inconsistencies exist as some research studies also found a decreased risk of PTB (Hendler et al., 2005) and longer gestation lengths in obese women (Arrowsmith et al., 2011).

It is important to note that there are many factors that impact on PTB ranging from socioeconomic status to obstetrical and gynaecological history with complex interactions of mother, foetus and environment that are all indirect and can be difficult to unravel (Goffinet, 2005; Murphy, 2007). Low maternal weight, previous preterm deliveries, anaemia and physical and emotional stress are factors that have been identified to be associated with the risk of PTB in Pakistan (Baig et al., 2013). Epidemiological factors such as young maternal age, older paternal age and shorter pregnancy intervals have all been identified as risk factors for PTB, along with environmental factors such as infections, excessive alcohol use and smoking (Murphy,

2007). NCDs have also previously been shown to impact PTB. Type 2 diabetes has been documented to increase the risk of PTB (López-De-andrés et al., 2020; Murphy, 2007; Wei et al., 2019; Yokomichi et al., 2022). Particular risk factors that have been identified as significantly increasing the risk of PTB with obese mothers are maternal age >35, ethnicity abdominal adiposity, type 1 diabetes, GDM, nulliparity, vitamin D deficiency and lower gestational weight gain (Fakhraei et al., 2022).

As discussed in section 1.5 other studies have found issues with dilation and longer labour lengths (Bhattacharya et al., 2007; Briese et al., 2011; Denison et al., 2008; Ellekjaer et al., 2017; Graves et al., 2006; T. S. U. Kiran et al., 2005; Pettersen-Dahl et al., 2018; Sebire et al., 2001; Sheiner et al., 2004). There have been 3 main reasons identified for non-elective CS sections: ineffective uterine contractility and longer labour length; obstructed labour either through malpresentation or pelvic anomaly and foetal stress (Cedergren, 2009). With ineffective uterine contractility and foetal stress associated with BMI but obstructed labour was not (Cedergren, 2009). Several other studies have found cervical dilation to be lowest in obese mothers (Maged et al., 2017), also exhibiting greater levels of arrested dilation (Verdiales et al., 2009) and longer labour lengths (Beyer et al., 2011; Kerrigan & Kingdon, 2010; Pettersen-Dahl et al., 2018; Sheiner et al., 2004). Although this is contested (Arrowsmith et al., 2011; T. S. U. Kiran et al., 2005) and that BMI and labour length varies among phase of labour and parity (Hirshberg et al., 2014). We have no information on labour duration, at what stage of labour the CS was carried out or reasons for the emergency CS. A further theory for increased risk of operative deliveries is from larger offspring having difficulty passing through the birth canal. Many studies have found macrosomia to be more common with obese mothers (Bhattacharya et al., 2007; Vats et al., 2021), with as much as a 2-fold greater risk for macrosomia (Kerrigan & Kingdon, 2010; T. S. U. Kiran et al., 2005) or large for gestational age babies (Sebire et al. 2001). In our study there was no association between BMI groups and birthweight so suggesting this is not the driver behind the increased risk of CS found in this study.

5.4.1.2 Obesity and the impact of NCDs

With Pakistan's high burden of NCDs, obesity and the established correlation between the two (Kazmi et al., 2022) we chose to look at 2 of the most common

NCDs associated with obesity and placental pathologies to understand their joint impact on adverse pregnancy outcomes in Pakistan. Neither Type 2 diabetes nor hypertensios had a significant effect on the mode of delivery. Several disagree with this finding as hypertension (Iftikhar et al., 2021; Z. Kiran et al., 2019; Malhamé et al., 2021), diabetes (J. Kitzmiller et al., 2018; Mackin et al., 2018), were found to increase operative deliveries. Similarly, studies based in Pakistan have found the same NCDs to be risk factors for increased CS but often these are all grouped as “pregnancy complications” to include diabetes, hypertension, respiratory disorders and thyroid problems that were also grouped with other conditions such as cardiac problems and anaemia (Amjad et al., 2020; Rasool et al., 2021). Interestingly no significant association was found with emergency CS or the NCDs as identified in the current study, however, it is key to note that the previously mentioned studies did not differentiate between elective and emergency CS, so it is difficult to make direct comparison.

Ante-natal care is an important factor that needs to be considered when trying to identify the key drivers behind adverse pregnancy outcomes. Studies have found that a lack of ante-natal care is a high-risk factor for PTB (Jiang et al., 2018) and important in identifying the risk of PTB and any influencing factors (Murphy, 2007). The WHO recommends at least 4 ante-natal visits and less than this has been found to significantly increase CS risk in Pakistan (Amjad et al., 2020). This could be because conditions such as NCDs can be captured and managed effectively with appropriate advice from physicians that are effective in improving birth outcomes and decrease the need for operative deliveries. Identifying exacerbations of diseases, the onset of gestational conditions such as pregnancy-induced hypertension and appropriate management of existing conditions like asthma (Bonham et al., 2018) are all important for reducing poor paediatric outcomes. Unfortunately, the current study did not have any information on ante-natal care of the women involved but would have been an interesting variable to include.

