**Bile acid receptors
role in susceptibility to and novel treatments for gestational diabetes mellitus**

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Bile acid receptors: role in susceptibility to and novel treatments for gestational diabetes mellitus

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Abstract

Numerous changes occur during pregnancy to accommodate the demands for fetal growth and development, including alterations in glucose, lipid and bile acid metabolism. Gestational diabetes mellitus (GDM) is a pathological condition that is becoming increasingly prevalent every year, increasing in frequency worldwide and affecting people of all backgrounds. There is limited understanding of the pathophysiology and progression of GDM, and improved treatments are needed.

This thesis hypothesises that the metabolites progesterone sulphates and bile acids influence glucose metabolism, thereby having a role in the development of GDM. Serum samples from four cohorts of women were used to assess this. Three cohorts (Cohorts 1, 2, 4) were derived from the third trimester and were either GDM or non-GDM, with cohort 4 also being separated by ethnicity (European or South Asian) and BMI (normal and obese). Cohort 3 comprised serum samples obtained from women in the first trimester who either went onto develop GDM or have uncomplicated pregnancies. Cohort 3 was also divided into low (≤ 25 kg/m^2) and high (≥ 35 kg/m^2) BMI. Additional experiments using *in vitro* studies of islets obtained from mouse and humans were performed to assess how glucose stimulated insulin secretion (GSIS) is affected by metabolites that were altered in GDM, and calcium signalling experiments further evaluated mechanisms of action.

Compared to non-GDM women, some progesterone sulphates were lowered in women diagnosed with GDM in the third trimester. Epiallopregnanolone sulphate (PM5S) was also reduced in the first trimester of women that subsequently developed GDM in the third trimester. *In vitro* islet experiments demonstrated that PM5S increases GSIS in mouse and human islets via transient receptor potential cation channel subfamily melastatin member 3 (TRPM3), an ion channel which allows influx of calcium ions into cells. This was further corroborated when HEK293 cells transfected with TRPM3 displayed concentration dependent increase in calcium concentration with PM5S. These findings demonstrate a link between progesterone sulphates and the development of GDM.

Using cohort 4, we assessed serum bile acid concentrations in GDM. Obese GDM European women displayed the most significant changes, showing an increase in primary, secondary, conjugated, unconjugated and 12α-hydroxylated bile acids. In contrast, obese GDM south Asian women have reduced serum concentrations of secondary bile acids. Furthermore our *in vitro* data demonstrate that TCA and TCDCA induce increased GSIS in mouse and human islets, although the mechanism of action remains inconclusive. Our data suggests that bile acids have a role in glucose metabolism and consequently the progression of GDM.

Takeda G-protein receptor 5 (TGR5), a bile acid receptor with the highest affinity to secondary bile acids, is known to be expressed in the gut, with the highest expression being at the terminal ileum and colon. TGR5 activity is implicated in the development of GDM and is a valuable therapeutic target. Work presented in this thesis addressed the hypothesis that bile acids and progesterone sulphates alter enteroendocrine signalling and GLP-1 secretion through TGR5 signalling in the gut. Mouse and human models of the colon were isolated and cultured to examine GLP-1 secretion in the presence of progesterone sulphates and bile acids. Both secondary bile acids and progesterone sulphates were shown to activate TGR5 to cause GLP-1 secretion in mouse crypt cultures and human colonoids. GLP-1 secretion is important in regulating and reducing glucose intolerance which in turn will improve GDM. These findings suggest that targeting bile acids, progesterone sulphates and TGR5 could improve glycaemic control in GDM.

COVID-19 Impact Statement

The pandemic had initially prevented my ability to carry out further experimental research. When KCL start to allow students back into the labs, it was a gradual process and had severe restrictions, which limited opportunities to complete my experimental work to an efficient standard and so did not complete all experiments I had planned. During the time I could not carry out experimental work, I focused my efforts on writing up a literature review, which has been published, and a scientific paper which is due to be published at the end of 2021. I also sought to put more emphasis on the clinical data I was beginning to generate and expand on this analysis. The pandemic had also severely impacted my mental well-being and I had struggled to cope with continuing my research and meeting my milestones, though I sought help for this to try and carry on as normal.

Statement of originality

The work described in this thesis was carried out by myself unless otherwise stated in the text.

Part of the introduction in Chapter 1 has been published as a review in: *Fan, H., Mitchell, A. and Williamson, C., 2021. ENDOCRINOLOGY IN PREGNANCY: Metabolic impact of bile acids in gestation. European Journal of Endocrinology,* 184(3), pp.R69-83. Please see Appendix 1 for full author accepted manuscript version. The definitive version is now freely available at DOI: 10.1530/EJE-20-1101. (2021).

Acknowledgements

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List of Abbreviations

αMCA	α-Muricholic acid
βMCA	β-Muricholic acid
ABCG5/8	ATP-Binding Cassette Transporters G5/8
ANOVA	Analysis of Variance
ASBT	Apical Sodium-Dependent Bile Acid Transporter
ATP	Adenosine Triphosphate
BAT	Brown Adipose Tissue
BMI	Body Mass index
BSA	Bovine Serum Albumin
BSEP/ABCB11	Bile Salt Export Pump
BSU	Biological Services Unit
CA	Cholic Acid
cAMP	Cyclic Adenosine Monophosphate
CDCA	Chenodeoxycholic Acid
ChREBP	Carbohydrate Response Element-binding Protein
cpm	Counts per minute
CTL	Control
CYP27A1	Sterol 27-hydroxylase
CYP7A1	Cholesterol 7α-hydroxylase
CYP8B1	Sterol 12α-hydroxylase
DCA	Deoxycholic Acid
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent assay
F/I	Forskolin/IBMX
FCS	Fetal Calf serum
FGFR4	Fibroblast Growth Factor Receptor 4

FGF19/15	Fibroblast Growth Factor 19/15
FPG	Fasting Plasma Glucose
FXR	Farnesoid X receptor
GCA	Glyco cholic acid
GCDCA	Glyco chenodeoxycholic acid
GDCA	Glyco deoxycholic acid
GDM	Gestational Diabetes Mellitus
GIP	Glucose-dependent Insulinotropic Polypeptide
GLCA	Glyco lithocholic acid
GLP-1	Glucagon-like-peptide 1
GLP-2	Glucagon-like-peptide 2
GLUT2	Glucose transporter type 2
GLUT4	Glucose transporter type 4
GOCA	Glyco obeticholic acid
GSIS	Glucose-Stimulated Insulin Secretion
GUDCA	Glyco ursodeoxycholic acid
HAPO	Hyperglycaemia and Adverse Pregnancy Outcomes
HCA	Hyocholic Acid
HDCA	Hyodeoxycholic Acid
HDL	High Density Lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HTRF	Homogeneous Time-Resolved Fluorescence
IADPSG	The International Association of Diabetes and Pregnancy Study Groups
IBMX	3-Isobutyl-1-Methylxanthine
ICP	Intrahepatic Cholestasis of Pregnancy
IL-6	Interleukin-6
IRS1	Insulin Receptor Substrate 1
ISO	Isosakuranetin
KO	Knockout
LCA	Lithocholic Acid
LDL	Low-density lipoproteins

LGA	Large for Gestational Age
LXR	Liver X Receptor
MDR3/ABCB4	Multidrug Resistance Protein 3
MEM	Minimum Essential Medium
MeOH	Methanol
MiG	Metformin in Gestational Diabetes
MOE	Molecular Operating Environment
NCS	Newborn Calf Serum
NICE	The National Institute for Health and Care Excellence
NS	No significance
NTCP	Sodium Taurocholate Co-Transporting Polypeptide
OATP	Organic Anion Transporting Polypeptides
OCA	Obeticholic Acid
OGTT	Oral Glucose Tolerance Test
OST α/β	Organic Solute Transporter α/β
OW	Overweight
PBS	Phosphate Buffered Saline
PC	Phosphatidylcholine
PEG	Polyethylene Glycol
PI3K	Phosphoinositide 3-kinase
PKA	Protein Kinase A
PKB	Protein Kinase B
PM2DiS	Allopregnandiol disulphate; 5α-pregnan-3α-20α-diol-3,20-disulphate
PM3DiS	Pregnandiol disulphate; 5β-pregnan-3α-20α-diol-3,20-disulphate
PM3S	Pregnandiol sulphate; 5β-pregnan-3α-20α-diol-sulphate
PM4S	Allopregnanolone sulphate; 5α-pregnan-3α-ol-20-one-sulphate
PM5S	Epiallopregnanolone sulphate; 5α-pregnan-3β-ol-20-one-sulphate
PMΔ5S	Pregnenolone sulphate; 5-pregnen-3β-ol-20-one-sulphate
PXR	Pregnane X receptor
RIA	Radioimmunoassay
RMSD	Root-mean-square deviation of atomic positions

ROC	Receiver Operating Characteristic
RPMI	Roswell Park Memorial Institute
S1PR2	Sphingosine-1 phosphate receptor subtype 2
SD	Standard Deviation
SEM	Standard error of the mean
SHP	Small Heterodimer Protein
SREBP-1c	Sterol Regulatory Element Binding protein 1c
T-αMCA	Tauro α-muricholic acid
T-βMCA	Tauro β-muricholic acid
T2DM	Type 2 Diabetes Mellitus
TBA	Total bile acids
TCA	Tauro cholic acid
TCDCA	Tauro chendeoxycholic acid
TDCA	Tauro deoxycholic acid
TGR5	Takeda G-protein receptor, also known as GPBAR1
TLCA	Tauro lithocholic acid
TNFα	Tumour Necrosis Factor-α
TOCA	Tauro obeticholic acid
TRP	Transient Receptor Potential
TRPM3	Transient Receptor Potential Melastatin-3
TUDCA	Tauro ursodeoxycholic acid
UCP-1	Uncoupling protein 1
UDCA	Ursodeoxycholic Acid
UPLC-MS/MS	Ultra-performance Liquid Chromatography-tandem Mass Spectrometry
UW	University of Wisconsin solution
VDR	Vitamin D receptor
WAT	White Adipose Tissue
WHO	World Health Organization
WT	Wildtype

Chapter 1: Introduction

1.1 Overview

Gestational diabetes mellitus (GDM) is a condition occurring during pregnancy that affects a significant proportion of pregnant women worldwide. Many of the complications are related to metabolic dysfunction, and the aetiology of many of the metabolic alterations that occur during GDM are not fully understood. The absence of a detailed understanding of underlying pathophysiological mechanisms present challenges for correct diagnosis and prediction of the onset of GDM, and for provision of the best treatments for prevention of the disease.

Bile acids are signalling molecules that play a significant role in normal physiology; metabolic bile acid changes during healthy pregnancy are necessary for normal fetal growth and development. Currently, there are few studies evaluating the effects of bile acids in GDM. A more thorough understanding of the metabolic gestational alterations that occur in bile acid homeostasis maybe key to further understand the progression of GDM, and possibly inform the development of therapeutic targets to treat GDM. Two of these targets include the bile acid receptors Farnesoid X receptor (FXR) and Takeda G-protein receptor 5 (TGR5). Both receptors are key in modulating normal metabolism and understanding how they affect GDM could be pivotal. Another group of hormones called progesterone sulphates have emerged as potential targets for influencing GDM; although studies exploring how progesterone sulphates interact with GDM are limited.

The work presented in this thesis explores the relationship between bile acids, progesterone sulphates and GDM. This includes looking at the mechanistic actions of the bile acid receptors, FXR and TGR5 as well as the changes in bile acid and progesterone sulphate serum concentrations in normal vs GDM pregnancies. Parts of this introduction draw upon a published review (Fan, Mitchell, & Williamson, 2021) included in appendix 1.

1.2 Bile acids

1.2.1 Synthesis and function

Bile acids are a group of cholesterol-derived steroids with an aliphatic side chain that are synthesised in the liver and exported into the bile. Before secretion, bile acids are conjugated with either glycine or taurine, increasing hydrophilicity and reducing cytotoxicity (Šarenac & Mikov, 2018). The primary functions of bile acids is to solubilise lipids by forming micelles to aid emulsification and facilitate absorption of fat by the gut (Ahmad & Haeusler, 2019). Recent research has demonstrated that bile

acids also have hormonal and metabolic functions, particularly in glucose and lipid regulation, and that they signal through the receptors FXR and TGR5 (Kiriyama & Nochi, 2019) in numerous cell types throughout the body.

1.2.2   Homeostasis and enterohepatic circulation of bile acids

Bile components, including bile salts are synthesised in the liver, exported into bile ducts and stored in the gallbladder until meal ingestion. High concentrations of bile acids are toxic and therefore production and excretion are tightly regulated. There are two main pathways for bile acid synthesis: the classical and alternative pathway.

In humans, the classical bile acid synthesis pathway results in the conversion of cholesterol into the primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA), and accounts for approximately 90% of bile acid synthesis. This hepatic-specific pathway involves at least 17 separate steps, and activity of the rate-limiting enzyme cholesterol 7α-hydroxylase (CYP7A1) determines the size of the bile acid pool (Chiang & Ferrell, 2018), while sterol 12α-hydroxylase (CYP8B1) increases CA synthesis and the CA:CDCA ratio (Chiang & Ferrell, 2018). The alternative bile acid synthesis pathway starts with hydroxylation of cholesterol by sterol 27-hydroxylase (CYP27A1) in extrahepatic sites to form 27-hydroxylcholesterol, which is then taken up by the liver and the majority converted to CDCA (Kiriyama & Nochi, 2019). Bile salts are formed by conjugation of bile acids with either taurine or glycine at a ratio of approximately 1:3 (Chiang & Ferrell, 2018), and transported into the bile canaliculi through the bile salt export pump (BSEP)(Kullak-Ublick, Stieger, Hagenbuch, & Meier, 2000). Other membrane-residing transporters that influence bile components include multidrug resistance protein (MDR3), a phosphatidylcholine (PC) floppase that transports PC from the inner to the outer canalicular membrane (and into the bile), and ATP-binding cassette transporters G5/8 heterodimer (*ABCG5/8*), which transport cholesterol into the bile canaliculi (Graf et al., 2003; Oude Elferink & Paulusma, 2007). From here, bile is transported to the gallbladder for storage. Figure 1.1 summarises the role of these hepatic pathways within the enterohepatic circulation of bile acids.

Ingestion of food stimulates release of bile from the gallbladder which facilitates the digestion and absorption of lipids and lipid-soluble vitamins. The gut microbiota in the ileum and colon deconjugate the primary bile acids, and further modify them through 7-dehydroxylation to produce secondary bile acids; lithocholic acid (LCA) is formed from CDCA whereas deoxycholic acid (DCA) is derived from CA (Figure 1.2). The gut microbiota can further modify bile acids by 7α/β-epimerisation to make ursodeoxycholic acid (UDCA), and more rarely by 3α/β-epimerisation, 5α/β-epimerisation or oxidation to produce iso-, allo-, or oxo-bile acids, respectively (Wahlström, Sayin, Marschall, &

Bäckhed, 2016). The bile acid pool in the terminal ileum comprises approximately 30% CA, 40% CDCA, 20-30% DCA and below 5% LCA (Šarenac & Mikov, 2018), although this varies between individuals as it is influenced by factors including nutrient availability and gut microbiota composition. Approximately 95% of the bile salts are reabsorbed, either through the apical sodium-dependent bile acid transporter (ASBT) at the distal ileum and colon, or through passive absorption of deconjugated or protonated uncharged conjugated bile acids along the length of the intestine (Dawson & Karpen, 2015). The remaining 5% is excreted in the faeces, and this loss is compensated by approximately 500mg/day *de novo* bile acid synthesis (Šarenac & Mikov, 2018). Reabsorbed bile acids are exported from ileal enterocytes into the enterohepatic circulation by the heterodimeric organic solute transporter α/β (OST α/β) on the basolateral membrane of the cells (Šarenac & Mikov, 2018). The bile acids are transported via the portal vein back to the hepatocytes through the sodium taurocholate co-transporting polypeptide (NTCP) or organic anion transporting polypeptides (OATP) (Šarenac & Mikov, 2018), reconjugated, and again exported into the bile duct (Figure 1.1).
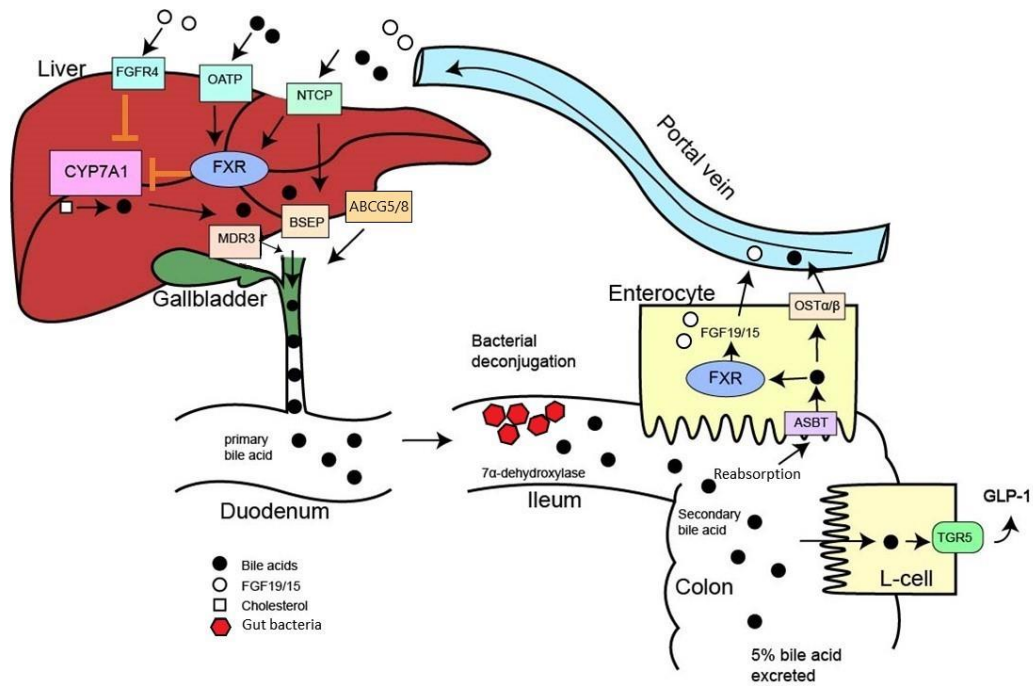
Figure 1.1: Enterohepatic Circulation of Bile Acids.

Schematic detailing the formation and export of bile acids into the intestinal tract. Bile acids are modified from primary to secondary forms by and deconjugation and 7α-dehydroxylase produced by gut bacteria. The majority of bile acids are reabsorbed through enterocytes (approximately 95%), with the remainder (~5%) excreted in the faeces. Reabsorbed bile acids in the L-cells activate TGR5 on the basolateral side which potentiates GLP-1 release. The reabsorbed bile acids are transported back to the liver via the portal vein, completing the enterohepatic cycle. FXR, Farnesoid X receptor; TGR5, Takeda G-protein coupled receptor 5; GLP-1, Glucagon-like peptide-1; ASBT, Apical sodium-dependent bile acid transporter; OST α/β, Organic solute transporter α/β; NTCP, Taurocholate co-transporting polypeptide; OATP, Organic anion transporting polypeptides; FGFR4, Fibroblast growth factor receptor 4; CYP7A1, Cholesterol 7α-hydroxylase; MDR3, Multidrug resistance protein; BSEP, Bile salt export pump; ABCG5/8, ATP-binding cassette transporters G5/G8 heterodimer; FGF19/15, Fibroblast growth factor 19/15.
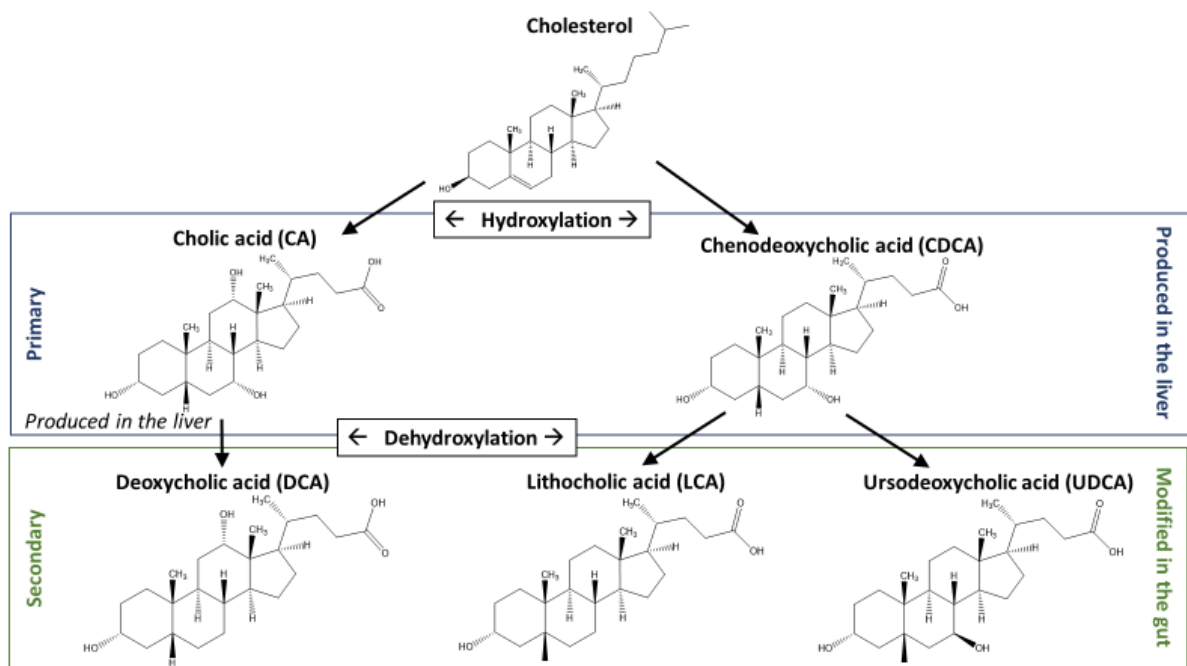
Figure 1.2: Bile acid structures.