5.4.2 Diet groups

Interestingly, previous research has found no association between maternal protein intake and PTB (Cohen et al., 2001). Intervention studies regarding protein have involved energy manipulation alongside – either balanced or restricted, however,

neither have shown any correlation or strong evidence that this reduces the rates of premature births birth (Villar et al., 2003). The same is true in intervention studies based in LMICs (Lassi et al., 2020). In parallel to this there has been no evidence of protein intake having an impact on CS rates (Villar et al., 2003). Although there was no evidence carbohydrate intake had any significant in the current study, carbohydrate diets have been shown to increase the length of gestation, but this was specific to overweight and obese women (Tanner et al., 2021). Very few studies have investigated the effect of specific food group consumption on pregnancy outcomes, those that have mainly focus on macronutrients and or micronutrient intakes or specific deficiencies. Interestingly, though, research to determine nutritional and macronutrient intakes at 3 timepoints in pregnancy found specific food groups to influence different outcomes at different stages of pregnancy. Fat, protein and energy intake for example were found to have significant negative correlations with labour duration but only in phase G2 (28th-35th week of gestation), however, in phase G3 (36th-38th week) only polysaccharide intake was significant. G3 also saw the most negative correlations with pregnancy length, with overall energy, MUFA and carbohydrate intake having the strongest correlations. Pregnancy length was also correlated with birth weight and length, a potential explanation for the reduced pregnancy length with greater energy and macronutrient intakes could be these increased intakes, increase birthweight and length and influence the earlier onset of labour but not to the extent of being preterm (Najpaverova et al., 2020). This data suggests that the source and quantity of food may be influential in a pregnancy stage dependent manner and as food choices may change throughout pregnancy, a much more detailed approach may be needed to unpick these relationships.

What was evident from the current study is that none of the food groups were found to have any significant effect on preterm birth or mode of delivery. This was surprising as dietary fats play a very important role in pregnancy and labour (Mcgregor et al., 2001). Cholesterol is biosynthesised in the mother, placenta and foetus into steroid hormones involved in the maintenance of pregnancy and initiation of labour (Pasqualini, 2005). There have been several studies investigating the impact of fish consumption or fish oil supplementation on pregnancy outcomes. Although there has been no evidence it adversely affects the risk of CS there is evidence fish oil reduces the risk of PTB (Villar et al., 2003). A Norwegian study found that there was

no association with a Mediterranean diet and PTB but consuming fish more than twice a week was found to reduce PTB rates (Haugen et al., 2008). It was suggested that the reduced PTB risk was due to increased consumption of n3 PUFAs, and interestingly, studies that looked omega 3: omega 6 ratios or overall fat intake were more likely to pick out associations (Abu-Saad & Fraser, 2010). Furthermore, research also provides evidence that a diet high in in- 6 PUFA increases the risk of PTB (M. Elmes et al., 2005). Essential FAs and metabolites are involved in the production of eicosanoids including prostaglandins which are important during pregnancy and labour (Mcgregor et al., 2001). N3 PUFAs are precursors to less potent 3-series prostaglandins and anti-inflammatory products and so an increase of these in a mother's diet, through fish and fish oil consumption, could reduce inflammation and increase the length of gestation (Mcgregor et al., 2001). In Chapter 4 we identified the MUFA, OA, to have a negative effect on prostaglandin production and inflammatory cytokines in placental cells. More detailed analysis into dietary fat consumption is needed to assess the relationship with diet and pregnancy outcomes fully.

5.4.3 PDGN dietary adherence

Adherence to the recommended guidelines also had no significant impact on adverse pregnancy outcomes.

PDGN adherence score was found to be significantly associated with low birth weight. Greater adherence to the guidelines, resulting in a reduced risk of delivering an LBW baby. This is expected and agrees with many previous studies. Although low birth weight has been shown to be reducing globally, South Asia remains one of the most prevalent regions of LBW babies (Blencowe et al., 2019). This is believed to be due to poor maternal nutrition in these settings (World Bank et al., 2005). 13.5% of women within this study gave birth to LBW infants, which is lower than the reported average of 21.4% (Pusdekar et al., 2020). A systematic review of intervention programs in LMICs found protein energy balance diets (25% of the energy from the diet coming from protein) to reduce the incidence of LBW by 40% and food distribution programs to be very effective (Lassi et al., 2020). Pregnancy weight gain has also been identified as a modifiable behaviour as the most important risk factor, those who gave birth to LBW infants had significantly reduced weight gain

throughout pregnancy (Bailey & Byrom, 2007). As agreed by several other studies (Fakhraei et al., 2022; Kominiarek & Peaceman, 2017). However, in this study once adjusted for confounding factors this was no longer significant. Surprisingly, none of the women in the study achieved a maximum score of 5, indicating that they were not meeting the recommended guidelines. It was also evident that not one individual in the study consumed the recommended level of grains and approximately half of the participants did not consume enough vegetables and meat or pulses. Unfortunately, the food frequency questionnaire only used 'daily consumption' as the most frequent consumption measure and the PDGN recommends 3 or 4 servings per day so our scores could be underestimating the consumption of some food groups.

Modest changes to maternal diet during pregnancy can impact an offsprings ability to withstand external stressors as well as altering the pace and balance of fetal growth (Jackson et al., 2003). The quality of the diet consumed during pregnancy is extremely important to support the mother and growth of foetus throughout pregnancy. Overall intake of energy, protein, fats, vitamins and minerals are needed for successful growth and development of the offspring, along with the continued health of the mother (Bhutta et al., 2013; Mousa et al., 2019). However, excess calorie and macronutrient consumption during pregnancy as well as deficiencies can equally be an issue (Marangoni et al., 2016). Many provide evidence that minerals and vitamins such as calcium, zinc, iron, folate and magnesium have protective effects on the risk of PTB (Villar et al., 2003). Most studies have focused on PTB and birthweight as outcomes for nutritional interventions and observations, but very few look at CS or mode of delivery. One of the few studies is one in Australia that found no association between the quality of diet during pregnancy and risk of CS delivery (Szewczyk et al., 2020). Studies that focussed on CS and mode of delivery as are specific to pre-pregnancy BMI or gestational weight gai. Excessive gestational weight gain increases the risk of PTB and CS (Kominiarek & Peaceman, 2017). Similarly, overweight and obese women are more likely to have greater gestational weight gain, but it is an important factor across all BMI categories (Kominiarek & Peaceman, 2017). It is difficult to ascertain which has a greater effect as they both have very similar risk factors and studies have been inconsistent in their findings (Kominiarek & Peaceman, 2017). Nutritional status during pregnancy is determined by the nutritional status of the mother but also influenced by maternal body composition

and weight gain during pregnancy (Kind et al., 2006). Further research is needed to fully understand the interplay between BMI, gestational weight gain and diet composition and how this can affect labour and pregnancy outcomes to allow for better intervention methods and effective treatments. Effective interventions are also likely to be required much earlier on in pregnancy or even during the preconceptual period to have significant and meaningful effects (Jackson et al., 2003).

5.4.4 Other important findings

A final yet very important finding from investigating both the effects of obesity and diets on pregnancy outcomes in Pakistan is that there is a huge number of women undergoing CS. It is evident that over half the women gave birth via an operative delivery, with 67.5% by elective CS (34.3%) closely followed by emergency CS (33.2%). The rates of CS across different areas of Pakistan vary hugely within the published literature– ranging from as low as 14% (Amjad et al., 2020) 21.4% (Hafeez et al., 2014), 34% (Iftikhar et al., 2021) and 69.7% (Rasool et al., 2021). This big variation in CS rates depend on the characteristics of the region or hospital each study was carried out. Interestingly, it has been found that higher socioeconomic standing, more urban areas (Rasool et al., 2021) and private hospitals to increase the risk for CS (Amjad et al., 2020; Padmadas et al., 2000). In other countries such as India the same trend persists (Singh et al., 2018). It is also worth noting the rates here may be inflated due to all the women exhibiting at least one NCD, which could impact clinical decisions and delivery of the baby. Regardless, the rates in this study far exceed the maximum 15% recommended by the World Health Organization (World Health Organisation, 1985). The drivers behind CS rates are complex and vary between countries. These drivers can be both medical and non-medical. Examples of medical reasons include prolonged and obstructed labour, fetal malpresentation, multiple pregnancies and maternal disorders. One of the most common is a previous CS (Elnakib *et al.* 2019). These can be from changes in the population for example, increases in older mothers or of multiple pregnancies but also include non-clinical factors such as generational shifts in work and family responsibilities, physician factors, organisational, economic and social factors (World Health Organisation, 2018b). To try and unravel the reasons behind high CS rates, one study asked women via a questionnaire about their knowledge and influences behind their choice to have a CS. All women said they would have a CS if their physician recommended it (Rasool et al., 2021). Reasons for mothers favouring CS over other types of birth without

medical indication have been documented as being a fear of labour pain, pelvic floor damage and repercussions and it's often perceived in these women to be safer for them and the baby as it can be portrayed as easier and more convenient. For healthcare practitioners, a fear of being sued for malpractice and to a much lesser extent convenience and planning are drivers behind agreeing to an elective. In many private health care settings, there are increased numbers of CS compared to the public sector if CS can generate more revenue for the hospital. Furthermore, in resource poor settings, lack of skilled professionals has been attributed to the high rates of CS in tertiary care hospitals (Betrán et al., 2018).

5.4.1 Limitations

One of the key limitations of the study was that both data sets were collected from a private health care setting, so caution is warranted when drawing conclusions from the data, especially regarding CS. Pakistan has a privatised health system and therefore the CS rates are often much higher due to increased profits, however, Aga Khan University hospital is a non-profit hospital, that has 4 secondary care facilities in Karachi and Hyderabad and receive a large number of referrals from urban and rural areas of the Sindh and other provinces. All the women selected for the study had a previous NCD recorded so there was no control group for comparison.

There were also several limitations of the diet component of the study. The very small number of participants made it hard to make confident conclusions due to being very underpowered. Unfortunately, the food frequency questionnaire lacked a lot of detail and only included daily as the most frequent consumption of a specific food and relied on participants recalling how often they ate something over the course of a year which could lead to inaccurate recollection. There is also no information on quantities consumed, so it is difficult to interpret this data with nutritional content as this could be hugely variable. This is limiting in terms of the dietary guidelines as even those who consumed daily still may not be hitting the recommended daily amount and in addition to this we also have no conception of overconsumption of any of these foods. A much larger study including a detailed food diary over the course of a week at multiple intervals would be a much more powerful tool in identifying any potential links that may have been missed due to the lack of detail here.

5.5 Conclusions

Rates of PTB were considerably high within this population, fitting with the current data. Obesity was found to have no impact on PTB rates and this is widely contested in the literature. Obesity was found to increase the risk of induction and emergency CS in this study. Many other studies across the globe have also found this, although, the drivers and reasoning behind this remain unclear. Including NCDs in the logistic regression model has a significant effect on pregnancy outcomes and the magnitude of obesity on some of these outcomes. No specific food groups were found to impact any pregnancy outcomes investigated, despite previous research identifying potential, particularly with omega 3 PUFAs and PTB. Diet quality was also found to have no significant impact. Alarmingly, no women in the study were found to be fulfilling the daily requirements set out by the PDGN. Further work is also needed to understand the drivers behind the increasing number of CS and effective interventions with doctors, mothers, and potential therapies to reduce the very high CS rates. Although this study did not find solid links between maternal factors and pregnancy outcomes, we have demonstrated that they have modulating effects and the potential to explain some discrepancies.

6 Overall Discussion

The previously established diet induced maternal obesity model showed chronic exposure to a HFHC diet prolonged labour duration, compromised uterine contractility, and reduced levels of circulating progesterone and $PGF_{2\alpha}$ (Elmes et al., 2011; Muir et al., 2016). Yet, there has been no investigation to determine how this dietary HFHC induced obesity affects the placenta. As the placenta is the site of communication between the maternal and foetal interface during pregnancy, with a clear and distinct role in producing pro-labour mediators (Vannuccini *et al.*, 2016), it is important to understand how it may be implicated in obesity. Thus, the first objective of this thesis was to understand the impact a HFHC diet has on the placenta and how this may contribute to dysfunctional labour. Furthermore, it is important to assess how closely the phenotype of the HFHC fed rat placenta matches that of an obese woman to understand if the model exhibits further translation.