Pathways displaying the formation and structures of primary and secondary bile acids in their unconjugated form, derived from cholesterol.

1.2.3   Metabolic actions

The emerging role of bile acids in the homeostasis of metabolic substrates has shifted the focus of bile acid research into looking at cellular targets of bile acids. Bile acids have been found to have multi-organ targets and a variety of receptors (Vítek & Haluzík, 2016). The two most notable, and those towards which the bile acids have the greatest affinity, are FXR and TGR5.

FXR plays an essential role in not only bile acid metabolism, but also in lipid and glucose metabolism (Ahmad & Haeusler, 2019). FXR is expressed in key metabolic organs like the liver, intestine and pancreatic β-cells, enabling its involvement in the control in metabolism (Ahmad & Haeusler, 2019). Likewise, TGR5, a G-protein-coupled receptor that is widely expressed contributes most notably to glucagon-like-peptide 1 (GLP-1) secretion via activation in the gut, influencing glucose homeostasis by promoting insulin secretion (Christiansen et al., 2019; Kuhre et al., 2018; Pathak et al., 2018). Bile acid activation of these receptors could be key targets for modulation of metabolism and may also help explain the pathogenesis of metabolic diseases. Their influence in glucose and lipid metabolism is further detailed later in this chapter.

1.2.4   Bile acids in Pregnancy

Pregnancy is associated with a number of metabolic adaptations to facilitate fetal growth. Serum bile acid concentrations are raised in pregnancy compared to non-pregnant adults, resulting in a gestational hypercholanaemia. There is also a gradual increase in serum bile acids as gestation progresses, although for most women this remains within the normal reference range (McIlvride, Dixon, & Williamson, 2017). The concentrations of CA and CDCA are also reported to change as gestation advances (Carter, 1991; Dai et al., 2018; Fulton, Douglas, Hutchon, & Beckett, 1983; Larsson, Palm, Hansson, & Axelsson, 2008; B. Zhu et al., 2019). Conjugated bile acids, in particular taurine conjugated, have also been reported to be higher in pregnant women (Castaño et al., 2006; Fulton et al., 1983).The composition of the maternal gut microbiome may also provide some answers to the bile acid alterations in gestation with some studies reporting a gradual reduction of *Bacteroidetes* and increase in *Firmicutes* as pregnancy progresses, similar to the changes in microbes reported in obesity (Collado, Isolauri, Laitinen, & Salminen, 2008; Koren et al., 2012). In a separate study, advancing gestation was associated with enhanced microbial bile acid deconjugation (secondary to an increase in *Bacteroidetes*-encoded bile salt hydrolase), reduced ileal bile acid uptake and therefore lowered FXR induction in enterocytes (Ovadia, Perdones-Montero, et al., 2019), resulting in increased hepatic bile acid synthesis. Along with reduced ileal FXR activity, studies of pregnant mice showed reduced FGF15 and reduced expression of bile acid transporters late in

gestation (Moscovitz et al., 2016; Ovadia, Perdones-Montero, et al., 2019). More detailed studies that take account of individual variation in gestational phenotypes are required to delineate the alterations in specific enterotypes with advancing gestation.

Pregnancy hormones have also been shown to influence bile acid homeostasis. Studies in mice demonstrated that, as serum bile acid levels increase during gestation, FXR expression is suppressed. This was associated with the downregulation of bile acid transporters such as BSEP, NTCP and OATP, particularly exemplified in the late state of pregnancy (Aleksunes, Yeager, Wen, Cui, & Klaassen, 2012; Milona, Owen, Cobbold, et al., 2010). Hormones such as progesterone and oestrogen, whose concentrations increase as gestation progresses, contribute to the changes in bile acid metabolism. Oestrogen and its metabolites inhibit FXR and BSEP, and increase CYP7A1 activity in animal studies (Chen et al., 2015; Milona, Owen, Cobbold, et al., 2010; Parini et al., 2000) Similarly, BSEP and NTCP are inhibited by sulphated progesterone metabolites and whilst progesterone sulphates exert partial agonism towards FXR, this prevents bile acid binding and reduces overall activation of FXR (Abu-Hayyeh et al., 2010, 2013; Vallejo, Briz, Serrano, Monte, & Marin, 2006) Therefore, both oestrogen, progesterone and their metabolites contribute to raised bile acids during normal pregnancy.

Bile acid composition and concentration may also differ in gestational disease states compared to uncomplicated pregnancies, particularly in the metabolic disorders intrahepatic cholestasis of pregnancy (ICP) and GDM which will be later discussed in this chapter.

1.3 Progesterone Sulphates

Recently, there has been a rise in the interest of sulphated progesterone metabolites and their interaction with bile acid metabolism and transport. Structurally, they are similar to bile acids, in that progesterone sulphates have a steroid backbone and side chains primarily coming from carbon rings A and D. Their similar structure allows similar interactions to the receptors that bile acids can interact with. Progesterone sulphates have been shown to competitively inhibit Na+-dependent bile acid uptake and prevent bile acid efflux by inhibiting BSEP in hepatocytes, which may be a contributing factor to the pathogenesis of hypercholanemia in normal pregnancy (Abu-Hayyeh et al., 2010; Vallejo et al., 2006). Levels of progesterone and its metabolites have been found to significantly increase during pregnancy (Kancheva et al., 2007). More notably, progesterone sulphates have also been found to be markedly higher in ICP as well. Progesterone sulphates are usually circulating at very low concentrations in non-pregnant individuals. There is an 80-fold increase in the third trimester in total allopregnanolone sulphate (PM4S) and epiallopregnanolone sulphate (PM5S) to concentrations that have a greater physiological impact in pregnant compared to

non-pregnant individuals (Abu-Hayyeh et al., 2010; Sjövall, 1970). These findings have prompted recent research to evaluate how progesterone sulphates impact gestational diseases and the possible metabolic effects of these hormones. Figure 1.3 shows the different structures of the progesterone sulphates discussed in this thesis.

PM3S
Pregnandiol sulphate

5β-pregnan-3α,-20α-diol-3-sulphate

PM2DiS
Allopregnandiol disulphate

5α-pregnan-3α,-20α-diol-3,20-disulphate

PM3DiS
Pregnandiol disulphate

5β-pregnan-3α,20α-diol-3,20-disulphate

PM4S
Allopregnanolone sulphate

5α-pregnan-3α-ol-20-one-sulfate

PM5S
Epiallopregnanolone sulphate

5α-pregnan-3β-ol-20-one-sulphate

PMΔ5S
Pregnenolone sulphate

5-pregnen-3β-ol-20-one-sulphate

Figure 1.3: Progesterone sulphate structures.

1.4 Glucose Metabolism

1.4.1 Glucose production and homeostasis

Circulating plasma glucose concentrations are maintained tightly; it is necessary to prevent elevation of glucose to high, and potentially toxic, levels, whilst also maintaining sufficient glucose to be available for use by organs such as the brain as a constant energy source. Glucose levels are increased through either dietary sources, through the production by gluconeogenesis in the liver or converting stored glycogen stores into glucose by glycogenolysis. When plasma glucose concentrations become too high, it is taken up into adipose and skeletal cells. The hormones insulin and glucagon are key in these processes. Glucagon increases glucose production whilst insulin suppresses glucose production if levels become too high, in addition to promoting uptake into cells.

Both insulin and glucagon are released from the islets of Langerhans. Islets of Langerhans are groups of cell located and randomly distributed within the pancreatic tissue, with the total number estimated between 3.2 and 14.7 million islets in human pancreas (Da Silva Xavier, 2018). Islets consist of α cells (glucagon producing), β cells (insulin producing), δ cells (somatostatin producing), γ or PP cells (pancreatic polypeptide producing) and ε cells (ghrelin producing) (Da Silva Xavier, 2018). These cells function to have distinct regulatory roles in energy homeostasis and are crucial to ensuring normal metabolic processes. The inability of islets to produce insulin, or make sufficient amounts, to control blood glucose are the causes of diabetes mellitus. Inter-species differences have also been noted, with both murine and human islets having differing cellular composition and architecture (Kim et al., 2009), which is key to note for the translatable applicability of studies using rodent islets.

1.4.2 Pancreatic β-cell function and insulin signalling

Both insulin and glucagon are released from the pancreas. Insulin is produced and stored in the β-cells and is released when glucose enters β-cells. β-cells are highly sensitive to glucose concentrations and any changes in glucose homeostasis affect β-cell function. Glucose enters the cells through the glucose transporter 2 (GLUT2) where it is turned to pyruvate through glycolysis. The pyruvate enters the mitochondria to produce ATP through the Krebs cycle. The increase in ATP production closes potassium channels preventing potassium leaving cells and as a result, causing membrane depolarisation and an influx of calcium ions through the now open voltage gated calcium channels. The increase in intracellular calcium stimulates the release of insulin from the secretory granules into the circulatory system, allowing insulin to act at various organs to lower systemic glucose levels. Though glucose is the main stimulus for insulin secretion, other biochemical

metabolites such as amino acids and fatty acids can also cause insulin secretion (Fu, R. Gilbert, & Liu, 2012).

The primary function of insulin is to stimulate the uptake of glucose into adipose and muscle whilst also inhibiting hepatic gluconeogenesis. Once absorbed, the glucose is either converted into triglycerides via lipogenesis or glycogen via glycogenesis. When insulin is bound to the insulin receptor, a tyrosine kinase receptor, autophosphorylation of the tyrosine residues is triggered which in turn phosphorylates and activates the insulin receptor substrate. Subsequently, this cascades and activates phosphoinositide 3-kinase (PI3K), inducing the activation of protein kinase B (PKB). Activated PKB facilitates the transport of glucose transporter type 4 (GLUT4) to the cell membrane and permits the diffusion of glucose into muscle and adipose cells (Saltiel & Kahn, 2001). PKB also starts the process of glycogenesis by phosphorylating and inhibiting glycogen synthase kinase which allows glycogen synthase to stay active and continue synthesising glycogen from glucose (X. Fang et al., 2000). Lipogenesis is also promoted by insulin in adipose tissue by increasing the transcription factor of sterol regulatory element binding protein 1c (SREBP-1c), whereby SREBP-1c promotes expression of lipogenic genes (Wang, Viscarra, Kim, & Sul, 2015). Expression of SREBP-1c also leads to increased expression of glucokinase thereby promoting glycolysis (Foretz, Guichard, Ferré, & Foufelle, 1999).

1.4.3   Gestational changes to glucose metabolism

Metabolic changes also occur during pregnancy to accommodate the demands of the fetus. Glucose metabolism during normal pregnancy is characterised by enhanced hepatic gluconeogenesis and impaired insulin sensitivity, resulting in higher circulating glucose concentrations during the third trimester. Insulin resistance is normally compensated for by an increase in the size and number of pancreatic islets, thereby enhancing glucose-stimulated insulin secretion (GSIS) (Hill & Szlapinski, 2020) and preventing the pregnant individual from going into a diabetic state.

These changes in glucose homeostasis and insulin sensitivity in the third trimester are a result of the rising hormones during pregnancy. Pregnancy hormones such as progesterone and oestrogen are at their highest during the third trimester, although the precise mechanisms by which these hormones influence glucose metabolism remain elusive. Oestradiol acts on the β-cells to enhance GSIS, and is also believed to be involved in developing maternal insulin resistance and glucose intolerance (Masuyama & Hiramatsu, 2011; Nadal, Alonso-Magdalena, Soriano, Ropero, & Quesada, 2009). Another suggested mechanisms of action is oestradiol binding directly to insulin and the insulin receptor to cause insulin resistance (Root-Bernstein, Podufaly, & Dillon, 2014). Elevated levels of

progesterone have also been implicated in contributing to decreased insulin sensitivity, increased insulin resistance and glucose intolerance (Masuyama & Hiramatsu, 2011). One mechanism through which this occurs is through inhibition of insulin induced GLUT4 translocation. Progesterone prevents GLUT4 translocation by suppressing the phosphoinositide 3-kinase-mediated pathway inhibiting Akt phosphorylation and decreasing insulin-induced phosphorylation of Cbl signalling proteins, causing reduced cellular glucose uptake (Wada et al., 2010). Human placental lactogen, also increased throughout pregnancy, also induces insulin release and causes peripheral insulin resistance (Barbour et al., 2007). Other hormones such as adiponectin and leptin have also been suggested to contribute to insulin resistance during pregnancy, suggesting that the reduction in insulin sensitivity is multifactorial (Skvarca, Tomazic, Blagus, Krhin, & Janez, 2013).

1.5 Lipid Metabolism

The metabolic process of lipogenesis to generate and store as fats is a key function of insulin as previously mentioned. Activation of SREBP-1c by insulin drives the production of lipids and upregulates genes involving fatty acid synthesis, thereby promoting lipogenesis. Lipogenesis encompasses both fatty acid and triglyceride synthesis where a majority of this process occurs at adipose and liver tissues. The liver also contributes to the synthesis and metabolism of cholesterol. Fatty acids are produced primarily from excess carbohydrates. Much like glucose metabolism, a series of reactions occur from the breakdown of glucose to fatty acids. Pyruvate is produced from glycolysis, converted to acetyl-CoA, and then to citrate in the Krebs cycle in the mitochondria. Acetyl-CoA can be carboxylated to malonyl-CoA by acetyl-CoA carboxylase before fatty acid synthase converts the malonyl-CoA into long- and short chain fatty acids (Wakil, Stoops, & Joshi, 1983). Triglycerides are formed from the esterification of three fatty acids to a glycerol backbone, also produced from glycolysis (Kersten, 2001). Triglycerides are stored in the liver and adipose tissue until the body requires fatty acids as an energy source, which the triglycerides then break down via lipolysis (Wallace & Metallo, 2020). Cholesterol is also synthesised from acetyl-CoA and through a series of enzymatic reactions, the rate-limiting step being the catalytic effects of 3-hydroxy-3-methylglutaryl-CoA reductase. Excess cholesterol is stored as cholesteryl esters by acyl-CoA:cholesterol acyltransferase (Chang, Li, Chang, & Urano, 2009).

1.5.1   Gestational changes to lipid metabolism

Lipid metabolism also changes during normal pregnancy. Serum lipids, particularly triglycerides, and low-density lipoproteins (LDL)-cholesterol increase as pregnancy progresses. Lipid storage through

increased *de novo* lipogenesis and inhibition of lipolysis is promoted in the early stages of pregnancy (Dathan-Stumpf et al., 2019; Zheng et al., 2018). Serum plasma concentrations of triglycerides, free fatty acids and cholesterol peak in the third trimester, at which point lipolysis is also increased (Diderholm, Stridsberg, Ewald, Lindeberg-Nordén, & Gustafsson, 2005). Insulin resistance is typically seen in pregnancy, which contributes to the stimulation of fatty acid synthesis, and increased lipid release into the serum, including free fatty acids, triglycerides, cholesterol and phospholipids. The increase in free fatty acids contributes to the insulin resistance and enhances lipolysis (Zeng, Liu, & Li, 2017). These changes are necessary to meet the physiological demands of fetal development and for energy storage for labour and lactation (Di Cianni, Miccoli, Volpe, Lencioni, & Del Prato, 2003).

Many aspects of the changes in lipid metabolism during gestation are yet to understood. Several hormones have been identified to contribute to key mechanisms for the changes seen in pregnancy. High oestrogen levels in the third trimester of pregnancy stimulate hepatic lipogenesis and reduce clearance of circulating triglyceride-rich lipoproteins by decreasing the lipase enzymes which would normally hydrolyse triglycerides (Zeng et al., 2017). The increasing progesterone concentrations during pregnancy also stimulates the deposition of fats (Kalkhoff, 1982). Human placental lactogen is also elevated in late pregnancy which also enhances lipolysis in adipocytes (Freemark, 2010). Much like the changes in glucose metabolism seen in pregnancy, the metabolic changes in lipids and fat storage are multifactorial.

1.6 Bile acid receptors

Bile acid activation of FXR and TGR5 is well documented and both receptors have roles in bile acid, lipid and glucose metabolism. The regulation of cholesterol by bile acids is also most notable. Daily synthesis of bile acids regulates the plasma cholesterol concentration, thereby ensuring this does not become too high. Catabolism of cholesterol to bile acids is regulated by CYP7A1 expression (Figure 1.1); high CYP7A1 expression leads to depletion of hepatic cholesterol and increased hepatic LDL receptor expression to replace the lost cholesterol by harvesting the circulatory cholesterol (Kalaany & Mangelsdorf, 2006). Further examples of how bile acid at a molecular level affect metabolism through the bile acid receptors have been well-documented. Understanding bile acid activation of FXR and TGR5 may provide key insights into the pathogenesis of metabolic disease states.

1.6.1 FXR

FXR is a nuclear receptor expressed mainly in the liver, intestine and kidneys, and is essential to regulating the metabolism and synthesis of bile acids. The primary bile acid CDCA is the most potent FXR ligand (CDCA>LCA=DCA>CA; Figure 1.4) (Kalaany & Mangelsdorf, 2006). Hepatic FXR activation promotes transcription of small heterodimer protein (SHP), which represses transcription of CYP7A1, thereby reducing hepatic synthesis of bile acids (Šarenac & Mikov, 2018) (Figure 1.1). FXR also upregulates the expression of MDR3 and BESP, promoting efflux of bile acids to further prevent bile acid build-up within hepatocytes. Intestinal FXR activation via transintestinal bile acid flux, induces the expression of fibroblast growth factor 19 (FGF19/FGF15 in mice) which is secreted by the intestinal epithelial cells. FGF19 is transported in the portal vein and binds hepatocyte fibroblast growth factor receptor 4 (FGFR4)/beta-Klotho to cause repression of CYP7A1 transcription, further downregulating bile acid synthesis (Ahmad & Haeusler, 2019) (Figure 1.1).

FXR also influences in lipid and glucose metabolism. Through transcriptional regulation, FXR activation stimulates β-oxidation of fatty acids and decreases lipid levels in the serum and liver (Savkur, Bramlett, Michael, & Burris, 2005). Activation of hepatic FXR with agonists in diabetic/obese mice or rats fed a high-fat diet, reduced serum and liver triglycerides and lipids. Hepatic expression of genes involving fatty acid synthesis, lipogenesis and gluconeogenesis were also reduced (Cipriani, Mencarelli, Palladino, & Fiorucci, 2010; S. Y. Han et al., 2021; Liu et al., 2018). This demonstrates the importance in lipid metabolism and that use of FXR agonists has the potential to improve metabolic abnormalities.

Studies have also shown that activation of FXR has a beneficial effect on glucose metabolism. At a molecular level, FXR has been shown to interact with carbohydrate response element-binding protein (ChREBP) and regulate its transcriptional activity. ChREBP is a key regulator of metabolism and activation stimulates several genes involving both glucose and lipid metabolism in several tissues such as liver, adipose tissue and pancreatic β-cells (Herman et al., 2012; Iizuka, Bruick, Liang, Horton, & Uyeda, 2004; Noordeen et al., 2010). FXR binds to ChREBP and transrepresses the expression of genes involved in glycolysis in human hepatocytes (Caron et al., 2013). *In vivo* experiments show FXR agonistic treatment or FXR overexpression lowered blood glucose levels in diabetic mice (Zhang et al., 2006). Pathak and colleagues demonstrated that FXR agonists improve glycaemia and reduce diet-induced weight gain in mice (Pathak et al., 2018), and another study demonstrated that bile acid activation of FXR in mice repressed gluconeogenic gene expression (Ma, Saha, Chan, & Moore, 2006). Through FXR activation, mice fed a CA diet had a reduced expression of phosphenolpyruvate carboxykinase, the rate-limiting enzyme in gluconeogenesis (De Fabiani et al., 2003). FXR-null mice develop elevated serum free fatty acids, alongside impaired glucose and insulin

tolerance, and elevated serum glucose levels. Activation of FXR with agonists in wild-type (WT) mice decreased serum glucose and Fxr$^{-/-}$ mice showed impaired GSIS and are glucose intolerant (Bellafante et al., 2020; Ma et al., 2006; Popescu et al., 2010; Zhang et al., 2006). Other studies, however, have shown beneficial effects of inhibition or deletion of FXR (Jiang et al., 2015; Li et al., 2013). Mice with intestine-specific FXR knockout (KO) had improved oral glucose tolerance and lower body weight (Li et al., 2013; Xie et al., 2017). These contradictions could be explained by the differential effects of FXR activation in the liver versus the intestine. Activation in the intestine appears to hamper glycaemic control, recent studies have shown that FXR inhibits GLP-1 production using murine colonic biopsies, colonoids of WT and FXR KO and GLP-1 secreting L-cell lines. FXR KO mice also displayed elevated plasma GLP-1 levels (Ducastel et al., 2020; Trabelsi et al., 2015). It is also important to note that FXR expression is found in peripheral tissues including adipose tissue, islets of Langerhans and adrenal glands (Lefebvre, Cariou, Lien, Kuipers, & Staels, 2009), and could contribute to glucose and lipid metabolism via actions in these tissues. *In vivo* and *in vitro* experiments involving animal islets demonstrated activation of FXR by bile acid stimulated insulin secretion (Düfer et al., 2012; Schittenhelm et al., 2015). In adipocytes, FXR appears to play a role in differentiation, promotes adipogenesis and lipogenesis (Rizzo et al., 2006; Shinohara & Fujimori, 2020; Van Zutphen et al., 2019).