This thesis found the HFHC diet to decrease total SFA and PUFA but increase MUFA, specifically OA and desaturase enzyme activities. A similar, but less distinct pattern was seen in obese human placenta, although no changes to total fatty acids were seen a significant decrease in stearic acid (the second most abundant FA) and no increase in MUFAs or OA but BMI positively correlated with increased SCD18 activity estimates. PPARγ was seen to increase in HFHC fed rats but no downstream effectors were seen to change, and no differences observed in humans. Due to increased levels of OA seen in the placenta and the potential anti-inflammatory effects seen in other tissues, a trophoblastic cell line was treated with increasing doses of OA and pro-labour mediators were measured. OA at the highest doses was found to significantly reduce PGE_2, $PGF_{2\alpha}$ and IL-6 production in vitro. These findings suggest obesity alters the placenta's ability to produce key signalling molecules that are increased dramatically at term and have a large impact on myometrial contractions.

Finally, this thesis showed obesity's effect on pregnancy outcomes can be modulated by the presence of NCDs which are often co-occurrent. An important finding, particularly in LMICs where obesity, NCDs and poor pregnancy outcomes are prevalent. The dietary study did not identify any patterns associated with pregnancy outcomes, potentially due to the small sample size but it was clear no mother

adhered to the recommended dietary guidelines, which could be an important area to address.

Chapter 3 of this thesis demonstrated HFHC feeding to induce obesity, can elicit significant placental changes. Fatty acid analysis of placental tissue using GCMS and mRNA expression analysis using RTqPCR was performed on placental tissue from both the rat model and human placentae of women with different BMI. Despite an increased consumption of SFA, the obese rat dams saw a decrease in total SFA in the placenta, along with a decrease in both n3 and n6 PUFAs. A large increase of total MUFAs was seen, attributed mostly to a doubling of OA, along with an increase in desaturase enzyme activity estimates. These findings could indicate a protective response from the placenta, to reduce the inflammatory and lipotoxic effects from excessive SFAs as demonstrated in liver, muscle and trophoblast cell culture models (Sampath and Ntambi, 2008; Ricchi et al., 2009; Natarajan et al., 2021). In terms of gene expression, a significant increase in PPARγ was seen in the HFHC fed rats, suggesting an increase in lipid accumulation and a potential anti-inflammatory effect. However, no downstream effectors were seen to change with diet.

In combination, OA and PPARγ were hypothesised to have an anti-inflammatory effect within the placenta. Evidence of this was demonstrated in chapter 4, where OA at physiological ranges was found to reduce the production of pro-labour mediators in trophoblast tissue. PGE_2, $PGF_{2\alpha}$ and IL-6 were all found to decrease with increasing doses of OA. The increased OA within the placenta, as a potential result of high SFA in the diet or circulation, could therefore decrease production of pro-labour mediator's in the placenta. Decreased production of PGE_2, $PGF_{2\alpha}$ and IL-6 within the uterine cavity that could lead to disruption of contractions. The placenta and membranes are the main source for PG production (W. jiao Li et al., 2021) and so the increase in OA within the placenta of these rats could explain the reduced circulating $PGF_{2\alpha}$ identified in the original pilot study (Elmes et al., 2011). PGE_2 and $PGF_{2\alpha}$ target the myometrium during parturition, generally increasing its contractile ability (W. jiao Li et al., 2021). Reduction in their production from the placenta could directly affect the contractile ability of the myometrium as seen in this rat model. The positive feedback loop and synergistic action of PGs and inflammatory cytokines in the cervix and myometrium would be further reduced as both expressions are decreased by

OA. Although a mechanism was not identified this could involve NFkB and PPARγ signalling.

This model utilised a first trimester trophoblast cell line. The HTR8-SVneo cell line used are considered to be a closer model of trophoblast cells as they were established through immortalisation via transfection of a viral plasmid, compared to JEG3 or BeWo cell line which are cloned from a primary choriocarcinoma (Weber *et al.*, 2013). However, this transfection may make them more like a progenitor trophoblast cell compared to a differentiated syncytiotrophoblasts or extra villous trophoblast (Weber *et al.*, 2013). A key difference with primary term trophoblast isolations is they are senescent and so are only viable up to one passage (Vidal *et al.*, 2024). Primary trophoblast cells isolated from term placenta would provide a more effective model for investigating the effect of OA on the pro-labour mediators. Furthermore, comparison of these from women of different BMIs may also give a greater understanding of the problem. As discussed in section 1.11.2.3 high maternal BMI has been demonstrated to alter lipid metabolism and processing in pregnant women. In humans increased BMI has been associated with increased expression of FATP 1, 2 and 3 (Lager et al., 2016; Hirschmugl et al., 2017) and a decrease in FATP 4 (Hirschmugl *et al.*, 2017). Along with increased storage, specifically in TGs, reduced ocidation rates and increased de novo synthesis (Saben et al., 2014; Gázquez et al., 2018). These could have modulating effects on how OA is taken up, processed and metabolised which could further impact the alterations seen and give a clearer picture of OA in the obese placenta.

Minimal differences were observed in the human placenta, with no change in total FAs but there was a decrease in stearic acid, the second most abundant SFA. Alongside, this there was an increase in SCD18 activity with increasing BMI, which the enzyme responsible for OA production despite no significant change in OA itself with BMI. No changes in PPARγ were identified either. There are a few explanations for these difference between the rat and human placentae in our study. It is important to note that the rats were kept in a very controlled environment with the only change being the difference in the fat intake and composition of the diet. The human participants from this study had no information collected on their diet, lifestyle and only very brief information on their medical history, all of which could have vast

impacts on their placental tissue. Other studies have found increases in PPARγ in placentas from obese women (Calabuig-Navarro *et al.*, 2017) and 288 genes have been identified as being differentially expressed in the placenta of obese women compared to lean, including NFκB and PPAR signalling (Saben *et al.*, 2014).

One of the main discrepancies between the hypothesis proposed here and other studies looking at the obese placenta, is the role of inflammation. We propose an anti-inflammatory action of OA in response to excess SFAs, which results in decreased pro-inflammatory cytokines and decreased prostaglandin production. However, other studies have proposed obesity as a pro-inflammatory disease. Diet induced obesity has been shown to increase TNF (Crew, Waddell and Mark, 2016) and IL-6 in rodent models (Ludidi et al., 2023; M. Wang et al., 2022). In humans a greater influx of neutrophils have been observed in foetal and maternal tissues at term (Scott *et al.*, 2022) and increased CD68 and CD14 positive cells in placentas of obese women, which can lead to increased cytokine expression (Challier *et al.*, 2008). Furthermore, studies in obese women have identified higher levels of TLR4, IL-6 and IL-8 mRNA have in placental tissue and IL6, IL8 and CRP in plasma (X. Yang et al., 2015). Other inflammatory regulators have been demonstrated to be altered in human tissue leading to a pro-inflammatory environment (Oliva et al., 2012; Lim and Lappas, 2015; Liong, Barker and Lappas, 2017). Low level inflammatory signals seen in obese humans has been suggested to be driven by TNF produced in adipose tissue which can activate NFκB and in turn produce more TNF, creating a positive feedback loop (M. Yuan et al., 2001).

However, these changes are not consistent across all studies. For example, Roberts et al. (2011) found only IL-6 to increase in the placenta of obese women and no change in IL-1b, IL-8 nor any change in CD68 and CD41 positive cells. In rodent models, where mice were fed a high fat diet to induce obesity, they found no change in placental macrophage infiltration (Ingvorsen *et al.*, 2014) and no increase in IL-6 in the placenta, despite increased circulating levels (Kretschmer *et al.*, 2020). In a non-human primate model, a western diet saw significantly higher CD68 positive cells but no difference in NOTCH1 and IL6 in the placenta and maternal or foetal visceral fat (Nogues *et al.*, 2021). Furthermore, it has been demonstrated an increase in only one inflammatory receptor (NT3, a modulator of inflammatory cell migration) but

decreases in TNF receptor 6 and STAT3 and NFκB and TLR4 levels unaltered (Gohir *et al.*, 2019). Even in surrounding adipose tissue (gonadal white adipose tissue – gWAT) there was no evidence of increases in IL-6 at the protein or mRNA level (Kretschmer *et al.*, 2020) and no large increase in pro-inflammatory factors at labour seen in obese dams as there was in lean counterparts (Appel *et al.*, 2017). In a non-human primate model feeding a western diet, an increase in macrophages and cytokines were seen in the placental, however, these were not significant (Sugino *et al.*, 2022). Circulating levels of cytokines is not a common phenomenon in all pregnancies complicated with maternal obesity (Kelly, Powell and Jansson, 2020). Even in human studies obesity has been demonstrated not to increase IL-6, TNF or IL-1b (Musa *et al.*, 2023) and no increase in inflammation within the placenta (Loardi *et al.*, 2016) but an increase in vascular lesions instead (Kovo *et al.*, 2015). A potential explanation for these discrepancies was suggested by Layden et al. (2023), stating selection bias is a key problem in sampling placenta for histopathological studies and in their study, controlling for this, they found obesity to have a protective effect against acute placental inflammation. This is further evidenced by obesity not influencing any downstream signalling pathways of SAPK/JNK, ERK 1/ 2 and JAK2 induced by pro-inflammatory cytokines, suggesting that changes may be a protective mechanism to counteract the maternal hyper inflammatory environment (Nogues *et al.*, 2021).

In our rat study we did not find evidence of an increased inflammatory state in the placenta. Lower n6 PUFA and SFAs were found, along with an increase in PPARγ (anti-inflammatory) and no change in the mRNA expression of NFκB, compared to lean dams. Unfortunately, we were unable to measure cytokine levels, however, this would have been very useful to confirm in our study. In humans, we saw no changes indicative of increased inflammation and again saw a reduction in one of the SFAs. Diet specific changes are especially relevant here. As discussed previously, SFAs are pro-inflammatory and palmitic acid has been suggested to be responsible for placental inflammation caused by obesity via NLRP3 signalling (Shirasuna *et al.*, 2016) but we saw a decrease in palmitic acid in the rat and no change in the human. Stearic acid has also been identified to act through NLRP3 (Myatt and Maloyan, 2016) and stearic acid was seen to decrease in the HFHC fed rats and in the obese human. TLR4 increases are associated with maternal obesity and is driven mainly through palmitic acid (Reyna *et al.*, 2008) and strong correlations have been identified with TLR4 and

inflammatory cytokines including IL-6 and IL-8 in circulation and in the placenta (X. Yang et al., 2016). Timing is a very important aspect to look at in these changes as gestational age differences have been seen in rodents with no significant differences observed at GD15.5 but at GD17.5 alterations were seen (D. W. Kim et al., 2014). There are big changes associated with labouring compared to non-labouring tissues and these are mostly to do with inflammation processes (Lee *et al.*, 2010). This argument is strengthened by a metabolomics study which found no evidence for lipotoxicity in the placenta in association with labour dystocia in obese women (Carlson *et al.*, 2020).