Due to their involvement in glucose and lipid metabolism, it is plausible that a reduced activity in FXR activity during pregnancy could impact the changes in metabolism during pregnancy. Targeting FXR could be key for therapeutics or further understanding the pathophysiology of metabolic gestational diseases.

FXR order of potency

CDCA	>	LCA	=	DCA	>	CA
$EC_{50} - 10\ \mu M$		$EC_{50} - 30\text{-}50\ \mu M$		$EC_{50} - 30\text{-}50\ \mu M$		$EC_{50} - 500\ \mu M$

TGR5 order of potency

LCA	>	DCA	>	CDCA	>	CA
$EC_{50} - 500\ nM$		$EC_{50} - 1\ \mu M$		$EC_{50} - 4.4\ \mu M$		$EC_{50} - 7.7\ \mu M$
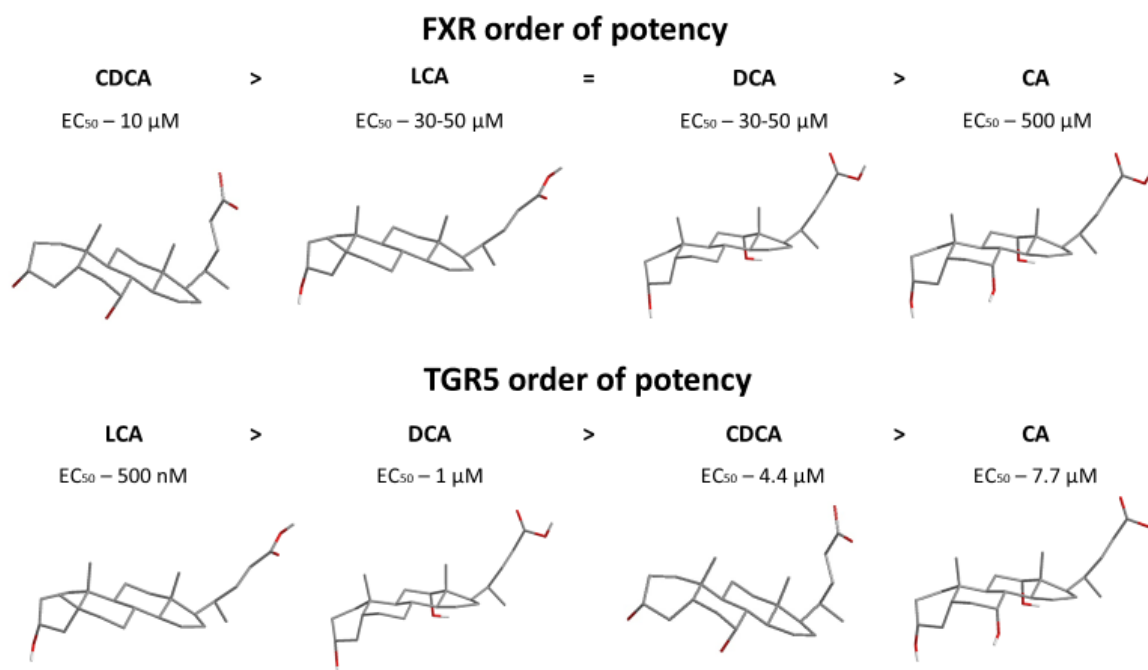
Figure 1.4: Order of potency of bile acids for FXR and TGR5.

Potency of bile acids to FXR and TGR5. CDCA and LCA most potent to FXR and TGR5 respectively, with CA being least potent for both receptors. 3D structures created using PerkinElmer, Chemdraw. EC50 refers to the concentration that gives half-maximal response.

1.6.2   TGR5

Bile acids also bind and activate TGR5, a cell surface G-protein-coupled receptor widely expressed in humans and animals. However, the most potent bile acid ligands for TGR5 differ to those that activate FXR (LCA>DCA>CDCA>CA; Figure 1.4) (Kawamata et al., 2003). When activated, TGR5 stimulates adenylyl cyclase to increase concentrations of cyclic adenosine monophosphate (cAMP), activating protein kinase A (PKA) and exerting cytosolic effects including calcium mobilisation and activating cellular signalling cascades such as nuclear factor κB, extracellular signal-regulated kinases and Akt/protein kinase B pathways (Chávez-Talavera, Tailleux, Lefebvre, & Staels, 2017; Guo, Chen, & Wang, 2016). Often, the signalling pathways of TGR5 are influenced by cell type and conditions. Figure 1.5 details the different actions of TGR5. Most notable is its expression in the enteroendocrine L cells. As previously mentioned, TGR5 activation causes secretion of gut hormone GLP-1 and PYY in the small intestine and colon, which promotes insulin secretion and reduce appetite (Bala et al., 2014; Christiansen et al., 2019; Kuhre et al., 2018; Pathak et al., 2018)(Figure 1.1). TGR5 activation of glucagon-like-peptide 2 (GLP-2) has also been demonstrated, with GLP-2 primary effects being implicated in growth and repair of the intestinal mucosa and gut motility (Parker et al., 2012). Activation of TGR5 receptors at the pancreatic islets causes release of insulin and improves insulin sensitivity and glycaemic control (Kumar et al., 2016, 2012; Maczewsky et al., 2019).

TGR5 activation also plays a role in lipid metabolism and energy expenditure. White and brown adipose tissue (WAT and BAT, respectively) are two major adipose tissues in the body. WAT is adapted for storage of surplus fatty acids derived from the diet in the form of triglycerides and for subsequent release under conditions of negative energy balance in the body. WAT is also known to contribute to the inflammatory response that occurs in obesity (Hirosumi et al., 2002; Hotamisligil, Shargill, & Spiegelman, 1993) In contrast, BAT is a highly vascularised, mitochondria-rich organ containing uncoupling protein 1 (UCP-1), which generates heat by uncoupling the mitochondrial proton gradient (Symonds, Aldiss, Pope, & Budge, 2018). TGR5 agonism causes remodelling of white adipocytes to give a more brown adipocyte-like phenotype, thus increasing β-oxidation and energy expenditure (Velazquez-Villegas et al., 2018; Zietak & Kozak, 2016). The expression of TGR5 on BAT has also demonstrated that when bile acids activate TGR5 on BAT in mice and in human BAT *in vitro*, increased thermogenic activity occurs thereby increasing energy expenditure (Broeders et al., 2015; Watanabe et al., 2006). Improved glucose metabolism and energy consumption are induced by the cAMP/PKA pathway in TGR5-activated skeletal muscle, alongside promoting muscle cell differentiation and hypertrophy to increase muscle strength and function (Huang et al., 2019; Kobayashi et al., 2017). Overexpression in skeletal muscle-specific TGR5 mice also improved glucose

clearance in glucose-intolerant mice (Sasaki et al., 2021). TGR5 expression is also found in several immune cells such as monocytes, macrophages and Kupffer cells; its activation exerts anti-inflammatory activities including inhibition of the production of pro-inflammatory cytokines and induction of differentiation of anti-inflammatory immune cells (Chávez-Talavera et al., 2017; Ichikawa et al., 2012; Yoneno et al., 2013) Many metabolic diseases have an inflammatory component, including diabetes; thus TGR5-mediated regulation of immune cell function warrants further investigation.

Much like FXR, a reduced TGR5 activity during pregnancy could result in metabolic changes causing gestational diseases. TGR5 could be a key target for therapeutics and understanding the mechanisms of the pathology of metabolic gestational diseases.
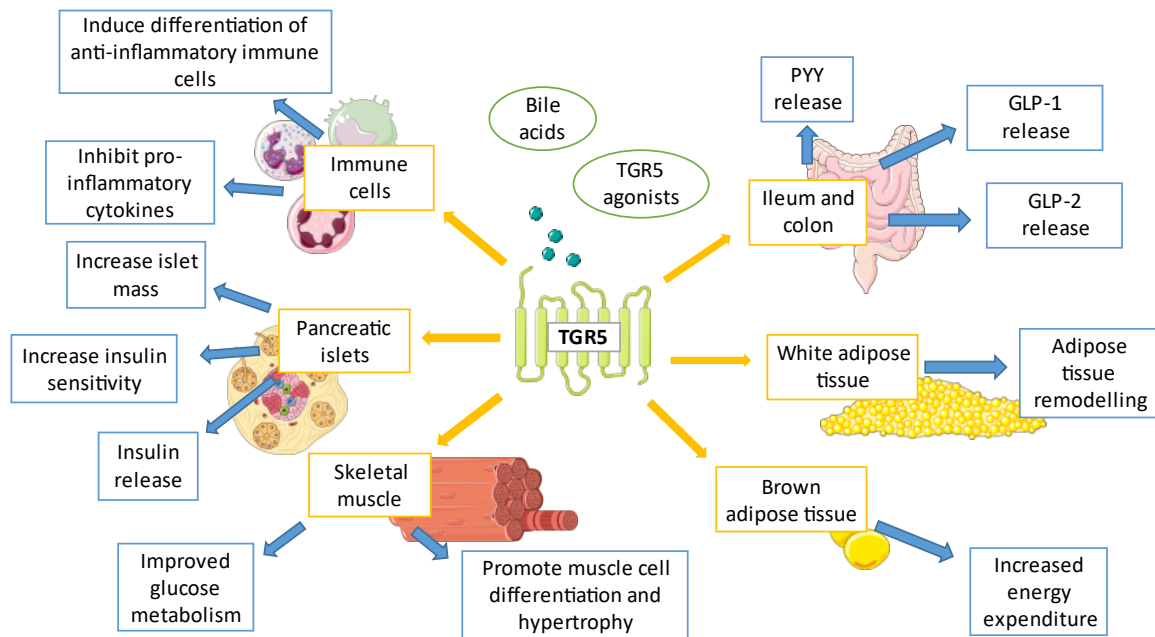
Figure 1.5: TGR5 effects at site of action.

Figure displaying some of the tissues TGR5 is expressed on and the effects it causes when activated by bile acids or other TGR5 agonists.

1.6.3 Other receptors

Other receptors have been reported to have affinity towards bile acids and have potential metabolic effects. The primary function of the pregnane X receptor (PXR) is to detect foreign substance and protects the body by promoting transcription of genes involved in removing and metabolising toxic substances. PXR is highly expressed in the liver and intestine, with the most potent bile acid ligand being LCA (Juřica, Dovrtělová, Nosková, & Zendulka, 2016). Strong evidence exists for ligand-activated PXR playing a role in lipid and glucose metabolism (Jie Gao & Xie, 2012), though there are contradictory outcomes. Whilst some studies have shown PXR activation mediates lipogenesis, suppresses β-oxidation and induces hyperglycaemia (Hakkola, Rysä, & Hukkanen, 2016; Zhou et al., 2006), others have reported that PXR activation improves glucose homeostasis and insulin sensitivity (Sultana et al., 2018). Species and gender-specific differences are thought to explain these variable results. However, research on metabolic regulation through bile acid bound PXR activation is limited, and further investigations may reveal new roles for bile acids acting on PXR.

The vitamin D receptor (VDR), when bound to vitamin D, mediates calcium and bone metabolism, innate and adaptive immune system and cardiovascular function. LCA can agonise VDR in the lower intestine, particularly in the ileum (Ishizawa, Akagi, & Makishima, 2018). However, the physiological relevance of LCA modulation of VDR function remains unclear. Recent research has pointed towards the ability of LCA to provide an immune protective effect at the epithelium via VDR activation (Yao et al., 2019). VDR has also been reported to have a role in maintaining glycaemia and lipid metabolism. A recent *in vitro* study demonstrated that an LCA derivative, LCA propionate, protects pancreatic β-cells from dedifferentiation (Neelankal John et al., 2018). Another study also reported activation of VDR, using $1,25[OH]_2D_3$, represses hepatic SHP, increasing CYP7A1 in mouse and humans which in turn reduces cholesterol (Chow et al., 2014). It remains to be seen whether LCA can regulate glucose metabolism via VDR.

The liver X receptor (LXR) is a nuclear receptor which has two isoforms: LXRα, which is highly expressed in tissues with high metabolic activity, including the liver, small intestine and adipocytes, and LXRβ, which is expressed ubiquitously (Juřica et al., 2016). Unlike FXR, activation of LXR increases transcription and activity of CYP7A1, increasing bile acid formation and reverse cholesterol transport, thus decreasing plasma cholesterol levels (Juřica et al., 2016). FXR and LXR activation thus finely tune lipid, glucose and bile acid metabolism (Kalaany & Mangelsdorf, 2006). Though primary bile acids are not regarded as agonists for LXR, some minor secondary bile acids have agonistic properties, such as hyocholic acid (HCA) and hyodeoxycholic acid (HDCA) (De Marino et al., 2017; Song, Hiipakka, & Liao, 2000). Both HCA and HDCA are found at low levels in the serum and the

intestinal tract, which raises questions as to whether their activation of LXR is of physiological relevance.

Alongside TGR5, other G-protein coupled receptors have also been documented to be activated by bile acids. Bile acids are known to interact with the muscarinic receptors, M_1-M_5 (Kiriyama & Nochi, 2019). Muscarinic receptors are responsible for the physiological effects of acetylcholine, which acts as a neurotransmitter in the brain or neuromuscular junctions and also mediates the parasympathetic system (Kiriyama & Nochi, 2019). There is increasing evidence that positive allosteric modulation of M_3 muscarinic receptors improves glucose homeostasis and promotes insulin release (Gautam et al., 2006; Hauge-Evans et al., 2014; L. Zhu et al., 2019), and mice lacking M_3 in pancreatic β-cells displayed impaired glucose control and reduced insulin release, producing a diabetic phenotype (Gautam et al., 2006).

Sphingosine-1 phosphate receptor subtype 2 (S1PR2), expressed in a variety of tissues, is another G-protein coupled receptor (Kiriyama & Nochi, 2019) for which bile acids are also ligands. When activated, S1PR2 mediates numerous cell functions including increasing cell permeability, promoting immune cell function, muscle contraction and neuron migration (Adada, Canals, Hannun, & Obeid, 2013). Research into the role of S1PR2 in metabolic function has demonstrated that its activation lowers glucose levels and upregulates lipid metabolism (Adada et al., 2013; Nagahashi et al., 2015).

These receptors have all been detailed with the potential of influencing metabolism by bile acids. However, our studies primarily will focus on the metabolic effects of FXR and TGR5 due to the greater effect bile acids have on these receptors.

1.7 TRPM3

1.7.1   Prevalence and Role

Transient receptor potential melastatin-3 (TRPM3) is a cation channel belonging to the melastatin family, a subgroup of the transient receptor potential (TRP) superfamily. Much like the other TRP channels, TRPM3, structurally, has six transmembrane domains and a pore domain, which is located between the fifth and sixth domain (Thiel et al., 2017). The structure of TRPM3 can be seen in Figure 1.6. TRPM3 is highly permeable to calcium (Nilius & Flockerzi, 2014), though TRPM3 has also been found to be permeable to Mg^{2+} and Zn^{2+} (Oberwinkler et al., 2005; Wagner et al., 2010). Agonists like pregnenolone sulphate (PMΔ5S) or CIM0216 can stimulate TRPM3 to allow an influx of calcium into cells, influencing calcium cell signalling and homeostasis (Drews et al., 2014; Lesch, Rubil, & Thiel, 2014; Majeed et al., 2010; Thiel et al., 2017; Wagner et al., 2008). Splice variants of TRPM3 exist,

which they mainly differ from their Ca^{2+} permeabilities, with most of the studies carried out on the variants which are more permeable to calcium (Frühwald et al., 2012; Grimm et al., 2003; Lee et al., 2003). TRPM3 is expressed in various tissues, including kidney, liver, brain, nervous system, reproductive organs, smooth muscle and pancreas (Fonfria et al., 2006; Grimm et al., 2003). The channel likely has tissue-specific roles, though TRPM3 still remains poorly characterised, with the Ca^{2+} permeable properties of TRPM3 investigated the most. To date, no research investigating TRPM3 and pregnancy has been carried out. TRPM3 is expressed in the ovaries and remains to be seen if the hormones, which are increased during pregnancy, can influence TRPM3 activation and upregulation.
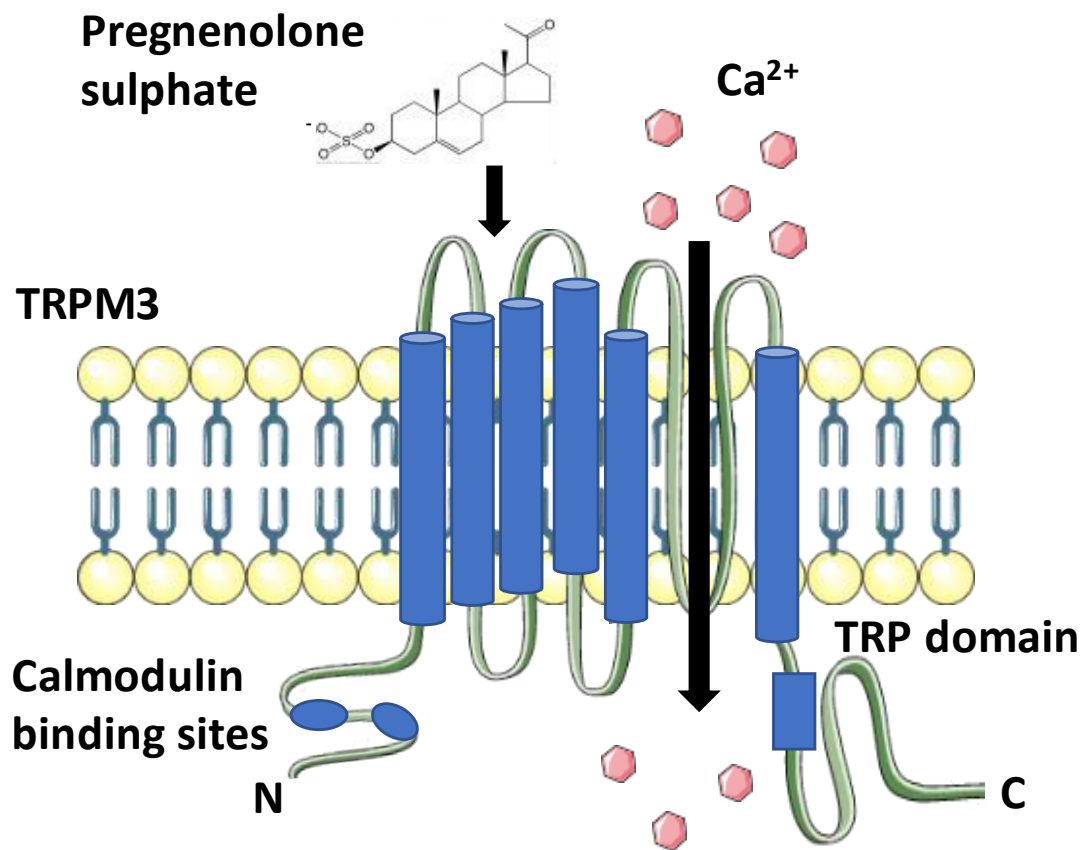
Figure 1.6: TRPM3 structure.

Structure of TRPM3 showing the 6 transmembrane domains and pore domain, which allows the flow of calcium into the cell when activated by agonists such as pregnenolone sulphate.

1.7.2   Progesterone Sulphate Metabolic capabilities

The progesterone sulphate PMΔ5S is a well-established activator of TRPM3 (Drews et al., 2014; Majeed et al., 2010; Thiel et al., 2017). TRPM3 expression in numerous tissues is likely to display tissue-specific roles and its metabolic capabilities have begun to unravel. Pancreatic β-cells have been shown to express TRPM3 in humans (Marabita & Islam, 2017). TRPM3 has therefore been shown to enhance glucose-stimulated insulin secretion by pregnenolone sulphate in numerous *in vitro* models (Becker et al., 2020; Thiel, Müller, & Rössler, 2013; Wagner et al., 2008). This mechanism of action is most likely through Ca^{2+} influx through TRPM3 causing an intracellular signalling cascade and membrane depolarisation (Becker et al., 2020; Thiel et al., 2013). However not all progesterone sulphates can activate TRPM3. The structural differences between the progesterone sulphates dictate their ability to bind to TRPM3. The three key structural parts in showing the highest affinity for TRPM3 are: the orientation of the hydrogen on carbon 5, whether there is a hydroxyl or keto group at carbon 20 position, and the orientation of the sulphate group on carbon 3 (Drews et al., 2014; Majeed et al., 2010). These differences can be seen in Figure 1.3. Majeed *et al.,* and Drews *et al.,* have suggested for strong TRPM3 stimulation, the β configuration of the sulphate group on carbon 3 is required. Establishing which progesterone sulphates activate TRPM3 strongly and correlating this with the differences in serum levels of specific sulphated progesterone metabolites in healthy and individuals with metabolic disorders could be key to understanding the role of progesterone sulphates in gestational diseases.

We are interested in seeing how the other progesterone sulphates affect the activation of TRPM3, particularly ones that are significantly raised or decreased in gestational diseases and may help explain the pathophysiology of metabolic disorders. Further investigations are also needed to establish whether TRPM3 channels participate in GSIS in humans, which maybe key to understanding gestational diseases like GDM.

1.8 Gestational Diabetes Mellitus (GDM)

1.8.1   Epidemiology

GDM is characterised by the pathological development of insulin and hyperglycaemia during pregnancy, which resolves following delivery. Due to the lack of consensus and diagnostic standard for GDM worldwide, there is a large variation in the prevalence of GDM which makes it challenging to compare across countries and regions. Some regions, such as South America and Africa, have fewer studies available to estimate GDM prevalence, which warrant further research in these areas making further comparative assessment difficult.