The final chapter investigated the combined effects of obesity and NCDs due to the profound link between obesity and NCDs (GBD 2015 Obesity Collaborators et al., 2017; Felisbino-Mendes et al., 2020), particularly in Pakistan (Kazmi *et al.*, 2022). The effect of obesity on pregnancy outcomes was attenuated through the inclusion of NCDs, however, the risk of emergency CS remained significant for obese mothers. Results from other studies are varied, finding NCDs (Bonham et al., 2018; Fazel et al., 2018; Iftikhar et al., 2021; T. S. U. Kiran et al., 2005; Malhamé et al., 2021) and obesity (Bhattacharya et al., 2007; Denison et al., 2008; Briese et al., 2011) to increase operative deliveries. However, discrepancies are seen with specific outcomes such as induction of labour and birthweight. Variation seen between studies can be accounted for in the variables looked at, for example, grouping all NCDs together (Amjad *et al.*, 2020; Rasool *et al.*, 2021). It was evident from this study, that within a setting where the prevalence of NCDs and obesity are high, there are a huge number of CS. To address the huge number of negative pregnancy outcomes, particularly in LMICs where there is a greater burden of obesity, NCDs and poor pregnancy outcomes, further work is needed to comprehensively understand the drivers behind this in order to come up with effective solutions to reduce them.

Diet is also hypothesised as a key factor in pregnancy outcomes. Although no effect of diet was demonstrated to have a significant effect in our study, possibly due to the small sample size, it was evident that none of the mothers included in the study ate according to dietary guidelines. Others have shown evidence of fish oil reducing PTBs (Villar *et al.*, 2003) and n6 PUFAs in the diet can increase this risk (Elmes et al., 2005). There is still a strong argument that issues with pregnancy outcomes may be more

related to diet than obesity itself. A diet high in saturated fat has been shown to be important for inflammatory signalling within adipose tissue and linked an 'obese' gene expression profile without an obese phenotype (Van Dijk *et al.*, 2009). Baboons fed a western diet for a year, did not have significant increases in body weight or fat mass so are not classed as obese (estimated around 3 years on this diet to induce obesity) but they did have some characteristics such as elevated serum TG, HDL, LDL/VLDL etc but not blood glucose (Sugino *et al.*, 2022). Epigenetic changes were observed in the placenta and foetus of these animals, along with increased macrophage infiltration but not an increase in cytokines. So, despite the absence of maternal obesity, a western diet had significant consequences for the placenta and foetus, suggested to be driven partly due to alterations observed in the gut microbiome (Sugino *et al.*, 2022). Furthermore, specific food groups have been found to influence outcomes at different stages within pregnancy (Najpaverova *et al.*, 2020).

There are a variety of diet models used to induce obesity within animal models. The most commonly used in rodents is a high fat model, however, this does not closely match a typical western diet, especially related to the proportion of energy from protein and fats. The average estimate from the US is around 14% from protein and 35% energy from fat, however, diet models often feed around 20% energy from protein and feeding in excess 40% energy from fat is considered to be too high (Christians *et al.*, 2019). These diets are often associated with reduced foetal growth which is often not seen in obese human pregnancies and so could explain the discrepancies (Christians *et al.*, 2019). FA composition of the diet is also important as demonstrated by Draycott et al. (2019), who fed high/low LA accompanied with high/low total fat consumption and saw differences in FA profile of the placenta with both amount of fat and LA having modulating effects. Alternative models for diet induced obesity include a cafeteria diet or high fat and high carbohydrate/ sucrose with aim to better model a western or obesogenic diet.

Dietary fats are not the only important factor to consider. Obesogenic diets are not solely associated with increased fat and cholesterol but also increased refined sugars and processed carbohydrates (Renault *et al.*, 2015). Particularly as the 'carbohydrate insulin model' proposes that obesity results from increased dietary carbohydrates

driving excess insulin secretion causing adipose tissue to accumulate, increasing hunger and decreasing energy burnt when exercising (Hall *et al.*, 2022). Glucose particularly, has a modulating effect on FAs. For example, when measuring free FA concentration within serum of pregnant women and an oral glucose load significantly decreased the total free FA in serum after 2 hours (Villa *et al.*, 2009). Placental explants cultured in high glucose media, had no change in FA profile, abundance or uptake but did see an increase in TG fractions down to reduction in b-oxidation (Hulme *et al.*, 2019). The placenta alters its physiology in response to the integration of nutritional and endocrine signals dependent on the nature and severity of the maternal environment (Mandò *et al.*, 2018). In obesity this can be factors such as nutritional status, and maternal glycemia (Mandò *et al.*, 2018). Glucose tolerance changes throughout pregnancy and as pregnancy progresses an increase in maternal insulin resistance within the feotal-palcental unit occurs, teamed with an increase in insulin production from pancreatic hyperplasia which is driven by oestrogen and progesterone (Jeve, Konje and Doshani, 2015). Insulin sensitivity and type 2 diabetes are common co-morbidities with obesity (Roland *et al.*, 2020) and obesity is commonly seen with GDM (Ruiz-Palacios *et al.*, 2017). Insulin can affect free FA profiles on a whole body level through altering lipid mobilisation, particularly in type 2 diabetes (Sobczak, Blindauer and Stewart, 2019). Although insulin cannot cross the placenta, it can bind to receptors and induce IP3/AKT and RAS/MEK/ERK signalling and alter metabolism, specifically AKT signalling can promote lipid accumulation within the placenta (Ruiz-Palacios *et al.*, 2017). GDM in particular, has been demonstrated to cause methylation changes within the placenta along with miRNA changes and interestingly PPARα showed lower methylation values (Lizárraga *et al.*, 2023) and so could mean increased expression. Insulin resistance and hyperinsulinemia, alongside obesity, has observed gene expression changes characteristic of impaired energy metabolism (Reichetzeder, 2021). A dietary intervention study aimed at improving eating and activity for obese mothers suggested that placental lipid droplets may reflect placental FA synthesis from excess glucose associated with insulin resistance as opposed to maternal TG uptake from serum (Gázquez *et al.*, 2018). Furthermore, Villa et al (2009) found the oral glucose load to decrease total FAs in serum to a much greater extent in pre-eclamptic mothers, who exhibit much more insulin resistance. Placental macrophages treated with MetaC (a cocktail of glucose, insulin and SFA palmitic acid as a model for