With many women choosing to have children at a later stage of their life and maternal obesity rates increasing worldwide every year, the prevalence has been rising, regardless of the diagnostic criteria with current figures ranging from 1 to >30% depending on the country (McIntyre et al., 2019). The last 20 years has seen an increase in GDM prevalence by 10-100% in multiple race and ethnic groups (Ferrara, 2007), which continues to rise. The international diabetes federation suggests 1 in 6 (16.8%) are affected by diabetes during pregnancy in the world (Egan & Dunne, 2020). The largest prevalence seen is in the middle east, north African (median 15.2%; range 8.8-20%), south east Asian (median 15%; range 9.6-18.3%) and east Asian (median 10.3%; range 4.5-20.3%) countries. The region with the lowest GDM prevalence, though also having the widest, is Europe (median 6.1%; range 1.8-31%) (McIntyre et al., 2019). Within these regions there is also considerable variation observed; an explanation of these variations within a region could be due to the different GDM diagnostic criteria applied.

1.8.2   Screening and diagnostics

As previously mentioned, multiple screening and diagnostic tests exist which normally examine concentrations of glucose in the plasma or serum in defined circumstances, giving different degrees of maternal hyperglycaemia and maternal/fetal risk. Oral glucose tolerance tests (OGTT) remain the standard test for GDM. The UK does not routinely check for GDM and OGTT is usually carried out at 24-28 weeks gestation in individuals identified as high risk, which is based on factors such as body mass index (BMI), ethnicity and family history of diabetes. Current guidelines from The National Institute for Health and Care Excellence (NICE) state a standardised one-step OGTT approach which initially involves an overnight fast, with blood drawn at fasting in the morning. This is followed by an ingestion of 75-g of glucose and blood drawn at 120 minutes post-prandially, blood is sometimes drawn at the 60 minutes mark if using other criteria such as those from The International Association of Diabetes and Pregnancy study Groups (IADSPG) (American Diabetes Association, 2020; NICE, 2015). Diagnoses of GDM is made when either the fasting or 120 minutes plasma glucose threshold is met or exceeded, 5.6 and 7.8 mmol/L respectively. The threshold criteria for GDM diagnosis vary between different expert groups. However there is currently not enough research to show the best diagnostic method and there is insufficient evidence to suggest which is the best for diagnosing GDM (Farrar, Duley, Dowswell, & Lawlor, 2017).

There is a need for a universally agreed standardised method or identification of a different biomarker to diagnose GDM more effectively and to provide less of a diagnostic burden to the patient. Markers like HbA1c, which can be used for diagnoses of T2DM, cannot be used for GDM

diagnosis (NICE, 2015), and so careful consideration is needed for selecting suitable biomarkers. No early biomarkers exist for identification of GDM, before onset of disease. Identification of potential risk of developing GDM in the first trimester allows for early intervention and management in the early stages of pregnancy, reducing the potential effects of GDM and minimising exposure of the developing fetus to suboptimal conditions. Research for other biomarkers exists, with metabolic hormones, adipokines, inflammatory markers and urine biomarkers all having shown potential associations with onset of GDM (Brink, van der Lely, & van der Linden, 2016; Georgiou et al., 2008; Lorenzo-Almorós et al., 2019; Tenenbaum-Gavish et al., 2020).

1.8.3   Aetiology

No one cause can be identified for the onset of GDM. Although pre-existing features such as obesity contribute to the likelihood of developing GDM, multiple genetic, environmental and hormonal risk factors are implicated in its pathogenesis including age, ethnicity, family history of diabetes, smoking and genetic susceptibility (McIntyre et al., 2019). Obesity appears to contribute highly, with nearly half of all GDM cases being linked to being overweight or obese (Kim et al., 2010). With a global population with an increasing BMI from increased dietary fat intake, westernised diets consumed in developing countries and reduced physical activity are all likely to be pre-pregnancy factors to that contribute to development of obesity and subsequent GDM (McIntyre et al., 2019). Others have reported age as also a major contributing factor, with risk increasing linearly with age. Those >40 years of age have a 2/3-fold increased risk of GDM compared to women <30 years of age (Carolan, Davey, Biro, & Kealy, 2012; Y. Han et al., 2021; Solomon et al., 1997). Ethnicity also appears to play a role and should be noted that when comparing countries using the same diagnostic criteria applied, there is still considerable variability in the prevalence of GDM. In a cohort observed in Northern California, South Asian, South East Asian and East Asian women had higher rates of GDM compared to other ethnicities of the same BMI and the lowest was among non-Hispanic white and African-Americans (Hedderson et al., 2012). Similar findings in the same ethnicities have also been reported, with higher rates seen in Hispanic, South Asian, East Asian and South East Asian/Pacific Islander populations and lower risks in a European background (Carolan et al., 2012; Dooley, Metzger, & Cho, 1991; Laurie & McIntyre, 2020; Lawrence, Contreras, Chen, & Sacks, 2008; Read et al., 2021). Genes have been implied in the aetiology of GDM as family history of diabetes is often used to assess risk, though studies examining the genetic contribution to GDM are limited and often small in sample size. Genetic variation in *MTNR1B* in Asian and Hispanic populations is thought to be associated with an increased risk of GDM. A gene found to be involved with β-cell compensation for insulin resistance (Kwak et al., 2012; Ren et al., 2014). Other genes significantly associated with GDM risk

include: *TCF7L2*, *HNF1A*, *GLIS3*, *GPSM1*, *RREB1*, *SLC30A8*, *CDKAL1*, *IGF2BP2*, *KCNJ11* (E23K), *GCK* (-30G/A) and *IRS1* (Gly972Arg), of note, most of these genes are involved in regulating insulin secretion (Ding et al., 2018; Kwak et al., 2012; C. Zhang et al., 2013).

1.8.4   Pathophysiology and complications of GDM

GDM remains a complicated metabolic disorder and the precise underlying mechanisms have yet to be completed elucidated. While the metabolic adaptations of normal pregnancy, described in the previous section, occur in all pregnancies, GDM occurs when the islets cannot meet the heightened insulin demand, and the β-cells become defective, resulting in hyperglycaemia (McIntyre et al., 2019). GDM typically occurs in the third trimester when insulin resistance is at its highest and peripheral insulin sensitivity at its lowest, hence the reason for diagnosing at the third trimester (McIntyre et al., 2019). Pregnancy hormones are also at their peak in the third trimester and influence glucose metabolism as previously mentioned, thereby contributing to the insulin resistance. Inflammatory factors such as tumour necrosis factor-α (TNFα) and interleukin-6 (IL-6) also change during pregnancy and contribute to the pathophysiology of GDM, with high inflammatory factors linked with obesity (McIntyre et al., 2019). In particular, TNFα inversely correlated with insulin sensitivity in late pregnancy (Kirwan et al., 2002). This reflects the action of TNFα antagonising the actions of insulin by blocking the phosphorylation of the insulin receptor (Hotamisligil, Murray, Choy, & Spiegelman, 1994). Further research is needed to unravel the pathophysiology of GDM.

GDM is associated with both short- and long-term complications for both the mother and fetus. Shorter-term consequences include accelerated fetal growth, macrosomia, neonatal hypoglycaemia, and jaundice (Athukorala et al., 2007; Metzger et al., 2008; O'Sullivan et al., 2011). Longer-term complications include increased risk of developing T2DM in both the mother and offspring (Kawasaki et al., 2018; Leybovitz-Haleluya, Wainstock, Landau, & Sheiner, 2018; Lowe et al., 2018; Metzger et al., 2008). The weight gained, chronic inflammation and insulin resistance during GDM are important risk factors of developing T2DM postpartum. Weight gain post-partum was found to correlate with an increased risk of developing T2DM due to a higher prevalence of progressive insulin resistance and continued pancreatic β-cell dysfunction (Peters, Kjos, Xiang, & Buchahan, 1996). There was also an increased risk of developing other diseases such as metabolic syndrome, cardiovascular, kidney and liver diseases for the mother (McIntyre et al., 2019; Metzger et al., 2008). Though studies examining longer-term effects of GDM on offspring are relatively sparse, exposure to GDM is associated with the offspring having a higher risk of abnormal glucose metabolism and adiposity 10-

14 years postpartum (Lowe, Lowe, et al., 2019; Lowe, Scholtens, et al., 2019; Metzger, 2007). Another recent study examining 11-12 year old offspring of women with GDM determined that these children were also at increased risk of hyperglycaemia, diabetes and obesity (Blotsky, Rahme, Dahhou, Nakhla, & Dasgupta, 2019; Lowe et al., 2018), consistent with the other findings.

1.8.5   Glucose Metabolism in GDM

Changes in glucose metabolism during pregnancy are required to meet the fetal demand. However, these metabolic changes may progress more severely and are accentuated in women with GDM. In late pregnancy, hyperglycaemia in GDM is a consequence of β-cell failure to compensate by insulin secretion for the increased insulin resistance. The exact mechanisms underlying β-cell dysfunction and reduced β-cell mass are varied and complex in GDM. With reduced insulin secretion, decreased insulin-stimulated glucose disposal occurs before insulin sensitivity is also progressively decreased. This is evident through women with GDM having reduced insulin suppression of hepatic glucose production compared to non-GDM patients (Catalano et al., 1993). GLUT4 translocation is thought to also influence glucose metabolism in GDM. As previously described, insulin binds and auto-phosphorylates the insulin receptor β-subunit, thereby activating the insulin receptor substrate 1 (IRS1), signalling the distribution of GLUT4 to the cell surface. In late pregnancy the expression of IRS1 is decreased. This is further exacerbated in GDM pregnancies and autophosphorylation of the insulin receptor β-subunit is also lowered further, resulting in reduced glucose uptake (Friedman et al., 1999). Figure 1.7 details the differences in normal vs GDM insulin signalling and how insulin binding cascades to GLUT4 translocation. Insulin resistance is found to be reversed postpartum by increased expression of the IRS1 protein (Kirwan et al., 2004). Understanding the glucose pathophysiology and the management of glucose levels during GDM pregnancy is crucial. Maternal hyperglycaemia is associated with impaired glucose tolerance in offspring (Plagemann, Harder, Kohlhoff, Rohde, & Dörner, 1997; Silverman, Metzger, Cho, & Loeb, 1995). Lowering and having tight control of glucose to the desirable levels could reduce the severity of the complications seen in GDM.

1.8.6   Lipid Metabolism in GDM

Much like glucose metabolism, increased circulating lipids in late pregnancy are required to meet the requirements of the fetus and the physiological demands of pregnancy. Consistent with insulin resistance, GDM induces a state of dyslipidaemia and GDM women have higher serum triglyceride concentrations in all trimesters and lower high-density lipoprotein (HDL) cholesterol levels in the

second and third trimesters compared to normal pregnancy. No significant differences were found in total cholesterol or LDL-cholesterol levels (Koukkou et al., 1996; Ryckman et al., 2015; Wang et al., 2019). GDM women may have reduced fatty acid uptake and subsequently lower oxidation of fatty acids. The higher insulin levels in GDM contribute to this, as insulin has been shown to decrease fatty acid oxidation and lipolysis, as well as reesterification of those fatty acids (Boden, Chen, Desantis, & Kendrick, 1993). Hyperinsulinaemia in GDM pregnancy, particularly in obese individuals, gives rise to adipose tissue dysfunction, characterised by abnormal production of adipokines. Adipokines have been known to mediate insulin resistance, causing reduced insulin sensitivity and increase in concentration during pregnancy and are further increased in GDM pregnancies (Gutaj et al., 2020; Wójcik, Chmielewska-Kassassir, Grzywnowicz, Woźniak, & Cypryk, 2014). Some of the adipocytokines include leptin, TNFα, IL-6, visfatin, resistin and chemerin (Boyadzhieva, Atanasova, Zacharieva, & Kedikova, 2013; Fasshauer et al., 2007; Jahromi, Zareian, & Madani, 2011; Kasher-Merona et al., 2014; Kirwan et al., 2002; Nien et al., 2007). Other adipokines like adiponectin or apelin increase insulin sensitivity but are significantly reduced in GDM pregnancies (Boyadzhieva et al., 2013). Leptin, TNFα and adiponectin have been well established adipokines, shown to increase or decrease in GDM, though further studies are needed to establish the roles of some of the other adipokines in GDM as it remains unclear if they are significantly changed in GDM compared to normal pregnancy (Gutaj et al., 2020). An increase in fatty acid utilisation by GDM fetuses would contribute to an increased fat depot and accumulation. Childhood obesity correlated significantly with maternal BMI and body fat (Catalano et al., 2009). Therefore, the increased body fat in GDM women would be a significant risk factor for childhood obesity and controlling the lipid metabolism in GDM pregnancies would reduce the complications observed in GDM.
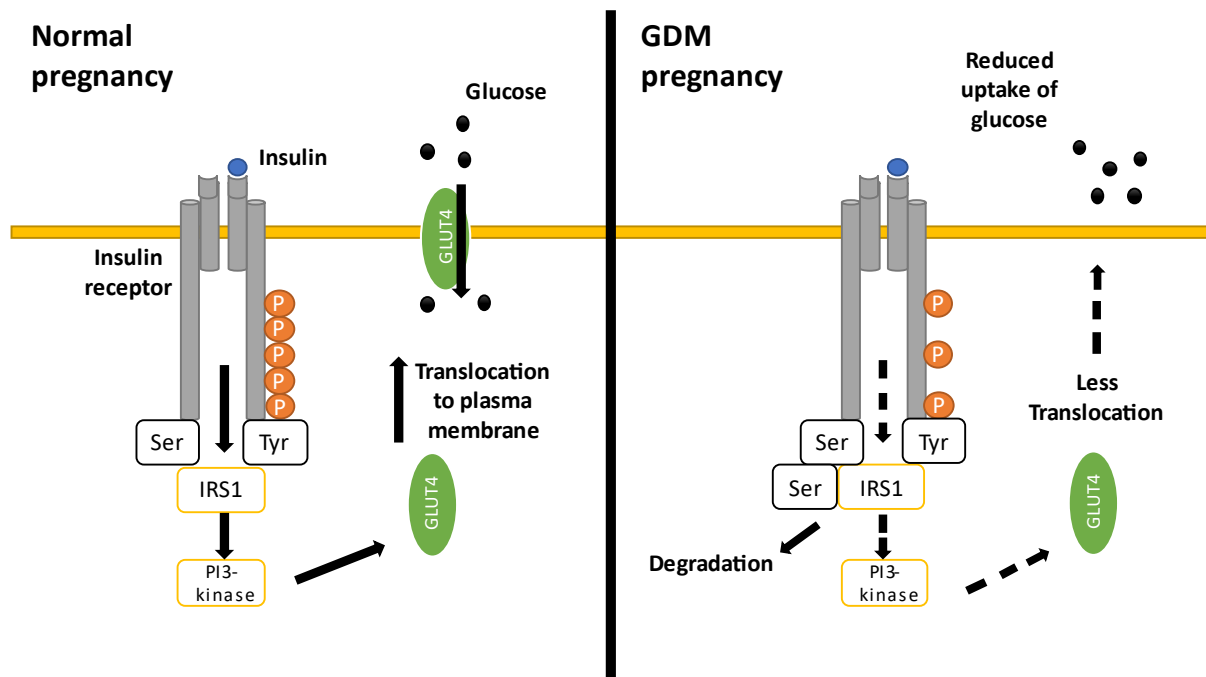
Figure 1.7: Insulin signalling in normal and GDM pregnancy.

Graphic showing in normal pregnancy, insulin activating the insulin receptor to cause phosphorylation of the β-subunit and of the tyrosine residues. This activates IRS1 which in turn activates PI3-kinase to causes a cascading event which results in the translocation of GLUT4 to the cell membrane. This translocation allows glucose to then enter cells. In GDM pregnancy, phosphorylation of the tyrosine residues is reduced, decreasing the activation of IRS1. PI3-kinase activity is therefore reduced and diminishes the translocation of GLUT4 to the cell membrane, preventing glucose entering cells and raising serum glucose levels. Multiple serine sites are also activated which further dampens the signal by destabilising and degrading IRS1.

1.8.7   Management

Initial treatment for GDM involves a lifestyle modification immediately after diagnosis, including dietary modification and exercise (American Diabetes Association, 2019). If hyperglycaemia has not resolved within 1-2 weeks, pharmacological treatment is initiated. Metformin and/or insulin are often given as first line treatments, with sulfonylureas sometimes given as an alternative, depending on different country guideline (American Diabetes Association, 2019). The metformin in gestational diabetes (MiG) trial demonstrated that mothers randomised to metformin, compared to insulin, had reduced maternal weight gain and gestational hypertension (Rowan, Hague, Gao, Battin, & Moore, 2008). However, the rate of large for gestational age (LGA) offspring was not affected and the children had more s.c. fat at 2 years of age at 2 years of age after maternal metformin treatment (Rowan et al., 2011). Furthermore, metformin use has been associated with greater childhood size, adiposity and inferior cardiometabolic health (Hanem et al., 2019). These studies have raised concerns that metformin, currently used by many women with GDM, does not adequately prevent adverse perinatal outcomes, and may have negative long-term effects on the metabolic health of the children (Barbour et al., 2018). However, a recent study has provided more reassuring data: the 3-5 year old children of obese women randomised to take metformin in pregnancy had lower gluteal and tricep circumferences, lower systolic blood pressure and improved left ventricular diastolic function compared to the children of obese women randomised to placebo (Panagiotopoulou et al., 2020). Thus, more research is required to establish whether maternal metformin treatment improves long-term cardiometabolic outcomes for exposed fetuses. Indeed, even insulin treatment (the 'gold-standard' pharmacological approach) was not shown to be of definitive benefit for GDM offspring in the most recent Cochrane review and was thought to possibly increase the risk of raised blood pressure compared to oral treatments (Martis et al., 2018).

The sulfonylurea, glibenclamide, has not been shown to be superior to insulin treatment in randomised trials (Langer, Conway, Berkus, Xenakis, & Gonzales, 2000), or as an add-on therapy to metformin (Reynolds et al., 2017). Sulfonylurea use is also linked with higher rates of LGA babies and neonatal hypoglycaemia compared with offspring of GDM women treated with insulin or metformin (Balsells et al., 2015). Thus, while the recommended treatments should be prescribed for women with GDM, the potential long-term effects for the child should be taken into careful consideration, and there is need for more effective intervention strategies to be developed, likely with consideration of individual risk factors as GDM is a heterogeneous disorder. However treatment for GDM does not seem to improve long-term effects seen in children (Landon et al., 2015; Shou, Wei, Wang, & Yang, 2019), although current postpartum studies evaluating children born from GDM women are of relatively short duration.

Lifestyle interventions have not shown consistent benefits to improve perinatal outcomes (Catalano & Demouzon, 2015). Though use of medications like insulin, metformin and sulfonylureas come with immediate encouraging outcomes, they come with the risk of side-effects and caution is required in its widespread use. Longer term follow-up studies are needed to evaluate use of these drugs during pregnancy. Lifestyle intervention studies before pregnancy are perhaps required to demonstrate that the focus on preventive efforts of GDM should be shifted to preconception, or very early in pregnancy.

1.9 Intrahepatic Cholestasis of Pregnancy and the link with GDM

Though a gradual rise in serum bile acids as gestation progresses is normal (McIlvride et al., 2017), for a small number of women these concentrations are elevated beyond this level, leading to intrahepatic cholestasis of pregnancy (ICP). ICP is the most common pregnancy-specific liver disease. Women with ICP most commonly present in the third trimester with pruritus and elevated serum bile acids, which can occur alongside raised liver transaminases. ICP accounts for roughly 1% of pregnancies in Europe and North America, with higher incidence in women of South Asian and South American ancestry, occurring most commonly in Chile and neighbouring countries (McIlvride et al., 2017). As well as pruritus, hypercholanaemia and abnormal liver function, maternal features of ICP include impaired glucose tolerance and dyslipidaemia (Martineau et al., 2015). ICP is associated with an increased risk of adverse perinatal outcomes including preterm birth, meconium stained amniotic fluid and stillbirth (Geenes et al., 2014; Glantz, Marschall, & Mattsson, 2004; Ovadia, Seed, et al., 2019). ICP has a complex aetiology with hormonal and genetic factors. Most women are diagnosed when the concentrations of both oestrogens and progesterone are at their highest in the later stages of pregnancy. Sulphated progesterone metabolites implicated in the pathogenesis of ICP, are elevated in women with ICP in the third trimester, but are raised before the onset of pruritus (Abu-Hayyeh et al., 2016). Genetic studies have demonstrated pathological variants in genes involved in bile acid synthesis and transport (particularly ABCB4 and ABCB11) in ICP (Dixon et al., 2017; Turro et al., 2020).

As well as total serum concentration, the bile acid profile is also altered. In normal pregnancy the CA/CDCA ratio is increased, and this is further amplified in ICP by a larger increase in CA (Brites, Rodrigues, Van-Zeller, Alexandra Brito, & Silva, 1998; Heikkinen, 1983). This ratio change increases the hydrophilicity of the bile acid pool, due to the extra hydroxyl group on CA. This further reduces FXR activation in ICP as CA is a less potent agonist of FXR (Figure 1.3), but is likely to be less harmful than if other bile acids were elevated as CA should exhibit cytoprotection over the more cytotoxic

hydrophobic bile acids (Pavlović et al., 2018). Activation of TGR5 by bile acids, or other agonists such as progesterone sulphates, may also play a role in the pruritus associated with ICP (Abu-Hayyeh et al., 2016; Alemi et al., 2013; Lieu et al., 2014). As well as maternal effects, bile acids have been directly implicated in fetal arrhythmias, with fetal PR interval elongation and abnormal calcium dynamics reported (Rodríguez et al., 2016; Sheikh Abdul Kadir et al., 2010; Vasavan et al., 2020; Williamson et al., 2001). FXR function has also been linked to the pathophysiology of ICP. FXR function is reduced in pregnancy due to the rise in oestrogen and its metabolites causing a cholestatic phenotype (Abu-Hayyeh et al., 2013; Milona, Owen, Cobbold, et al., 2010). While reduced FXR function is likely to occur in all pregnancies, in some women gestational changes will exacerbate susceptibility to hypercholanaemia to cause ICP.

UDCA is a hydrophilic secondary bile acid, normally used to treat a variety of cholestatic liver disorders. UDCA lowers serum levels of bile acids acting on BSEP, MDR3 and multidrug resistance-associated protein 4, which improves biliary secretion of bile acids (Beuers, 2006; Marschall et al., 2005). Other effects include protection of the liver from bile acid-induced apoptosis, anti-inflammatory actions and stabilisation of the 'biliary bicarbonate umbrella' (Beuers, Trauner, Jansen, & Poupon, 2015). UDCA treatment also alters the bile acid pool, constituting approximately 60% of total bile acid measurements in treated women and replacing more harmful bile acids (Manna et al., 2019). UDCA is a commonly used treatment for ICP, with studies demonstrating reductions in maternal features of ICP, such as itch, hypercholanaemia, elevated transaminases and adverse outcomes (Bacq et al., 2017; Kong et al., 2016). However, a recent trial demonstrated that UDCA did not reduce the frequency of a composite endpoint that perinatal death, spontaneous and iatrogenic preterm birth and admission to the neonatal unit for more than 4 h (Chappell et al., 2019). Ongoing research is evaluating whether UDCA may be a benefit to a subgroup of women with ICP, or only those at risk of specific adverse pregnancy outcomes.

Progesterone sulphates have also been suggested to contribute to the pathophysiology of ICP. As previously mentioned, progesterone sulphates have been shown to increase to significant levels in normal pregnancy (Abu-Hayyeh et al., 2010). Progesterone sulphates also contribute to a cholestatic phenotype in pregnancy by inhibiting bile acid uptake and preventing bile acid efflux. An increasing number of reports have also discovered progesterone sulphates being significantly increased in ICP pregnancies compared to normal pregnancies (Abu-Hayyeh et al., 2013; Meng et al., 1997b; Reyes & Sjövall, 2000). PM5S in particular was shown to be increased by 330% in ICP women (Abu-Hayyeh et al., 2013). Serum and urinary levels can be decreased by UDCA in ICP women (Glantz et al., 2008; Meng et al., 1997a). Progesterone sulphates were also found to be potential prognostic markers for ICP. To help predict the onset of ICP, progesterone sulphates were found to be significantly elevated

in the first trimester in women who went on to develop ICP (Abu-Hayyeh et al., 2016). Further studies of progesterone sulphates and ICP have also revealed their ability to bind to FXR and TGR5, with 5β-pregnan-3α-20α-diol-sulphate (PM3S) showing it can activate TGR5 (Abu-Hayyeh et al., 2016). TGR5 activation by progesterone sulphates also may explain the itch response commonly seen in ICP, which was shown to be elicited by intradermal injection of PM3S in mice and the response eliminated in Tgr5-KO mice (Abu-Hayyeh et al., 2016). PM5S also show partial agonism for FXR (Abu-Hayyeh et al., 2013), which would give reduced function to FXR and help explain the pathophysiology of ICP, as impaired FXR function would increase concentrations of bile acids. This was seen in vivo as PM5S exacerbated hypercholanemia in mice (Abu-Hayyeh et al., 2013). Further understanding of the progesterone sulphates is needed to explain its rise in normal and ICP pregnancies. Fully elucidating their mechanism of action could also help explain their pathophysiology in not only ICP, but other metabolic gestational diseases like GDM.

Women with ICP have an increased risk of developing GDM (Arafa & Dong, 2020; Liu et al., 2020; Majewska et al., 2019; Martineau et al., 2014), which is characterised by elevated postprandial plasma glucose concentrations and increased insulin resistance. ICP is also associated with dyslipidaemia, in particular having elevated serum triglyceride and LDL-cholesterol concentrations (Dann et al., 2006; Martineau et al., 2015), similar to GDM, and it is thought bile acids and their receptors may also play a role in the development of impaired glucose tolerance in pregnancy. Indeed, pregnant mice with CA feeding associated hypercholanaemia have abnormal islet morphology resulting in decreased β-cell proliferation and pregnant mice lacking FXR and TGR5 have impaired glucose homeostasis (Bellafante et al., 2020). This presents a rationale that the bile acids and their receptors could be key to understanding the pathophysiology of GDM and potential therapeutic targets could arise from further research of bile acids their receptors.

1.10 Bile acids and Progesterone Sulphates in GDM

The bile acid pool and composition are different in diabetic states, and there is evidence that the pool could increase in type 2 diabetes mellitus (T2DM). These changes could increase insulin resistance, affecting glucose metabolism and progress the pathogenesis of diabetes (Ahmad & Haeusler, 2019). Similar changes could occur in GDM, however currently studies evaluating changes in bile acid or progesterone sulphate concentrations in GDM are lacking.

1.10.1 Clinical studies

ICP and GDM have some similarities; both are gestational metabolic disorders associated with maternal dyslipidaemia. Although bile acids and their receptors have a greater impact on ICP, it is plausible that bile acid signalling also influences the risk of GDM, and modulation of bile acid pathways may be of benefit in both conditions.

There is an increasing research focus on the relationship between bile acids and the risk of T2DM, in particular the relevance of the CA:CDCA ratio and CYP8B1 function (Kaur et al., 2015; Zaborska & Cummings, 2018). However, studies of bile acids in GDM are comparatively limited. As described earlier in this chapter, elevated serum bile acids and changes in the bile acid pool also occur in normal pregnancy and are heightened in ICP. Furthermore women with ICP have an increased risk of developing GDM (Arafa & Dong, 2020; Liu et al., 2020; Majewska et al., 2019; Martineau et al., 2014). Total and individual bile acid species have been found to be higher in women with GDM in the third trimester (Jieying Gao et al., 2016). Elevated total bile acids were also found in women in their first trimester who went on to develop GDM (Hou et al., 2018, 2016). Consistent with this, a study examining changes in bile acids through each trimester found that total bile acid concentrations positively correlated with insulin resistance (Gagnon et al., 2021). Kong *et al* also suggested that women with higher serum total bile acid levels during early to mid-pregnancy have a higher risk of developing GDM (Kong et al., 2020). However, other studies found a reduction in bile acids in GDM women recruited from the first and second trimester (Dudzik et al., 2014; Li et al., 2018). Likewise, a study examining bile acids in GDM women in the third trimester found no differences in overall total bile acids, but significant reductions in the conjugated forms of DCA, glycodeoxycholic acid (GDCA) and taurodeoxycholic acid (TDCA) (Zhu et al., 2021). These differences could be due to ethnicity, the heterogeneous aetiology of GDM or variations in the method used to assay bile acids.

The composition of the bile acid pools also differs between these studies and some report alterations in the concentrations of minor bile acids that may not be of relevance to clinical metabolic phenotypes. This discrepancy between studies of bile acids in GDM warrants further investigation. It is important for future studies to use consistent measurement techniques in large cohorts of women with GDM, and to match the BMI in women with uncomplicated pregnancies, alongside ethnic group, gestational week of pregnancy and feeding/fasting blood sampling, as these factors all influence the concentration of specific bile acid species in the serum.

Similar to GDM, discrepancies in bile acid concentrations have also been found in T2DM. Total, individual and conjugated only bile acids have been found to be higher in T2DM, with higher bile acid levels also found to positively correlate with higher fasting plasma glucose and insulin

resistance, and negatively correlated with insulin sensitivity (Bennion & Grundy, 1977; Cariou et al., 2011; Vincent et al., 2013; Wang et al., 2020; Wewalka et al., 2014; T. Wu et al., 2015; Zhao et al., 2020; Zhu et al., 2020). Though others did not find changes in total bile acid levels, positive correlation between bile acid concentration and insulin resistance was found (Sun et al., 2016). One study found no changes in total bile acids but changes within the bile acid pool were found, with CDCA decreased in T2DM (Brufau, Bahr, et al., 2010). However, this study had relatively smaller patient cohorts compared to the other studies mentioned. Others had mixed findings, primary unconjugated bile acids were found to be inversely associated with diabetes incidence and found more frequently in non-diabetes, whilst conjugated primary and secondary bile acids were positively related to incidence of diabetes and had higher incidence in the diabetes group (Lu et al., 2021; Suhre et al., 2010). The inconsistencies found in studies in T2DM highlights the difficulties and needs for further research between bile acid composition and diabetes.

Currently, serum progesterone sulphates have not been investigated in GDM. Research, though also lacking, has been carried out linking progesterone sulphates and T2DM. One study found a correlation of PMΔ5S being reduced in patients with T2DM compared to healthy controls (Tagawa et al., 2002). The PMΔ5S -precursor, pregnenolone, were also found to be negatively correlated to a Chinese rural population with prediabetes and T2DM (Jiang et al., 2019). Though interestingly, during insulin-induced hypoglycaemia, PMΔ5S levels was also shown to increase in T2DM (Halama et al., 2019). Studies looking at the relationship between other progesterone sulphates and diabetes is limited. With recent studies showing significant relationships between ICP and progesterone sulphates, and women with ICP also having an increased risk of developing GDM, it is plausible that meaningful trends could be seen between progesterone sulphates and GDM.

1.10.2 FXR and TGR5 impact in GDM

Changes in bile acid composition are also seen in diabetic animal models similar to humans (Herrema et al., 2010; Nervi, Severín, & Valdivieso, 1978; Siow, Schurr, & Vitale, 1991; Subbiah, Yunker, Hassan, & Thibert, 1984; Uchida, Makino, & Akiyoshi, 1985; F. Zhang et al., 2019). Many of these studies have gone on to implicate FXR and TGR5 in contributing to the pathophysiology of diabetes. The studies mentioned previously showed changes in the bile acid pools in pregnancies diagnosed with GDM, this indicates FXR activity is altered in GDM. Due to their involvement in glucose metabolism, it is possible that changes in FXR and TGR5 activity could affect GDM susceptibility. Since both receptors play a role in regulation of glucose homeostasis, changes to normal receptor function are also likely to affect glucose metabolism. FXR expression has been

found to be reduced in rodent models of Type 1 and 2 diabetes (Duran-Sandoval et al., 2004). As previously mentioned, FXR^{-/-} mice demonstrated elevated glucose levels and insulin resistance (Ma et al., 2006; Zhang et al., 2006). Consistent with this, mice deficient of FXR or TGR5 develop gestational impaired glucose tolerance and FXR^{-/-} mice have insulin resistance in pregnancy (Bellafante et al., 2020). Currently there is a lack of studies evaluating the impact of these receptors on GDM pathophysiology and metabolism. Given that FXR activity is reduced in pregnancy and the role that TGR5 has in pregnancy is not known, it is plausible both FXR and TGR5 could contribute to the pathophysiology of GDM. Reduced activity of these receptors could be the link for the increased risk of developing GDM in ICP women.

1.11   Gut hormones

1.11.1 Gut physiology and changes in Pregnancy

As mentioned earlier in 1.2.4, as pregnancy progresses, alterations in the gut physiology occur. Normally, the gastrointestinal tract functions to process ingested food mechanically and chemically, extracting nutrients and excreting waste products. Along the small intestine and colon, enteroendocrine cells are stimulated to release hormones into the blood, to further stimulate or inhibit additional effects of metabolism or on gut motility. Quite often, these cells are directly stimulated by the digested nutrients in the gut lumen. This includes bile secreted into the gut, with the most notable effect being secondary bile acids activating TGR5 receptors and causing GLP-1 release (Norman & Henry, 2015). The gut microbiome is a key part of maintaining normal gut physiology and has a symbiotic relationship with the human digestive tract. The intestinal bacteria has a range of roles which include aiding digestion and metabolism, as well as synthesising nutritional products from the digested materials such as vitamins or short-chain fatty acids. One of the key roles the gut microbiota has is the transformation of primary bile acids to the secondary bile acids DCA and LCA, which not only have antimicrobial properties, but are also involved in aiding glucose and lipid metabolism (Clarke et al., 2014; Joyce & Gahan, 2014). The composition of the microbiome is considered a key indicator to overall health. Dysregulation of the microbiome is often associated with metabolic and inflammatory conditions. Recent research is aiming to manipulate the gut microbiome to combat metabolic related diseases (Clarke et al., 2014). Transplantation of gut microbiota from obese mice into germ-free mice altered the animals into an obese phenotype (Turnbaugh, Bäckhed, Fulton, & Gordon, 2008; Turnbaugh et al., 2006). The bacterial phyla which appear to dominate the gut microbiome are *Bacteroidetes* and *Firmicutes,* representing more than approximately 90% of the total community. Actinobacteria and Proteobacteria are also very much

widely present, subdominant, phyla making up a proportion of the rest of the microbiome (Magne et al., 2020). The *Firmicutes/Bacteroidetes* ratio appears to differ according to the metabolic status of an individual and correlates with glucose tolerance. Those with elevated levels of the *Firmicutes/Bacteroidetes* ratio is associated with obesity, insulin resistance and low glucose tolerance compared to healthy people (Larsen et al., 2010; Zhang & Zhang, 2013).

During pregnancy, there is decreased motility in the gastrointestinal tract due to the relaxation of the smooth muscles, increasing transit time (Longo, Moore, Canzoneri, & Robichaux, 2010). As previously mentioned, the composition of the microbiome during pregnancy is also altered with the *Firmicutes/Bacteroidetes* ratio positively correlating with gestational age (Collado et al., 2008; Koren et al., 2012). Ileal FXR activity has also been shown to reduce during pregnancy and microbial modification of bile acids is also altered during pregnancy (Ovadia, Perdones-Montero, et al., 2019), which may explain the increasing levels of GLP-1 seen in advancing gestation (Valsamakis et al., 2010). Understanding how the gestational signals can impact the gut microbiome and expression of FXR or TGR5 is important for understanding the pathophysiology of metabolic diseases in pregnancy.

1.11.2 GLP-1 and TGR5

Bile plays an important part in maintaining normal gastrointestinal physiology. As previously mentioned, TGR5 is stimulated by bile acids, with secondary bile acids having higher affinity towards TGR5 and a more potent effect on GLP-1 secretion (Christiansen et al., 2019; Kuhre et al., 2018; Pathak et al., 2018). GLP-1 is packed and stored in the intestinal L-cells, a majority of which are located primarily in the distal ileum and colon. These enteroendocrine cells are in direct contact with the lumen on the apical side and also to vascular tissue on the basolateral side, where the TGR5 receptors are found (Baggio & Drucker, 2007). Activation of TGR5 results in a G-protein signalling cascade, increasing cAMP to cause the secretion of GLP-1 in the secretory granules of L-cells (Chávez-Talavera et al., 2017; Guo et al., 2016). Bacterial transformation of primary bile acids to secondary bile acids is key to the activation of TGR5 and release of GLP-1. Lower or very high toxic levels of secondary bile acids are both linked to numerous gastrointestinal diseases (Joyce & Gahan, 2014), further indicating the importance of this process in the gut and the importance of the gut microbiome.

1.11.3 Gut hormone changes in GDM

It is likely that TGR5 signalling is affected during normal and GDM pregnancies, altering the amount of GLP-1 secreted in the enteroendocrine L-cells. During normal pregnancy fasting serum GLP-1 concentrations increase from the second to third trimester, which is thought to compensate for the increase in glycaemia and insulin resistance (Valsamakis et al., 2010). GLP-1 secretion is key for pancreatic β-cell adaptations. During normal pregnancy, islet and β-cell area increase in both mice and humans. A failure to adapt leads to the development of GDM. In GLP-1 receptor null mice, these islet adaptations are abolished, suggesting that GLP-1 is a key mediator in β-cell mass expansion and related adaptations in pregnancy (Moffett, Vasu, Thorens, Drucker, & Flatt, 2014). GDM pregnancies have reduced GLP-1 secretion (Mosavat, Omar, Jamalpour, & Tan, 2020; Sukumar et al., 2018), likely explaining failure of the islets to adapt during some GDM pregnancies. Altered TGR5 signalling could be responsible for this. With changes in the gut microbiome in normal pregnancy, promoting enhanced hepatic bile acid synthesis and increased microbial deconjugation and dihydroxylation of primary bile acids to LCA and DCA, the TGR5 receptor would be further activated, thereby influencing maternal metabolism (Ovadia, Perdones-Montero, et al., 2019). Pathological changes in the gut microbiome could be partly responsible for the development of GDM. Gut dysbiosis in women with GDM is associated with inflammation, glucose intolerance and adiposity. The gut microbiota profile often resembles those with T2DM (Hasain et al., 2020). Changes in the gut microbiome in GDM pregnancies could therefore also affect bile acid metabolism and alter TGR5 signalling. As well as targeting the bile acid receptors, the gut microbiota should be considered for future strategies in controlling the pathophysiology of GDM and as a possible area for therapeutics.

1.12   Hypothesis and aims

We hypothesise that bile acid and progesterone sulphate signalling via bile acid receptors at the enterocytes and islets influence gestational glucose metabolism. Furthermore, we predict that serum levels of bile acids and progesterone sulphates are altered in GDM women compared to normal pregnancies.

The aims of this thesis are to:

- Study the serum concentrations of progesterone sulphates in GDM women.

- Examine the serum concentrations of bile acids in different cohorts of GDM women.

- Investigate how progesterone sulphates and bile acids impact insulin signalling.

- To investigate whether enterocyte bile aid signalling influences glucose metabolism.

Chapter 2: Methods

2.1 Materials

Listed below are the materials and equipment used in the experiments described in this thesis.

2.1.1 Chemicals and reagents

Chemicals and reagents	Manufacturer
γ-globulin	Sigma-Aldrich, UK
A83-01	Tocris Bioscience, UK
Advanced DMEM/F-12	Gibco, UK
Agarose	Lonza, USA
B27 supplement	Gibco, UK
Boric Acid	Sigma-Aldrich, UK
BSA	Sigma-Aldrich, UK
CA	Sigma-Aldrich, UK
CDCA	Sigma-Aldrich, UK
Calcium chloride ($CaCl_2$)	Sigma-Aldrich, UK
Collagenase (C9407, C7657)	Sigma-Aldrich, UK
DAPT	Sigma-Aldrich, UK
DMEM (D6546)	Sigma-Aldrich, UK
DMEM (31966021)	Gibco, UK
DMSO	Sigma-Aldrich
DNA ladder, 100 bp	Invitrogen, UK
dNTP mix	KAPA Biosystems, USA
Dye, Orange DNA loading, 6X	Thermo Fisher Scientific, UK
EDTA	Sigma-Aldrich, UK
EDTA-free protease inhibitor cocktail	Roche, UK
Ethanol	Sigma-Aldrich, UK
Ethidium Bromide	Sigma-Aldrich, UK
FCS	Sigma-Aldrich, UK
Forskolin	Tocris Bioscience, UK

Fura-2 AM	Invitrogen, UK
G418	Gibco, UK
Gastrin	Sigma-Aldrich, UK
Glucose	Sigma-Aldrich, UK
GlutaMAX	Gibco, UK
GW4064	Sigma-Aldrich
HEPES	Sigma-Aldrich, UK
Histopaque 1077	Sigma-Aldrich, UK
Hydrochloric acid (HCl)	Sigma-Aldrich, UK
IBMX	Tocris Bioscience, UK
Igepal	Sigma-Aldrich, UK
INT-777	Cayman Chemical, USA
Iodine-125 (^{125}I) radionuclide	PerkinElmer, USA
Isosakuranetin	Extrasynthese, France
LCA	Sigma-Aldrich, UK
L-glutamine	Sigma-Aldrich, UK
Matrigel cell recovery solution	SLS, UK
Matrigel matrix, basement membrane	Corning, USA
Magnesium chloride (MgCl$_2$)	Sigma-Aldrich, UK
Magnesium chloride hexahydrate (MgCl$_2\bullet$2H$_2$O)	Sigma-Aldrich, UK
Magnesium sulphate heptahydrate (MgSO$_4\bullet$7H$_2$O)	Sigma-Aldrich, UK
mEGF	Gibco, UK
MEM (M2279)	Sigma-Aldrich, UK
mNoggin	Peprotech, UK
N-acetylcysteine	Sigma-Aldrich, UK
N2 supplement	Gibco, UK
NCS	Sigma-Aldrich, UK
Nicotinamide	Sigma-Aldrich, UK
PBS	Sigma-Aldrich, UK

PEG	Sigma-Aldrich, UK
Penicillin/streptomycin	Sigma-Aldrich, UK
PM3S	Steraloids, USA
PM4S	Steraloids, USA
PM5S	Steraloids, USA
Poly-D-Lysine	Gibco, UK
Potassium chloride (KCl)	Sigma-Aldrich, UK
Potassium dihydrogen orthophosphate (KH$_2$PO$_4$)	Sigma-Aldrich, UK
Probenecid	Tocris Bioscience, UK
Proteinase K	Invitrogen, UK
PMΔ5S	Sigma-Aldrich, UK
RPMI-1640	Sigma-Aldrich, UK
SB 202190	Sigma-Aldrich, UK
Sodium bicarbonate (NaHCO$_3$)	Sigma-Aldrich, UK
Sodium chloride (NaCl)	Sigma-Aldrich, UK
Sodium deoxycholate monohydrate	Sigma-Aldrich, UK
Sodium dodecyl sulphate	Sigma-Aldrich, UK
Sodium hydroxide (NaOH)	Sigma-Aldrich, UK
Sodium phosphate monobasic dihydrate (NaH$_2$PO$_4$•2H$_2$O)	Sigma-Aldrich, UK
TBE buffer, 10X	Invitrogen, UK
TCA	Sigma-Aldrich, UK
TCDCA	Sigma-Aldrich, UK
TDCA	Sigma-Aldrich, UK
TLCA	Sigma-Aldrich, UK
Trizma base (Tris)	Sigma-Aldrich, UK
Trypsin-EDTA	Gibco, UK
Tween-20	Sigma-Aldrich, UK
UDCA	Sigma-Aldrich, UK
Zeocin	Gibco, UK

2.1.2 Cell lines and plasmids

Cell line/Plasmid	Manufacturer/ Supplier
HEK293 cells (RRID:CVCL_U427)	Thermo Fisher Scientific, UK
L-Wnt3A cells	Gift from H.Clevers Laboratory, Hubrecht Institute, Netherlands
R-spondin1-producing 293T cell line (Fujii, Matano, Nanki, & Sato, 2015)	Gift from C.Kuo Laboratory, Stanford University, USA
TRPM3α2 plasmid (pcDNA3.1) -plasma insert sequence found in appendix 2	Gift from Dr Stephan Philipp, University of Saarland, Homburg, Germany

2.1.3 Primers

Primer	Sequence (5'-3')
mTGR5_common	AGAGCCAAGAGGGACAATCC
mTGR5_rec	GATGGCTGAGAGGCGAAG
mTGR5_WT	TGGGTGAGTGGAGTCTTCCT

2.1.4 Kits

Kits	Manufacturer
GLP-1 (Active) ELISA kit (EGLP-35K)	Merck, UK
Insulin HTRF kit (62IN2PEG)	Cisbio, USA
Taq DNA Polymerase kit	Invitrogen, UK

2.1.5 Equipment and analytical software

Equipment/software	Manufacturer/ Supplier
BioDoc-It Imaging System	UVP (Analytik Jena US), USA
ChemDraw (version 20.1)	PerkinElmer, USA
FlexStation 3 Multi-mode Microplate reader	Molecular Devices, USA
GraphPad Prism 8 software	GraphPad software Inc., USA
G-Storm PCR machine	Labtech, UK

Jouan CR4i centrifuge	Thermo Fisher Scientific, UK
Molecular Operating Environment, 2019.01	Chemical Computing Group ULC, USA
Nanodrop ND-1000 spectrophotometer	Wilmington, UK
PHERAstar FS microplate reader	BMG Labtech, UK
PowerPac 200	Bio-Rad, UK
Rotina 420R Centrifuge	Hettich Lab Technology, Germany
Sigma 1-14K Microfuge	Sciquip Ltd, UK
Soniprep 150	MSE LTD, UK
STATA 16	StataCorp LLC, USA
Universal 320 R	Hettich Lab Technology, Germany
Wizard^2 Automatic Gamma counter (2470)	PerkinElmer, USA

Table 2-1: Clinical and demographic characteristics of each patient cohort.