metabolic stress) further increased the activation of NLRP3 and subsequent release of IL-1b and apoptosis of palmitic acid alone (Rogers *et al.*, 2020). When this cocktail was used on BeWo cells specifically, it altered the pregnancy hormones HCGb and progesterone release (Rogers *et al.*, 2023). In a placental explant study high glucose media altered the uptake of oleic acid and altered placental TG, phospholipid and lysophospholipids compared to those in normal glucose media (Watkins *et al.*, 2023). The relationship between glucose, insulin and FA uptake are not entirely clear as Duttaroy and Jørgensen (2005) found no change to OA, EPA, DHA and AA uptake in BeWo cells when treated with insulin. These studies demonstrate that not only does excess glucose intake cause insulin resistance, which can affect lipid profiles and alteration of cellular lipid content but can also affect the metabolism of FAs, even at acute loads. As the increase in insulin resistance and dietary sugars have been associated with obesity (M. Yuan et al., 2001) it is important to consider these in fully understanding how maternal obesity may affect the placental metabolism and what impact this could have on the pro-labour mediators produced. Our rat model of maternal obesity was based on a high fat diet and no measure of glucose tolerance or insulin resistance was looked at. This could be a contributing factor to the differences seen between the rat and human tissue.

Another important consideration of overall diet is micronutrients. Rats with a severe iron deficiency were found to have an altered FA composition of erythrocyte membranes, particularly in relation to AA and OA (Tichelaar *et al.*, 1997). Selenium treated placental explants blunted the inflammatory response induced by LPS or TNF with specific reduction s in IL-6 and IL-1b through ERK phosphorylation (Nguyen-Ngo, Perkins and Lappas, 2022). These again demonstrate the importance of the modulating effects the diet can have on FA compositions and signalling within the placenta which could modulate the response to maternal obesity.

As discussed previously one of the limitations of this study is the placentas are taken from mothers who delivered by elective CS and so we have no indication of whether or not they would have a normal and successful labour. Not all obese women have difficult labours and so some of these women may have had no issues at all during delivery and so may not have any placental abnormalities. It could be that there is a sub type of obese women or women who have a specific phenotype in response to

diet, that have difficult labours and so it is important to identify these risk factors prior to term. Four subtypes of obesity heterogeneity have been classified, which include ‘metabolically healthy’ where there is an absence of other metabolic disorders such as diabetes or hypertension, ‘metabolically unhealthy’ whom have an increased BMI along with metabolic disorders as well as a subgroup with a BMI below 25 but other markers such as insulin resistance and abdominal/visceral adiposity (Mayoral *et al.*, 2020). This is particularly interesting as in the metabolically healthy obese a reduced inflammatory state has been observed and is proposed to be driven by a more favourable FA profile (Perreault *et al.*, 2014). An example of this in pregnancy is demonstrated by baboons maintained on a high fat diet for 4 years. 2 phenotypes were observed based on body weight and insulin resistance, described as diet sensitive or diet resistant. Some overall changes based on the high fat diet were observed such as decreased blood flow within the placenta, increased TGs and general increase in IL-1b and TLR4 but those described as diet sensitive had much greater placental pathology and increased 13 cytokines which the HFD saw no overall change in (Frias *et al.*, 2011). Furthermore, the diet sensitive baboons group saw increased frequency of adverse pregnancy outcomes including still birth (Frias *et al.*, 2011). Another similar study in pigs, all maintained on the same diet, were classified based on their back fat. Proteomic study identified changes in placental lipid accretion, oxidative stress and inflammation, suggested to be driven through increased JNK/ NFκB signalling in the placenta of obese sows (T. Liang et al., 2018).

For this to be investigated in humans placentae from obese and lean women with successful vaginal births should be compared to obese women who had more difficult births such as prolonged and dysfunctional labour and induction or emergency CS. In this regard, Birchenall et al (2022) conducted a metabolomics analysis of maternal blood, cord vein and cord artery to try and identify changes in metabolites between non-labouring and labouring mothers. This included steroid hormones and fatty acid composition and successfully identified changes in foetal-placental circulation associated with spontaneous labour. A similar study could be undertaken comparing obese women with normal births and those with complications to pull out any metabolomic changes. Human studies have identified a ‘metabolically healthy’ subset of obese individuals who tend to have better adipose tissue function, more insulin sensitive and reduce inflammatory environment

(Goossens, 2017). A similar concept could exist with maternal obesity. FA composition from maternal plasma at term and/or the initiation of spontaneous labour, along with the fatty acid composition of the placentas post-delivery could identify specific changes which may explain the differences experienced. Furthermore, gene and protein expression of the placenta should also be examined. PPARγ has been implicated in the differences observed in adipose tissue between metabolically healthy and unhealthy individuals and has been suggested to link to pathological placental disorders (Kwiatkowski *et al.*, 2021). Expression of key genes in the regulation of labour, along with PPARγ should be examined to understand how these may contribute to disordered labour. Conducting a dietary analysis through FFQ and validating this through fatty acid analysis of erythrocytes specifically (as an accurate marker of diet within the past week) could also help understand the drivers behind these metabolomic changes.