Study	HAPO Study (Cohort 1)		GDM Study (Cohort 2)		Early Pregnancy Study (Cohort 3)			
Groups	FPG ≤ 4.3 mmol/L	FPG ≥ 5.1 mmol/L	Control	GDM	Control BMI ≤25 kg/m²	GDM BMI ≤25 kg/m²	Control BMI ≥35 kg/m²	GDM BMI ≥35 kg/m²
Number of participants	94	93	64	25	50	50	50	50
Maternal age in years, median (range)	29.1 (25.4 - 33.6)	31.9 (29 - 34.9)	33 (30.8 - 38)	33 (31 - 36)	32.8 (29.3 - 34.5)	33.1 (30.2 - 37.5)	29.3 (26 - 34.1)	32.8 (29.6 - 36.1)
Gestational age of sample in weeks, median (range)	29.1 (28.5 - 29.7)	29.1 (28.6 - 29.7)	29 (28 - 30)	29 (28 - 30)	12.6 (12.1 - 13)	12.5 (12 - 13)	12.7 (12.2 - 13)	12.7 (12.3 - 13.1)
Ethnicity (Number (%))								
White	94 (100%)	93 (100%)	35 (54.7%)	14 (56%)	40 (80%)	30 (60%)	27 (54%)	17 (34%)
Black	0 (0%)	0 (0%)	17 (26.6%)	6 (24%)	6 (12%)	5 (10%)	20 (40%)	30 (60%)
Asian	0 (0%)	0 (0%)	5 (7.8 %)	2 (8%)	2 (4%)	13 (26%)	1 (2%)	2 (4%)
Mixed	0 (0%)	0 (0%)	3 (4.7%)	2 (8%)	2 (4%)	2 (4%)	2 (4%)	1 (2%)
Other	0 (0%)	0 (0%)	4 (6.3%)	1 (4%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
BMI, median (range)	21.7 (20.3 - 23.6)	25.6 (23.1 - 32.8)	28.7 (25.3 - 33.7)	31.6 (26.8 - 35.6)	22.1 (21.3 - 23.4)	22.3 (21 - 23.7)	37.5 (35.9 - 38.6)	37.9 (36.3 - 40.8)
Singleton pregnancies	-	-	62 (96.9%)	25 (100%)	-	-	-	-
Parity (Nullip vs Multip)	-	-	22 v 42	6 v 19	24 v 26	27 v 23	21 v 29	11 v 39
Previous GDM (Number (%))	-	-	41 (64.1%)	17 (68%)	-	12 (52%)	-	16 (41%)
Blood glucose in mmol/L, median (range) — 0 minutes	4.2 (4.1 - 4.3)	5.2 (5.1 - 5.5)	4.3 (4.2 - 4.6)	4.8 (4.5 - 5.3)	-	-	-	-
Blood glucose in mmol/L, median (range) — 60 minutes	6.6 (5.6 - 7.9)	8.7 (7.7 - 9.8)	7.6 (6.7 - 8.6)	9.9 (8.6 - 10.2)	-	-	-	-
Blood glucose in mmol/L, median (range) — 120 minutes	5.5 (4.9 - 6.3)	6.8 (5.9 - 8.2)	6.6 (5.8 - 7.2)	8.6 (8.1 - 9.1)	-	-	-	-

Study	PRiDE Study (Cohort 4)							
Groups	South Asian				European			
	Non GDM, Normal	Non GDM, Obese	GDM, Normal	GDM, Obese	Non GDM, Normal	Non GDM, Obese	GDM, Normal	GDM, Obese
Number of participants	66	80	12	55	72	80	34	80
Maternal age in years, median (range)	29.6 (26.7 - 32.2)	32.1 (29.2 - 35.7)	31.4 (29.3 – 34.6)	33.2 (29.1 - 37.2)	29.3 (25.8 - 34.8)	28.9 (26.3 - 32)	34.2 (30.9 - 36.3)	30.9 (28.3 - 35.4)
Gestational age of sample in weeks, median (range)	26.9 (26.1 - 27.9)	26.6 (26.1 - 27.4)	27 (26.3 - 28)	26.4 (23.1 - 27.3)	27.4 (26.9 - 28.2)	27.6 (26.6 - 28.3)	26.7 (25.7 - 27.9)	26.6 (26.1 - 27.6)
BMI, median (range)	20.7 (20.2 - 21.0)	31.9 (30.1 - 34.0)	21.5 (20.3 - 22.3)	31.5 (29.2 - 35.0)	19.7 (18.7 - 22.7)	46.4 (37.1 - 50.3)	23.1 (21.3 - 24.3)	42.5 (40.5 - 44.1)
Singleton pregnancies	65 (98.5%)	77 (96.3%)	11 (91.7%)	54 (98.2%)	72 (100%)	79 (98.8%)	34 (100%)	80 (100%)
Parity (Nullip vs Multip)	61 v 5	65 v 15	10 v 2	43 v 12	53 v 19	65 v 15	26 v 8	61 v 19
Previous GDM (Number (%))	2 (3.0%)	5 (6.3%)	3 (25%)	13 (23.6%)	5 (6.9%)	0 (0%)	13 (38.2%)	5 (6.3%)
Blood glucose in mmol/L, median (range) — 0 minutes	4.2 (4.0 - 4.4)	4.5 (4.3 - 4.7)	5.1 (4.9 - 5.2)	5.2 (5.0 - 5.5)	4.3 (4.1 - 4.5)	4.5 (4.3 - 4.8)	4.7 (4.2 - 5.2)	5.3 (5.1 - 5.7)
Blood glucose in mmol/L, median (range) — 60 minutes	-	-	-	-	-	-	-	-
Blood glucose in mmol/L, median (range) — 120 minutes	5.2 (4.5 - 5.7)	6.0 (5.2 - .6.8)	7.1 (5.5 - 8.6)	8.6 (6.9 - 9.8)	5.2 (4.5 - 6.2)	5.6 (5.1 - 6.7)	8.5 (6.5 - 9.3)	7.0 (5.9 - 8.7)

(-) indicates no data gathered for that cohort.

Table 2-2: Details of each donor human islets used in Chapter 3.

Age (years)	Gender	BMI kg/m^2
46	Female	29.03
38	Female	27
41	Male	25.35
47	Male	28.33
40	Female	27.01
43	Female	30

Table 2-3: Details of each donor human islets used in Chapter 5.

Age (years)	Gender	BMI kg/m^2
49	Female	37.89
35	Female	37.1
53	Male	22
49	Male	-
42	Male	-

(-) indicates no information gathered from that donor.

2.2 Methods

This section describes general methods and study designs that were common between chapters. Additional methods specific to each chapter are included in the relevant chapter.

2.2.1 Study samples and design

Progesterone sulphates and bile acids were measured in four separate patient cohorts for this thesis. Table 2-1 gives the demography of the participants in each cohort. All studies conformed to the 1975 Declaration of Helsinki guidelines. Sample sizes in 1, 3 and 4 were calculated using power calculations and a power of 0.9, using a significance level of 0.05, was achieved using previous metabolic studies. Cohort 1 was calculated from the Abu-Hayyeh et al., 2016 progesterone sulphate study. Cohorts 3 and 4 were calculated based on cohorts 1 and 2. A power of 0.9 in cohort 4 was achieved using the first 3 cohorts. Cohort 2 used all available samples from the study.

Cohort 1 comprised of 187 fasting samples from the Hyperglycaemia and Adverse Pregnancy Outcomes (HAPO) study archive (Belfast). The HAPO study was conducted in 15 centres in 9 countries, and aimed to determine the adverse outcomes associated with varying degrees of maternal glucose intolerance that were less severe than those seen in overt diabetes (The HAPO Study Cooperative Research Group et al., 2008). A standard OGTT was carried out at 24-32 weeks of gestation with 75g glucose and women were withdrawn if fasting plasma glucose level exceeds 5/8 mmol/L or 2 hour post load level exceeds 11.1 mmol/L. Samples used in this study were from women who were not diagnosed as having GDM, and were from women in the quartiles with the lowest fasting plasma glucose (FPG) (<4.3 mmol/L, 94 samples) or highest FPG (5.1 - 6.6 mmol/L, 93 samples). These samples were collected between 2000-2006 from women at the Royal Victoria Hospital, Belfast. Participants gave written informed consent, with ethical approval obtained from the Northern Ireland Regional Ethics Committee (RO1-HD34242, RO1-HD34243, RD04/0002756). Methods have been previously published (The HAPO Study Cooperative Research Group et al., 2008). Participants were excluded if they had any of the following criteria: age younger than 18 years, multiple pregnancy, plan to undergo delivery at another hospital, an uncertain date of last menstrual period and no ultrasonographic estimation between 6 and 24 weeks of gestational age, inability to complete the OGTT within 32 weeks gestation, if they had used *in vitro* fertilisation or conceived by means of gonadotropin ovulation induction, glucose testing before recruitment or diagnosis of diabetes during the pregnancy, participation in another study, infection with human immunodeficiency virus or hepatitis B or C virus, previous participation in the HAPO study, or inability to converse in the languages used on centre forms without an interpreter.

Cohort 2 comprised samples from 'A role for placental kisspeptin in β cell adaptation to pregnancy', described previously (Bowe et al., 2019). (The hypothesis of the original study was that plasma kisspeptin levels will be lower in pregnant women with GDM compared to those with normal glucose tolerance in the insulin resistant phases of pregnancy). Study participants were pregnant women between 26-34 weeks gestation attending for OGTT, and able to give informed consent to participate. Women were referred for an OGTT if they had a BMI at least 40 kg/m^2, GDM in a previous pregnancy, or a random plasma glucose ≥6.7 mmol/L. Those unable to give informed consent or with known major medical problems were excluded from the study. Consent and ethics were approved for the research by the London-Westminster research ethics committee of King's College Hospital (13/LO/0539). GDM was diagnosed according to the IADPSG criteria: 1 or more of fasting plasma glucose ≥5.1 mmol/L, following a 75g glucose challenge, 60 minutes ≥10.0 mmol/L, or 120 minutes ≥8.5 mmol/L. A total of 91 women participated in the study, and 89 were selected for analysis due to availability of serum samples. We analysed 25 GDM and 64 non-GDM fasting samples from this study for metabolite measurement.

Cohort 3 comprised samples collected at 11-13 weeks' gestation between 2010-2015 at King's College Hospital, London, UK. Invited participants gave written informed consent; the study was approved by the National Research Ethics Committee (02-03-033) (Nanda et al., 2012). Serum samples were collected and processed using standard methods from women attending their routine first trimester ultrasound scan. Samples were stored at -80°C for subsequent biochemical analysis. Screening for GDM in the hospital was based on a two-step approach. A random plasma glucose was measured in all women at 24-28 weeks' gestation. If the concentration of glucose was ≥6.7 mmol/L, an OGTT was performed within the subsequent 2 weeks. Diagnosis of GDM was made if FPG level was ≥6 mmol/L or if after 2 hours, was ≥7.8 mmol/L. Metabolite analysis was performed in serum samples taken at 11-13 weeks from 100 women that subsequently developed GDM, and 100 women who had an uncomplicated pregnancy (defined as pregnancies occurring without complications which resulted in live birth after 37 weeks' gestation of phenotypically normal neonates). The GDM samples were selected according to maternal BMI at 11-13 weeks' gestation; 50 samples per group from women with BMI ≤25 kg/m^2, or with BMI ≥35 kg/m^2. Each GDM sample was matched to one uncomplicated pregnancy sample for BMI group, with samples taken on the same or next day.

Cohort 4 comprise of samples collected from the Micronutrients in Pregnancy as a Risk Factor for gestational Diabetes and Effects on mother and baby (PRiDE) study, carried out in approximately 8-12 sites around the West Midlands region (Saravanan et al., 2021). The primary objective of this study was to determine differences in the risk of GDM in women with and without early pregnancy B12 insufficiency. They hypothesised that an imbalance of B12 and folate in early pregnancy is

associated with the risk of hyperglycaemia and GDM subsequently in pregnancy. Invited participants gave written informed consent and study approved by the National Research Ethics Committee (12/WM/0010). Women were identified for the study at less than 16 weeks gestation, at 18-45 years of age, who had risk factors for GDM according to the NICE criteria and will be referred for an OGTT at 26-28 weeks gestation. Any one of the following risk factors were considered high-risk group for GDM: BMI >30 kg/m^2, previous GDM, previous unexplained still birth or baby >4.5kg, first degree relative with diabetes, ethnic minority group, age >35 years, polycystic ovarian syndrome, or other local criteria followed by the participating sites to comply with routine practise. Exclusion criteria were as follows: pre-existing diabetes (Type 1 or 2), diagnosis of Vitamin B12 or folate deficiency in current pregnancy, previous pregnancy with a fetal neural tube defect, diagnosis of severe anaemia in current pregnancy (HB<10 g/dL) or prescribed Vitamin B12 injections within the last 6 months. During the OGTT, a fasting blood test was taken, followed by consumption of 75g glucose and glucose measured again at 2 hours. The modified NICE 2015 criteria were used to diagnose GDM (fasting plasma glucose ≥5.6 mmol/l and/or 2 hour plasma glucose ≥7.8 mmol/l). Serum samples prepared by routine methods were stored in -80°C until analysis. Fasting samples were selected according to BMI and ethnicity. The groups being European: non-GDM (n=80) and GDM obese (n=80) (>30 kg/m^2), non-GDM (n=72) and GDM (n=34) normal BMI (<25 kg/m^2) and South Asian: non-GDM (n=80) and GDM (n=55) obese (>27 kg/m^2), non-GDM (n=66) and GDM (n=12) normal (<23 kg/m^2). The BMI thresholds for both European and South Asian differ for normal and obese, to give the equivalence for risk of health conditions or mortality, hence the lower boundaries for South Asians. These thresholds are based on the recommendations of an world health organization (WHO) expert consultation in 2004 (Nishida et al., 2004) and of NICE guidance, published in 2013 (NICE, 2013).

2.2.2 Quantification of bile acids and progesterone sulphates by UPLC-MS/MS

Bile acids and progesterone sulphates were quantified using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) by the research group of Professor Hanns-Ulrich Marschall at the University of Gothenburg, and/or Karolinska Institute (Sweden), similarly to as previously described (Abu-Hayyeh et al., 2016), but using a Waters Xevo TQXS UPLC-MS/MS platform. The method is described below.

Internal standards (100 ng of d4-glycholic acid (GCA), d4-glycochenodeoxycholic acid (GCDCA), d4-GDCA, d4-glyco-UDCA (GUDCA), d4-LCA (all from Qmx Laboratories, Essex, UK), d5-CA (Toronto Research Chemicals, Toronto, Canada), and d4-TCA (TLC PharmaChem, Vaughan, Canada) were dissolved in 40 µl methanol (MeOH), added to 100 µl serum and vortexed. 800 µl of acetonitrile was added to precipitate proteins. After vortexing and centrifugation, the supernatant was dried in a stream of nitrogen and then first taken up in 125 µl MeOH, followed by 125 µl of an aqueous solution containing 40% MeOH, 0.02% formic acid, and 10 mmol/L ammonium acetate. Before injection, 75 µl of the sample was transferred to a new vial and 80 µl of the following added: three parts of MeOH and one part of an aqueous solution containing 40% MeOH, 0.02% formic acid, and 10 mmol/L ammonium acetate.

10 µl of this mixture was analysed on a ultra-performance liquid chromatography alliance 2695 system coupled to a Xevo TQ mass spectrometer (Waters, Manchester, UK) using a SunFire C18 (4.6 x 100 mm, 3.5 µm) column (Waters) and gradient elution with 0.01% formic acid and 5 mmol/L ammonium acetate in water. The mobile phase contained 0.01% formic acid and 5 mmol/L ammonium acetate in MeOH. Cone voltage was 60 V and collision energy 18 eV for unconjugated bile acids, 60 V and 29-43 eV for glycine conjugates and 88 V and 56-65 eV for taurine conjugates respectively. Analytes were detected using selected ion monitoring and quantified by comparison with the internal standards. The desolvation temperature was 650°C and the source temperature was 150°C. Selected reaction monitoring was used with dwell times of 100 ms. Analytes were quantified using deuterised internal standards except for progesterone sulphates for which d4-GUDCA was used. Results were calculated as response ($area_{analyte}/area_{internal\ std}$). Retention times and response curves of bile acids listed (Table 2-4) were evaluated from reference compounds obtained from Sigma; 5β-pregnan-3β-ol,20-one,3-sulfate (pregnandiol-3-sulfate), 5α-pregnan-3α-ol,20-one,3-sulfate (allopregnandiol-3-sulfate), and 5α-pregnan-3β-ol,20-one,3-sulfate (epiallopregnandiol-3-sulfate) were obtained from Steraloids, USA; 5β-pregnan-3α,20α-diol-3-sulfate, 5β-pregnan-3α,20α-diol-disulfate, and 5α-pregnan-3α,20α-diol-disulfate were from Sai Advantium, India. 5α-Pregnan-3β,20α-diol-disulfate was tentatively identified as the remaining isomer from its retention times and mass spectrum. 5β-Pregnan-3α,20α-diol-disulfate and 5α-pregnan-3α,20α-diol-disulfate coeluted at

all of the conditions tested. Using this system, less than 10% intra-assay variability was observed when rerunning the same sample.

It was not possible to assay PMΔ5S in the HAPO samples as these samples were analysed before the D4-labelled internal standard for unsaturated progesterone metabolites became available in 2018.

Table 2-4: Retention times of bile acids measured by UPLC-MS/MS

Compound	Ions	RT KI	RT (LC-flow 0.6 ml/min)
T-αMCA	514 > 79.9	2.56	2.56
T-βMCA	514 > 79.9	2.74	2.74
TUDCA	498 > 79.9	3.99	3.80
d4-GUDCA	452 > 73.9	4.19	4.03
GUDCA	448 > 73.9	4.19	4.03
TCA	514 > 79.9	5.77	5.39
d4-GCA	468 > 73.9	6.07	5.81
GCA	464 > 73.9	6.07	5.81
PM5S	396.8 > 96.9	6.14	5.83
Iso-UDCA	391 > 391	6.32	6.44
d4-UDCA	395 > 395	6.82	6.92
UDCA	391 > 391	6.85	6.94
HCA	407 > 407	7.01	7.11
HDCA	391 > 391	7.8	7.87
TCDCA	498 > 79.9	8.05	7.69
GCDCA	448 > 73.9	8.47	8.16
d5-CA	412 > 412	8.51	8.56
CA	407 > 407	8.51	8.58
TDCA	498 > 79.9	8.86	8.50
GDCA	448 > 73.9	9.28	8.97
TLCA	482 > 79.9	11.23	10.92
TOCA	526 > 79.9	11.58	11.27
d4-GLCA	436 > 73.9	11.65	11.41
GLCA	432 > 73.9	11.67	11.45
CDCA	391 > 391	11.71	11.81
GOCA	476 > 73.9	11.92	11.74
DCA	391 > 391	12.2	12.21
OCA	419 > 419	14.15	14.28
d4-LCA	379 > 379	14.32	14.45
LCA	375 > 375	14.34	14.47
αMCA	407 > 407		5.14
βMCA	407 > 407		5.63

HPLC-MS/MS: high-performance liquid chromatography-tandem mass spectrometry, RT: retention time, KI: Kovats Index, T: taurine-conjugated, G: glycine-conjugated, MCA: muricholic acid, UDCA: ursodeoxycholic acid, CA: cholic acid, PM5S: epiallopregnanolone sulphate, HCA: hyocholic acid, HDCA: hyodeoxycholic acid, CDCA: chenodeoxycholic acid, DCA: deoxycholic acid, LCA: lithocholic acid, OCA: obeticholic acid

2.2.3 Mouse colonies and maintenance

Female 8-12 weeks old, wild type C57BL/6J mice, purchased from Charles River, UK, were used for islet experiments in chapters 3 and 4. Tgr5$^{-/-}$ mice, maintained on a C57BL/6 background, were used for islet studies and crypt experiments in chapters 3, 4 and 5. Fxr$^{-/-}$ also maintained on a C57BL/6 background were used for islet studies in chapter 3 only. Fxr$^{-/-}$ and Tgr5$^{-/-}$ mice have been previously described in detail (Milona, Owen, Van Mil, et al., 2010; Thomas et al., 2009), and were provided by Professor Kristina Schoonjans, École Polytechnique fédérale de Lausanne, Switzerland. All mice were maintained in the Biological Services unit (BSU) at King's College London on a 12 hour light/ dark cycle. The BSU was maintained at 22-24°C with free access to food and water. Mice were acclimatized to the BSU for a minimum of one week prior to experiments. All experiments were performed in accordance with the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 and approved by King's College London's Animal Welfare and Ethical Review Body and the Home Office.

2.2.4 Mouse genotyping

Mouse ear biopsies were incubated overnight at 55°C with 150 µl lysis buffer (5 mM EDTA, 200 mM NaCl, 100 mM Tris, 0.2% sodium dodecyl sulphate) and 20 mg/ml proteinase K. Biopsies were then washed with 100% ethanol and centrifuged at 14462 g (Sigma 1-14K Microfuge) at 4°C for 30 minutes. Tubes containing the samples had the supernatant discarded and then were air dried before being resuspended in ultrapure water. DNA concentration was checked with nanodrop. 2 µl of 100 ng DNA were used in a master mix containing 10 mM dNTP, mTGR5_common, mTGR5_rec, mTGR5_WT, water and all components of the Taq DNA polymerase kit (Taq polymerase, 50 mM MgCl$_2$, 10x PCR Taq buffer) to a final volume of 25 µl. Tubes then placed in G-storm PCR machine and run for under the following conditions: 95°C for 5 minutes, followed by 40 cycles of 95°C for 30 seconds, 65°C for 30 seconds and 72°C for 45 seconds, finishing off at 72°C for 5 minutes. DNA was loaded with orange dye on 2% agarose gel (containing ethidium bromide) in TBE buffer, for 25 minutes at 100V. The gel was visualised using UV in a BioDoc-IT imaging system.

2.2.5 Mouse islet isolation

Mice were culled on the day of isolation and abdomen opened to reveal intestines and liver. The upper part of the duodenum was clamped and islets were isolated by inflating the pancreas by collagenase (C7657) (1 mg/ml), using a 30 gauge needle, through the common bile duct and separated from exocrine pancreatic tissue. Tissue contained in a 50 ml tube was then transferred to

a 37°C water bath for 10 minutes. Subsequently, MEM (10% FCS and 1% penicillin/streptomycin) was immediately added. Digested pancreas samples were centrifuged at 302 g for 75 seconds and the supernatant discarded. The pellet was resuspended in MEM and centrifuged as above. This was repeated and the resuspended pellet filtered through 425 µM stainless steel mesh. The tissue was centrifuged once more as above, the supernatant discarded, and the pellet resuspended in 10 ml histopaque. Using a pipette, 15 ml MEM was gradually added to form two distinct layers and centrifuged at 1170 g (Universal 320 R) for 24 minutes. Islets were then removed at the interface using a pipette and MEM filled to have a total volume of 50 ml. Tube containing islets were then centrifuged at 302 g for 90 seconds and 25 ml of media taken out. Islets were further purified through sedimentation, after waiting for 4 minutes, 10 ml of media was removed, and 10 ml of new MEM media was added in. This was repeated 4 times to purify islets with the final time, 10 ml of media was not added in. The top 10 ml of media was taken out and the remaining 15 ml containing the islets was put into a petri dish. Using a pipette, MEM was removed, and islets were incubated at 37°C, 5% CO_2 in 10 ml RPMI-1640 (10% FBS, 2 mmol/l glutamine, 100 units/ml penicillin/0.1 mg/ml streptomycin and 11 mmol/l glucose) overnight before experiments were performed.

2.2.6 Human islet isolation

Human islets were provided on the day of experimentation and already isolated. These isolated islets originated from heart-beating organ donors at the King's College Hospital Islet Transplantation unit, as previously described (Huang et al., 2004). The isolation process involves perfusing the pancreata with University of Wisconsin solution (UWS) (Raffinose (30 mmol/L), Lactobionate (100 mmol/L), hydroxyethyl starch (50 g/L), glutathione (3 mmol/L), allopurinol (1 mmol/L), adenosine (5 mmol/L), dihydrogen phosphate (5 mmol/L), hydrogen phosphate (20 mmol/L), magnesium sulphate (5 mmol/L), sodium (30 mmol/L), potassium (120 mmol/L)). Following pancreas digestion with liverase, islets were purified using UWS-Euro-sodium-diatrizoate medium. After established a 130 ml base medium (1095 g/ml), a linear density gradient medium of 1.065 to 1.095 was created. Up to 60 ml of digested tissue were suspended in 150 ml UW and top loaded onto gradient medium with the Cobe 2991 centrifuge spinning at 3,000 rpm. Fractions were collected from the gradient and aliquoted, stained with dithizone (25 µg/ml) to visualise islets and islet fractions pooled. The final islet equivalent number (IEQ) was derived on the basis of the counting and measuring of total islets in 100 µl (n=2) of the purified tissues and converted into the equivalent number of an idealised diameter of 150 µm. The viability of the islets was assessed by a fluorometric fluorescein diacetate assay, with viable cells stained green and dead cells red. Human islets were maintained at 37°C, 5% CO_2 in CRML-1066 medium supplemented with 2% human albumin, 4 mM glutamine, 2 mM HEPES

(pH 7.2-7.4) and 10 mM nicotinamide, prior to use. Consent for using islets for research has been obtained and studies approved by the Ethical committee of King's College Hospital (REC number: LREC 01-082). Islets were isolated between 2018-2019. Table 2-2 and Table 2-3 details the age, gender and BMI of each organ donor.

2.2.7 Islet static incubations

GSIS from islets were assessed in static incubations. Islets were preincubated in 2 mM glucose Gey and Gey buffer (111 mM NaCl, 5 mM KCl, 27 mM NaHCO$_3$, 1 mM MgCl$_2$•2H$_2$O, 0.22 mM KH$_2$PO$_4$, 0.28 mM MgSO$_4$•7H$_2$O), supplemented with 2 mM CaCl$_2$, 0.5 mg/ml BSA for 1 hour at 37°C, 5% CO$_2$. Groups of 5 size-matched islets were incubated for 1 hour at 37°C water bath in Gey and Gey buffer with the supplementations and either low glucose (2 or 3 mM) or high glucose (20 mM), with the reagents of interest. Incubation medium was stored at -20°C until assayed. Insulin content from collected medium was assayed using an in-house radioimmunoassay, apart from Fxr$^{-/-}$ islet experiment which were determined by insulin HTRF ELISA. The 20 mM glucose, acting as the positive control, checks if islets are stimulating significantly more than baseline (2mM) glucose levels and are working as normal. Reagents would be incubated with the same concentrations of glucose to see if this would make a difference and allow comparison to the non-treated islets.

2.2.8   Radioimmunoassay for insulin

The collected supernatant from static incubations was determined using an in-house radioimmunoassay (RIA) as previously described (Jones, Salmon, & Howell, 1988). Samples were diluted with borate buffer (133 mM boric acid, 10 mM EDTA, 67.5 mM NaOH, 2 g/2l BSA, pH 7.4) to a concentration optimal for detection. A standard curve was prepared by serial dilution of 10 ng/ml to 0.04 ng/ml rat insulin, in triplicate. A series of reference tubes for constructing the standard curve were also prepared in triplicate. These controls were measuring: non-specific binding to determine the binding of radiolabelled antigen tracer ^{125}I in the absence of the anti-insulin anti-body, maximum binding of the antibody as well as total γ emission of the radioactive tracer which contained only the diluted radioactive tracer antigen. The antibodies were raised against bovine insulin in Hartley guinea pigs, and the insulin standard was purified rat insulin. The radiolabelled antigen was diluted to give an approximate count of 10,000 counts per minute (cpm). Each sample, in duplicate, contained the diluted radioactive ^{125}I ligand and the anti-insulin antibody diluted 1:10. All samples and controls were incubated at 4°C for 48 hours to allow them to equilibrate.

After, 1 ml of the precipitant mixture (PBS, 1% γ-globulin, 15% PEG and tween-20) were added to each tube except those measuring total γ emission. The precipitate from the samples and controls were centrifuged at 3,000 g, 15 minutes, 4°C (Jouan CR4i). After centrifugation, the supernatant was aspirated. The γ-emission in the precipitate were detected using the Wizard[2] automatic γ counter. Insulin concentrations were calculated against the standard curve as insulin secretion ng/islet/hour.

Chapter 3: Investigation of progesterone sulphates in GDM

3.1. Introduction

GDM is a common gestational metabolic disorder, characterised by hyperglycaemia during pregnancy. GDM is growing problem, affecting 15% of pregnancies worldwide with higher rates in Asia (Feig et al., 2014; Lee et al., 2018) and its prevalence continues to grow as obesity rates rise. Multiple risks are associated with GDM for both the mother and baby. As well as maternal hyperglycaemia, GDM is associated with dyslipidaemia (Ryckman et al., 2015; White et al., 2017) and pre-eclampsia (Tobias et al., 2017). Fetal risks include large for gestational age infants, birth-related injuries such as shoulder dystocia and neonatal hypoglycaemia (Ovesen, Jensen, Damm, Rasmussen, & Kesmodel, 2015; The HAPO Study Cooperative Research Group et al., 2008). Furthermore, long-term consequences are associated with GDM for both mother and offspring such as development of diabetes and other obesity-related disorders (Bellamy et al., 2009; Saravanan et al., 2020).

There is a need for new ways to screen GDM to enable earlier diagnosis. Currently, diagnosis of GDM is through an OGTT at 28 weeks gestation, which requires a hospital visit and multiple blood draws (NICE, 2015). Biochemical features of GDM may develop earlier in pregnancy; first trimester hyperglycaemia is associated with large for gestational age infants and increased rates of caesarean section (Riskin-Mashiah, Younes, Damti, & Auslender, 2009). Finding early biomarkers will allow early intervention and reduce the risk of adverse outcomes associated with GDM.

One group of hormones of interest are sulphated progesterone metabolites. Whilst these metabolites circulate at very low, nearly undetectable levels, they are increased 100-fold during pregnancy (Abu-Hayyeh et al., 2013). Progesterone sulphates can also bind to receptors that influence lipid and glucose metabolism which could be key in understanding the pathophysiology of GDM. PM3S can activate TGR5 (Abu-Hayyeh et al., 2016), whilst PM5S has shown partial agonism for FXR (Abu-Hayyeh et al., 2013); both receptors shown to affect not only bile acid metabolism, but also glucose and lipid homeostasis. These receptors are of particular relevance to GDM as $Fxr^{-/-}$ and $Tgr5^{-/-}$ mice have impaired glucose tolerance in pregnancy (Bellafante et al., 2020). Furthermore PMΔ5S has been shown to activate TRPM3 in islets, causing insulin secretion (Drews et al., 2014).

Each of the progesterone sulphates has a distinct structure due to the different orientations of the side chain structures. These changes relate to orientation of the hydrogen on carbon 5, sulphation of the hydroxyl groups or which functional group, hydroxyl or keto, is attached at the carbon 20 position. These differences alter the conformation of the progesterone sulphates and therefore may determine their ability to bind to different receptors. Establishing the changes in specific

progesterone sulphates between GDM and normal pregnancy and demonstrating which receptors these metabolites activate is key to understanding whether progesterone sulphates play a role in the development or progression of GDM.

Very few studies exist that link progesterone sulphates with diabetes, and there have been none to study GDM. PMΔ5S and the PMΔ5S -precursor pregnenolone was found to be reduced in patients with T2DM and prediabetes (Jiang et al., 2019; Tagawa et al., 2002). Interestingly others found that during insulin-induced hypoglycaemia, PMΔ5S levels were increased in T2DM (Halama et al., 2019).

We hypothesise that specific progesterone sulphates are altered in GDM, and this in turn alters signalling through its receptors, leading to changes in glucose tolerance.

The aims of this chapter are to:

- Assess the serum concentrations of specific progesterone sulphates in normal vs GDM pregnancies.
- Investigate the effects of progesterone sulphates on isolated islets.

3.2. Materials and Methods

3.2.1. Human studies

Serum samples from Cohorts 1, 2 and 3 were quantified for progesterone sulphates by UPLC-MS/MS. Further details on the Cohorts and UPLC-MS/MS method can be found in chapter 2, pp.62, 64-65, 67-69.

3.2.2. Islet studies

Islets were isolated for static incubations, originating from female wildtype, Fxr$^{-/-}$ and Tgr$^{-/-}$ C57BL/6 mice and human islets from donated from King's College Hospital. Insulin content was determined using an in-house radioimmunoassay, apart from the Fxr$^{-/-}$ islet experiment which was determined by Insulin ELISA according to the manufacturer's instructions. The Fxr$^{-/-}$ islet experiments were carried out by Elena Bellafante. Details of the methods are given in chapter 2, pp.63, 70-73.

3.2.3. Cell Culture of HEK-293 cells

HEK293 cells with stably expressing TRPM3α2 plasmid were grown in DMEM AQ supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), FBS (10%) and G418 (0.5 mg/ ml) at 5% CO_2 and 37°C, maintained by Ivona Primicheru in Professor Stuart Bevans and Dr David Andersson labs, source and method of generation of TRPM3-transfected HEK293 cells found in Chapter 2.

3.2.4. Intracellular calcium assays

HEK293 cells expressing TRPM3 were plated in poly-D-lysine-coated 96-well black-walled plates (Corning Costar, USA), with 10,000 cells per well incubated overnight. The next day cells were loaded with 2.5 µM fura-2 AM, along with 1 mM probenecid for 1 hour. Dye loading and all experiments were performed in a solution containing 140 mM Nacl, 5 mM KCl, 10 mM glucose, 10 mM HEPES, 2 mM $CaCl_2$ and 1 mM $MgCl_2$, buffered to a pH 7.4 by NaOH. The progesterone sulphates PM3S, PM4S, PM5S and PMΔ5S, all added to give a final concentration of 0.5, 1, 2, 5, 10, 20, 50 and 100 µM, in their respective wells, were administered and responses read, after 240 seconds per well, through the FlexStation 3 Multi-Mode Microplate Reader at 37°C. PMΔ5S acted as the positive control in this experiment. Basal emissions ratios (340 nm/380 nm) were measured and then changes in dye emission ratio were determined every 6 seconds after compound addition. Experiments were performed in triplicate.

3.2.5. Homology modelling and ligand binding modelling

The modelling was achieved using the help of Professor Franca Franternali (King's College London, UK) and produced the images shown, pp. 89-93. Molecular Operating Environment (MOE) version 2019.01 was used to visualise the binding sites, superimpose and dock the structures of PMΔ5S and PM5S. Through MOE, the model of human TRPM3 was built through the "Homology model" program of the "protein" module.

The UniProt protein BLAST search of the PDB database found a suitable homologous template. Mouse TRPM7 (PDB ID: 5ZX5)(Duan et al., 2018) was identified as the top-scoring template for comparative modelling, and through UniProt, the primary structure of human TRPM3 was retrieved.

Using the "Align/Superpose" program of the MOE "Protein" module, human TRPM3 and mouse TRPM7 primary structures were globally aligned and manually improved, taking into account also the alignment produced by Schrödinger (https://schrodinger.com) BioLuminate alignment tools contained in the "Multiple Sequence View/Editor".

Ten different intermediate models were produced. Using the electrostatic solvation energy and a Generalised Born/Volume Integral (GB/VI) methodology (Labute, 2008), a top-scoring model was calculated, selected and energy-minimised.

Mouse TRPM7 was co-crystallised and solved with several cholesterol hemisuccinate molecules which share the same cyclopentanoperhydrophenanthrene scaffold of our investigated ligands, and so the cholesterol hemisuccinate molecules were transferred to the final human TRPM3 model, using the MOE Homology model" option "Used as environment".

Molecular docking was carried out on the refined human TRPM7 through the MOE "Dock" program of the "Compute" module, testing a database containing PMΔ5S and PM5S. Three different cholesterol hemisuccinate binding sites were sequentially tested using the following settings: placement "Triangle Matcher", score "London dG", number of retained poses per ligand "30", refinement "Rigid Receptor", score "GBVI/WSA dG", number of retained poses per ligand "5". Both the empirical scoring functions, the London dG and the GBVI/WSA (Naïm et al., 2007), were used for scoring the produced docking poses, expressed as kcal/mol and estimated an approximate binding free energy value for each generated complex.

3.2.6. Statistical analysis

Data presented as mean ± standard error of the mean (SEM) for all human and islet studies in chapter 3. Statistical analysis was performed using GraphPad Prism 8. Data was assessed for normality using the Shapiro-Wilk normality test, if not normally distributed, the data was transformed using the logarithmic function. When only two groups were being compared, student's t-test or Mann-Whitney U test was used. If more than two were being compared, ANOVA was used followed by Tukey's post-hoc analysis, the parametric post hoc test, or a Kruskal-Wallis test followed by a Dunn's post hoc test, the non-parametric multiple comparisons test. The post hoc multiple comparisons tests compare every mean with every other mean, this determines which of the specific means were significant from the others. Spearman rank correlation coefficient was used to evaluate the strength of two variables in the data. A $P < 0.05$ was considered significant after multiple corrections testing to reduce. Logistic regressions were performed using STATA 16.

3.3. Results

3.3.1. Serum progesterone sulphate concentrations from three GDM Cohorts

Serum progesterone sulphates were assayed from 3 cohorts. Cohort 1 (participants in the HAPO study from Belfast) analysed women from the highest and lowest quartiles of FPG concentrations. The higher FPG (5.1-6.6 mmol/L) had significantly lower concentrations of PM3S (P=0.0268), PM3DiS (P=0.0186), PM5S (P=0.0012) and PM4S (P=0.0051), when compared to the lower FPG group ($\leq$ 4.3mmol/L) (Table 3-1). Cohort 2, samples from a Kisspeptin-GDM study, also demonstrated similar consistent findings to women in Cohort 1. Women with GDM had a 1.6-fold decrease in PM5S (P=0.0475) and a 1.35-fold decrease in PMΔ5S (P=0.0104) when compared to those with normal glucose tolerance (Figure 3.1A).

Early pregnancy, 11-13 weeks samples were used to determine if progesterone sulphates were significantly reduced in the first trimester in women that subsequently developed GDM. Cohort 3 demonstrates that women with normal BMI who went on to develop GDM did not have any significant differences in progesterone sulphate levels compared to women with comparable BMI and uncomplicated pregnancies (Figure 3.1B). However, in the group with BMI $\geq$35 kg/m^2, serum PM5S concentrations were significantly reduced (P=0.0340) in women who subsequently developed GDM compared to those that did not (Figure 3.1C).

Table 3-1: Progesterone sulphates are reduced in Cohort 1: HAPO study.

Progesterone sulphate	FPG≤4.3 mmol/L	FPG≥5.1 mmol/L	Significant
PM3S	2.13 ± 2.35	1.44 ± 1.82	*
PM3DiS	2.17 ± 1.09	1.81 ± 0.97	*
PM2DiS	1.94 ± 2.27	1.46 ± 1.95	NS
PM5S	5.12 ± 6.28	2.62 ± 3.79	**
PM4S	1.13 ± 1.43	0.63 ± 0.90	**

Serum Samples from HAPO were assayed using UPLC-MS/MS for progesterone sulphates: PM3S, PM3DiS, PM2DiS, PM5S, PM4S. Serum samples show reduced progesterone sulphates in women with high FPG, n=93, compared to low FPG, n=94. Significant differences are indicated by: * $P < 0.05$, ** $P < 0.01$, as determined by T-tests. Data expressed as mean ± SD. NS; no significance.