If clear changes can be identified from the above study that can be linked to potential dietary patterns, a dietary intervention within these women during their pregnancy could be a viable option for reducing their risk. Clinical trials aimed at improving healthy eating indexes saw that a higher quality diet (less total fat) during pregnancy indicate it may improve insulin profile and signalling in the placenta (E. C. Francis et al., 2022). A systematic review found maternal lifestyle interventions to be successful in regulating negative epigenetic effects and a less inflammatory environment in the placenta from maternal hyperglycaemia (Panagiotidou *et al.*, 2024). Although this is not fully supported, as another human random control trial found that lifestyle and dietary intervention was not associated with any clinically important markers including maternal inflammation (Moran *et al.*, 2013). There is, however, no evidence that these types of interventions can cause negative outcomes for the epigenome or general health of the offspring, with results from studies stating positive or neutral effects of the interventions (Panagiotidou *et al.*, 2024). Dietary alterations could be particularly important during the first and second trimester due to the increased adipose uptake of FAs and greater fat deposits (Herrera et al., 2006; Herrera, Maiti and Smith, 2023). Adipose tissue composition then largely influences the maternal FA plasma levels and consequently placental FA composition due to the increased lipolytic activity during the third trimester (Herrera, 2002; Amusquivar and Herrera, 2003). Amusquivar et al (2003) demonstrated in a rat study that the alterations in the

FA composition of the diet could alter the adipose tissue composition and significant correlations between LCPUFAs in maternal plasma and the placenta were seen. Evidence has shown these intervention approaches may work but the information must be specific to the individual and their lifestyle to elicit the greatest effects, and particularly in the first trimester to really capitalise on weight management during pregnancy (Langley-Evans, Pearce and Ellis, 2022). Pre-pregnancy period is a good opportunity for safe weight management interventions and advice should be given on the effect their BMI may have on the risks during pregnancy and childbirth (Poston *et al.*, 2016). During pregnancy those with a BMI>30 should be advised to avoid high gestational weight gain (Poston *et al.*, 2016). The international federation for obstetrics and gynaecology (FIGO) has stated nutritional interventions before, during and after pregnancy have the potential to have a vast impact on the obesity and NCDs worldwide due to the simple to assess and modifiable risk factor (Hanson *et al.*, 2019).

It is clear that chronic exposure to a HFHC diet significantly alters the placental fatty acid profile and gene expression. This could have implications on the production of pro-labour mediators as demonstrated through cell culture model. This in turn may affect the uterine environment at term, impeding the contractile ability of the myometrium. Unfortunately, a mechanism or signalling pathway behind the action of OA on PGs and inflammatory cytokines could not be identified. Future work will aim to establish the impact of OA under more labour like conditions and identify a signalling route through inhibitors focusing on NFκB and PPARγ. To consolidate the relationship between OA, dietary SFA levels and obesity, a comprehensive dietary study teamed with fatty acid analysis and gene/ protein expression of obese women in successful and complicated labours needs to be carried out.

6.1 Conclusion

In conclusion, placental changes induced by a HFHC diet may contribute to the disrupted contractions and prolonged labour seen within this model of maternal obesity. Changes within obese human placental tissue were limited but could be due to large differences in the diet or a specific subset of women who have labouring problems. Our dietary analysis study was not large enough to pull out any relationships with diet and birth outcomes, but others have. Finally, we demonstrate

the importance of accounting for common comorbidities such as NCDs when understanding a woman's risk for negative pregnancy outcomes.

6.2 Future work

Chapter 3 established changes in the rat that were not as clear in the human and a potential difference for this was the rat tissue was in active labour and the human tissue taken from elective CS. There is some evidence indicating altered fatty acid metabolism pathways between labouring and non-labouring placenta (Birchenall, Welsh and López Bernal, 2022) and so comparing human term labouring and elective CS FA profiles would be useful to compare. This chapter also identified increases in mRNA of PPARγ in the rat but with no clear effective pathway identified through looking at mRNA. Activity assays of PPARs and NFκB in HFHC and control and differing BMI human placentae would be useful to confirm functional alterations and their impacts. Along with multiplex assays to assess cytokine production within the placenta.

Chapter 4 established increased levels of OA decreased PG and IL-6 production from a trophoblast cell line. Confirming these findings in a more labour like environment, using dexamethasone, and using primary trophoblast cells from term placenta would further confirm this hypothesis. To specifically understand the role of PPARγ better, trophoblast cells in culture could be treated with agonist and antagonist and measure markers of inflammation (for example interleukins) both secreted and intracellular. Co-cultures of smooth muscle cells and endothelial cells have been developed to mimic the decidua and myometrium boundary (Ke *et al.*, 2020). In this study they used transwell system and cultured HTR8SVneo cells on the opposite side in order to measure invasion of these cells (Ke *et al.*, 2020). A similar model using 2 planar chambers separated by a semipermeable synthetic membrane, which allows for signal propagation but are too small for cells to move through have been used (Richardson *et al.*, 2020). Placenta-on-a-chip has predominantly been used to look at the transport between mother and foetus for example with drug delivery, with just 2 cell types and a channel between (Park *et al.*, 2023). As detailed in Richardson et al. (2020), these models can have multiple layers and have been used from amnion through to decidua. This could be setup with trophoblast cells, endothelial and smooth muscle cells to establish whether increased OA within these cells would have

impactful alterations on the myometrium which could affect contractions. Measurements such as PGs and inflammatory cytokines could be measured in the media as well as looking at key changes in the myometrium cells such as measuring CAP expression. As discussed in section 4.4 The use of PPARγ antagonists and agonists could also be used to better understand their role in inflammation specifically.

The metabolomics study discussed in 6.1 to identify potential biomarkers or a subset of women more at risk of dysfunctional labour would be beneficial. This could help identify the specific drivers of disrupted and prolonged labour. Combined with a more extensive, detailed FFQ study with a much larger sample size may also help to identify dietary patterns that could contribute to dysfunctional labour and inform nutritional advice. It would also be important to collect more information on risk factors and details associated with CS to get a clearer picture on the role obesity plays in this specifically.

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