A

B

C
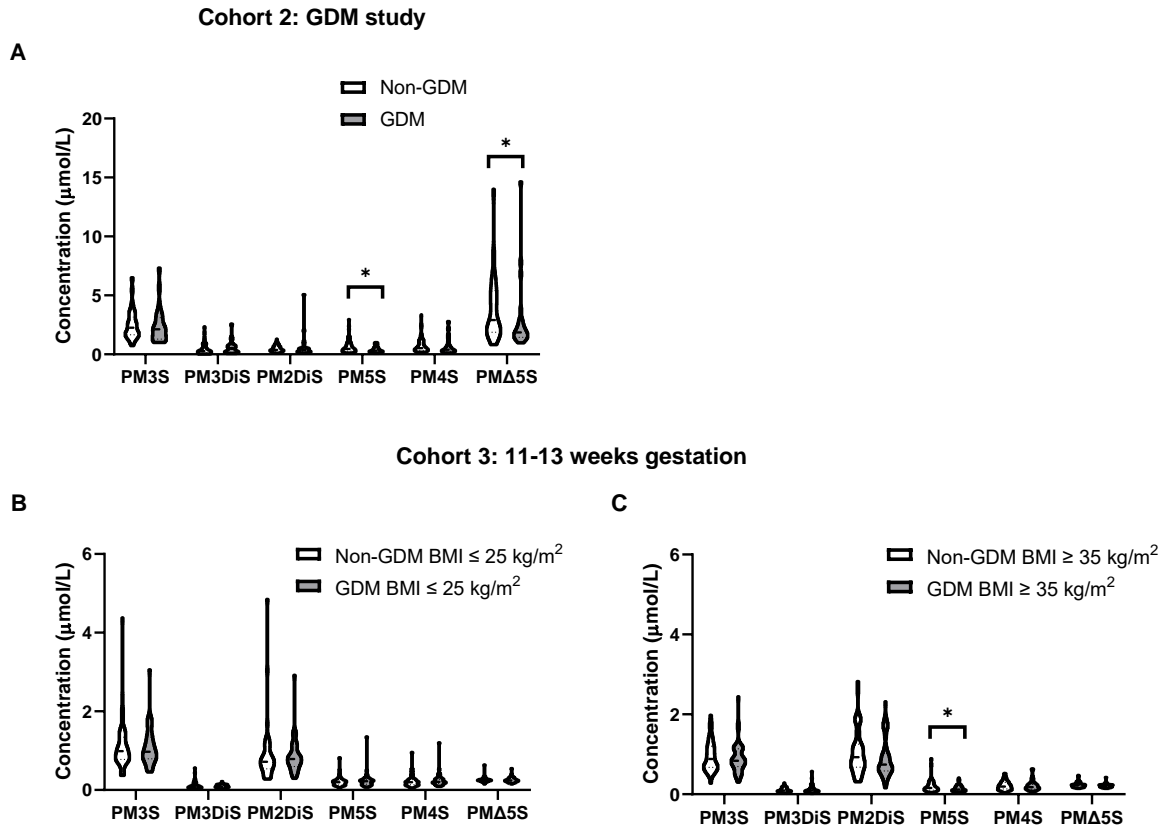
Figure 3.1: Progesterone sulphates are reduced in GDM.

Serum samples from Cohorts 2 and 3 assayed using UPLC-MS/MS for the following progesterone sulphates: PM3S, PM3DiS, PM2DiS, PM5S, PM4S and PMΔ5S. **(A)** Displays the results in Cohort 2 from women with GDM (n=25) and controls (n=64). **(B)** and **(C)** were serum samples from the early pregnancy group, Cohort 3, which were separated according to BMI and whether they subsequently developed GDM or have uncomplicated pregnancies. **(B)** were women with BMI ≤25 kg/m^2 and **(C)** women with BMI ≥35 kg/m^2, n=50 per group. Significant differences are indicated by: * $P < 0.05$, as determined by T-tests, if data was not normally distributed, Mann-Whitney U test was used. Data expressed as mean ± SEM.

3.3.2. Logistic regression and correlations

Correlations were carried out between progesterone sulphate concentrations and BMI in Cohort 2 to establish if there was a relationship between maternal BMI and progesterone sulphate levels. There was a negative correlation between BMI and concentrations of PM5S (Rho -0.3, P=0.02) and PM3S (Rho -0.42, P= <0.01) in women in Cohort 2 (Table 3-2).

Logistic regression was performed on the biochemical markers: PM5S, PM3S and the PM3S/PM5S ratio to determine if they could predict onset of GDM using the data from the early pregnancy samples. Whilst we found a significant difference in PM5S concentration in the high BMI group, the area under the receiver operating characteristic (ROC) curve was 0.6, suggesting that it is not a strong predictive biomarker for GDM (Table 3-3).

Table 3-2: BMI Correlations with progesterone sulphates in women with GDM (Cohort 2).

Cohort 2 – BMI correlation		
Progesterone Sulfate	**P**	**Rho**
PM5S	0.02	-0.3
PM3S	<0.01	-0.42
PM3S/PM5S ratio	0.8	0.03

Spearman's rank correlation coefficient (Rho) was used to assess correlations.

Table 3-3: Logistic regression analysis of PM5S, PM3S and PM3S/PM5S ratio as predictors of GDM at 11-13 weeks gestation.

11-13 week samples BMI ≤ 25			
Biochemical Marker	**OR (95% CI)**	**P**	**Area under ROC curve (95% CI)**
PM5S	0.82 (0.46-1.45)	0.49	0.46 (0.34-0.57)
PM3S	0.98 (0.51-1.86)	0.94	0.50 (0.38-0.61)
PM3S/PM5S ratio	0.76 (0.40-1.44)	0.40	0.44 (0.33-0.56)

11-13 week samples BMI ≥ 35			
Biochemical Marker	**OR (95% CI)**	**P**	**Area under ROC curve (95% CI)**
PM5S	1.66 (0.99-2.77)	0.05	0.60 (0.49-0.72)
PM3S	0.76 (0.38-1.53)	0.44	0.46 (0.35-0.58)
PM3S/PM5S ratio	1.55 (0.89-2.68)	0.12	0.59 (0.48-0.70)

3.3.3. Effect of progesterone sulphates in islets

As PM5S was significantly reduced in the serum of women with diagnosis of GDM and in early pregnancy samples from women with BMI ≥35 kg/m^2 who subsequently develop GDM, we first focused our investigations on how PM5S alters islet insulin secretion.

Challenging WT mouse islets with 20 mM glucose significantly increased GSIS by 2.3, 4.0 and 4.2-fold respectively (Figure 3.2A, B, C). Addition of 50 µM PM5S did not affect insulin secretion at low glucose concentrations. Though in samples exposed to high glucose concentrations, PM5S increased GSIS further when compared to exposure to glucose alone in all 3 graphs, i.e. 2.2, 3.8 and 2.6-fold respectively (Figure 3.2A, B, C), suggesting PM5S augments insulin release in response to high glucose concentrations. As previously mentioned, progesterone sulphates can bind to TGR5 and FXR, and both are expressed in the pancreatic β cells. However, when Tgr5$^{-/-}$ and Fxr$^{-/-}$ islets were treated with PM5S, there were no significant differences in GSIS observed compared to WT islets (Figure 3.2A, B).

TRPM3 is activated by PMΔ5S, which has similar key orientations and 3D structure to PM5S. We investigated whether PM5S binding to TRPM3 was responsible for increasing GSIS at high glucose concentrations. Isosakuranetin (ISO), a TRPM3 antagonist, was co-incubated with PM5S in mouse and human islets. ISO alone did not significantly change GSIS at low or high concentrations of glucose compared to no treatment. However, ISO in 20 mM glucose did not significantly increase GSIS compared with ISO in 2 mM glucose. Co-incubation of ISO with PM5S abolished the PM5S-augmented GSIS at high glucose concentrations (Figure 3.2C). Similar observations were made in human islets. A 2-fold increase in GSIS was observed at high glucose compared to low and was further elevated when incubated with PM5S by 2.2-fold. Like with the mouse islets, ISO alone did not alter GSIS but co-incubation with PM5S abolished the PM5S-induced increase in GSIS in high glucose concentrations (Figure 3.2D). Inhibiting the TRPM3 channel by ISO prevented the PM5S-stimulated increase in GSIS, in high glucose levels, in both mouse and human islets.

In addition to investigating the mechanistic effects of PM5S on islets, PM3S and PM4S were also assessed in murine islets to see if insulin secretion is altered. When incubated alone in high glucose concentrations, PM3S and PM4S had no effect on GSIS compared to 20 mM glucose only (Figure 3.2E).
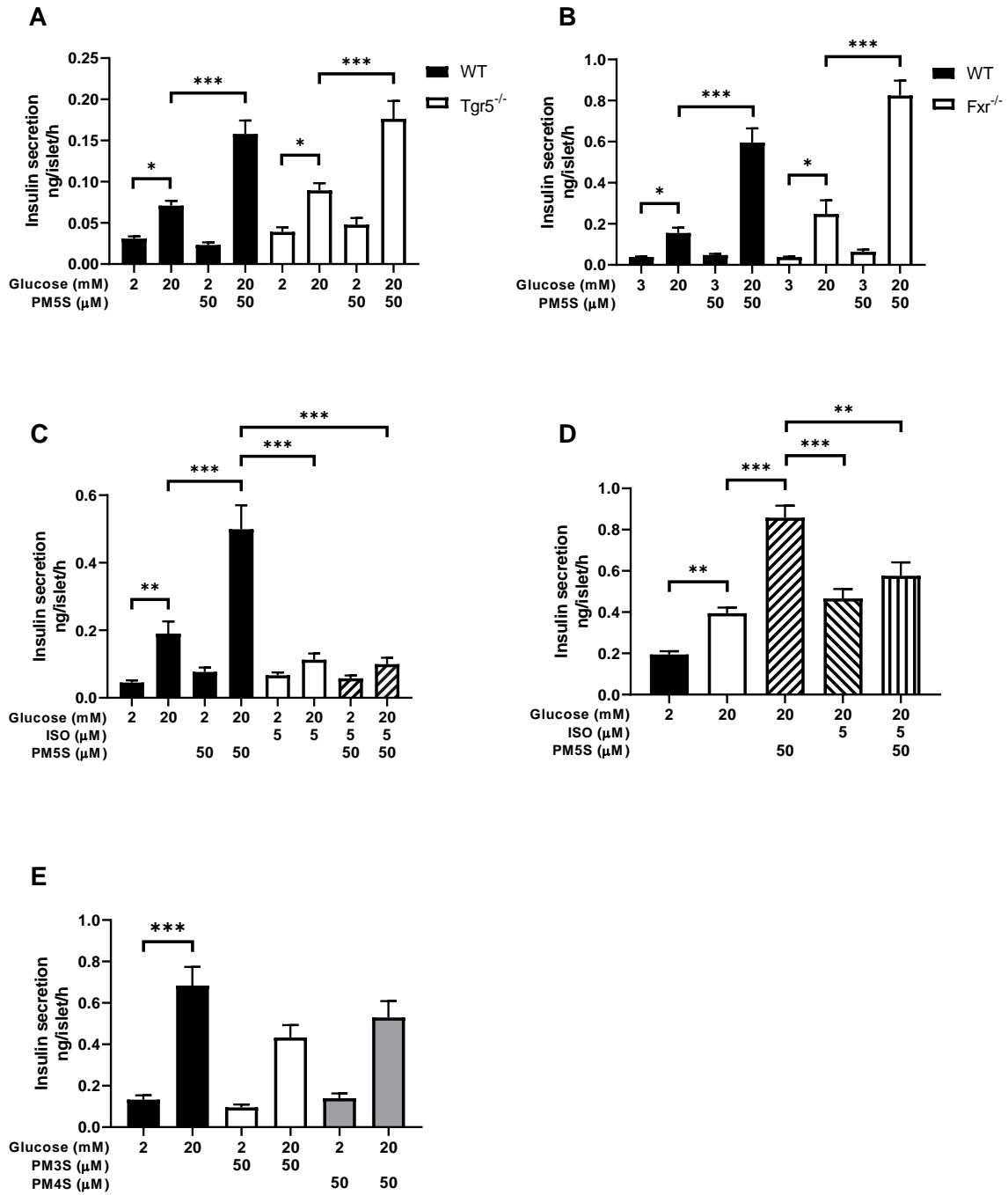
Figure 3.2: PM5S increases GSIS in murine and human islets via TRPM3.

Islets isolated from mice or humans were isolated and incubated with PM5S at low or high concentrations of glucose. Insulin secretion in response to PM5S were first assessed in islets isolated from **(A)** WT and Tgr5$^{-/-}$ mice or **(B)** WT and Fxr$^{-/-}$ mice. **(C)** WT mouse islets were then incubated with the TRPM3 antagonist ISO with and without PM5S. **(D)** In human islets, ISO and/or PM5S were similarly incubated at high glucose concentrations. **(E)** WT mouse islets were incubated with PM3S and PM4S to further assess how different progesterone sulphates affect insulin secretion. Significant differences are indicated by * $P<0.05$, ** $P<0.01$, *** $P<0.001$ as determined by one-way ANOVA followed by Tukey's multiple comparisons test or Kruskal-Wallis test followed by Dunn's multiple comparisons test if data is not normally distributed. Data expressed as mean ± SEM, each graph n=3-6 independent experiments.

3.3.4. Effects of calcium concentrations in transfected HEK293 cells in response to progesterone sulphates

TRPM3 activation causes calcium entry into cells and therefore changes in intracellular calcium concentrations were assessed in response to different progesterone sulphates using HEK293 cells transfected with TRPM3. These cells were loaded with fluorescent calcium indicator dye, Fura-2. PMΔ5S, our positive control, is an established agonist of TRPM3, as previously mentioned in Chapter 1, and was found to be reduced in GDM (Figure 3.1). PMΔ5S caused a concentration-dependent increase of intracellular calcium that starts to plateau at 20 µM (EC$_{50}$ value = 6.2 µM, Bmax = 3.9 ΔF) (Figure 3.3A, E, Table 3-4). Similarly, PM5S also caused a concentration-dependent increase of calcium that had a comparable concentration-response curve to PMΔ5S (EC$_{50}$ = 7.8 µM, Bmax = 3.3 ΔF), also reaching maximal response at 20 µM (Figure 3.3B, E, Table 3-4). When the HEK cells were incubated with PM3S, no changes in calcium concentration was observed at any concentration (Figure 3.3C, E, Table 3-4). When incubated with PM4S, partial agonism was shown. PM4S started to increase intracellular calcium concentration at around 10 µM but did not evoke the same magnitude of calcium response as seen with PMΔ5S or PM5S. PM4S gave approximate EC$_{50}$ values of 11.7 µM and average maximal ΔF value of 2 at 100 µM (Figure 3.3D, E, Table 3-4).
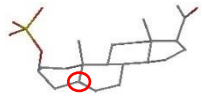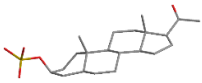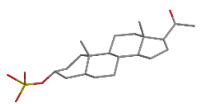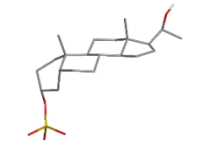
The structural differences between each of the progesterone sulphate tested can be observed in Table 3-4. PMΔ5S and PM5S are structurally similar in shape and so are likely bind to TRPM3 in similar ways, meaning they would have similar agonistic properties.
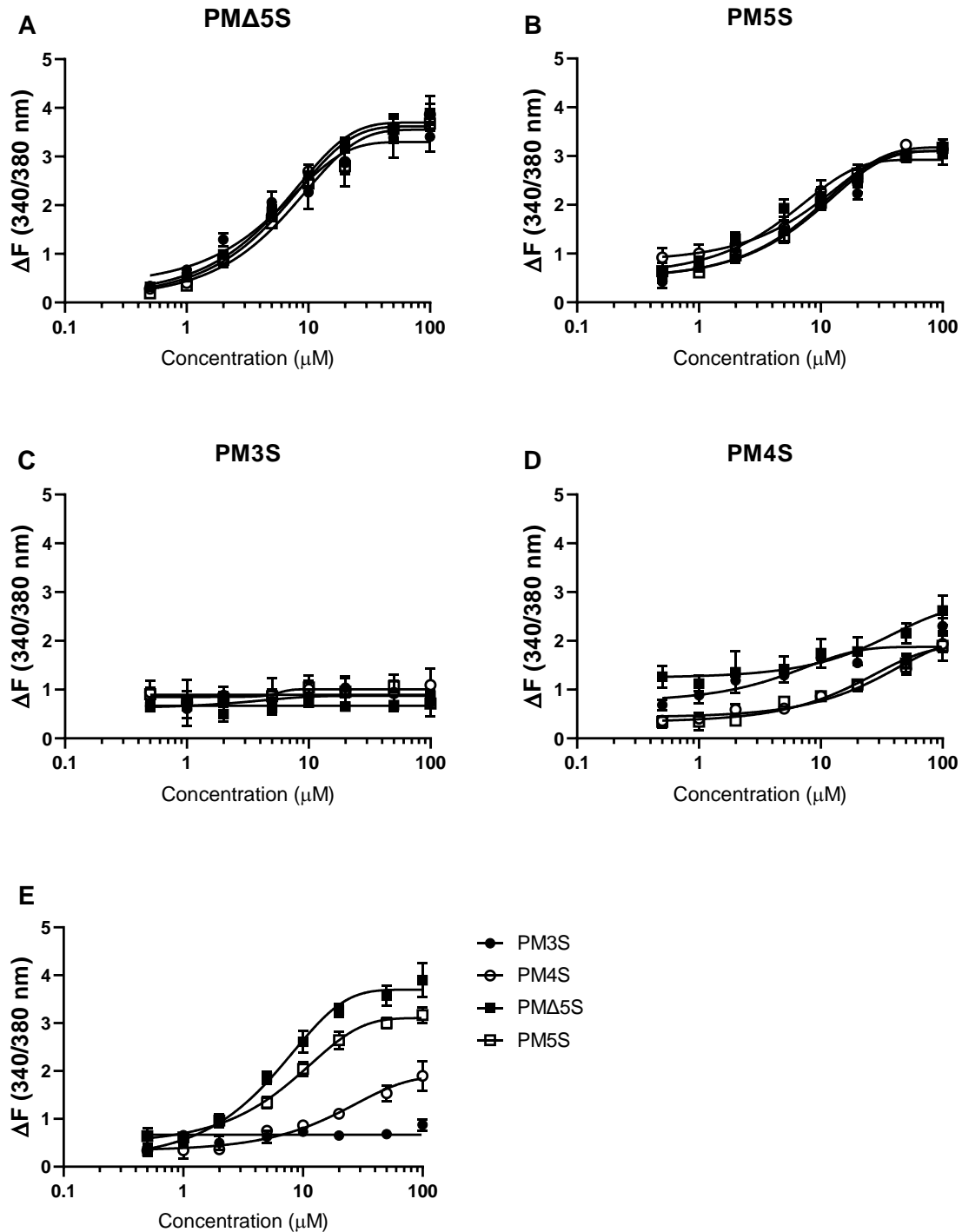
Figure 3.3: Ca2+ concentration is altered by some progesterone sulphates.

HEK cells transfected with TRPM3 were loaded with Fura-2 to measure changes in intracellular calcium concentration. Increasing concentrations of progesterone sulphates were given. **(A-D)** displays 4 replicate experiments for each progesterone sulphate investigated; **(A)** PMΔ5S, **(B)** PM5S, **(C)** PM3S, **(D)** PM4S. **(E)** Curves are representative examples of each data set show against each other. n=4 independent experiments for each progesterone sulphate.

Table 3-4: TRPM3 response upon activation by progesterone sulphates in transfected HEK cells.

Progesterone sulphate	Structure	EC_{50} in µM	B_{max} in ΔF	Average maximal response in ΔF	Predictive response in normal pregnancy ΔF	Predictive response in GDM pregnancy ΔF
PMΔ5S, Pregnenolone sulphate, 5-pregnen-3β-ol-20-one-sulphate		6.2	3.9	3.5	1.5	0.8
PM5S, Epiallopregnanolone sulphate, 5α-pregnan-3β-ol-20-one-sulphate		7.8	3.3	3.2	1.1-1.6	0.6-1.0
PM4S, Allopregnanolone sulphate, 5α-pregnan-3α-ol-20-one-sulphate		11.7	X	2.0	0.7-0.9	0.4-0.7
PM3S, Pregnandiol sulphate, 5β-pregnan-3α,-20α-diol-3-sulphate		X	X	X	X	X

Names, structures, and approximate calcium responses of each progesterone sulphate investigated. Predictive responses refer to the ΔF values obtained according to the mean concentrations of each study cohort in Figure 3.1. Orientation of sulphate group and of the hydrogen group on carbon number 5, circled in PMΔ5S, affects the 3D orientation of each progesterone sulphate. Structures of progesterone sulphates created using PerkinElmer ChemDraw. PM4S are approximate values as a full dose response could not be determined. X indicates that no response value could be obtained from the experiments.

3.3.5. Computational TRPM3 modelling and binding models

A tetra-protomeric model of human TRPM3 was built (Figure 3.4) through comparative modelling on the crystallographic solved structure of mouse TRPM7 homologue. This was then checked with the "Protein Geometry" program of the MOE "protein" module. The phi and psi angles of the final model show very good distribution as reported in the Ramachandran plot (Figure 3.5).

Molecular docking results list approximate binding free energy, expressed as kcal/mol, for all tested ligands on the three TRPM7 putative binding sites identified by cholesterol hemisuccinate (Table 3-5). PMΔ5S and PM5S share the same cyclopentanoperhydrophenanthrene scaffold of cholesterol, so structural data from mouse TRPM7 can be exploited for our purposes. A detailed analysis of the docking poses generated in the three human TPRM3 binding sites allowed us to disregard poses that do not share the same binding mode of cholesterol hemisuccinate, scoring the remaining ones according to their binding free energy values.

Cholesterol hemisuccinate binding modes with all 3 binding sites depicted are shown in Figure 3.6A-C. Figure 3.6D-F shows the amino acid residue interactions on TRPM7 with cholesterol hemisuccinate at each of the sites. Cholesterol hemisuccinate superimposed on PM5S at all 3 sites shows the most suitable poses by PM5S (Figure 3.6G-I). Figure 3.6G and Figure 3.6H show the first 2 sites with similar binding positions that the ligands can achieve. At site 3, no suitable position is found for superimposition (Figure 3.6I). Furthermore, 3D images show that progesterone sulphates, PMΔ5S and PM5S, share very similar binding modes to cholesterol hemisuccinate when docked in two of the three available binding sites (Figure 3.7). At the third site, progesterone sulphates do not show the same binding mode compared with the arrangement of cholesterol hemisuccinate. Supporting this, cholesterol hemisuccinate, PMΔ5S and PM5S share similar amino acid residues at both sites. Most notable interactions at site 1 were methionine, lysine and tyrosine, with a set of hydrogen bonds formed between ligands and the active sites; on the other hand asparagine and tryptophan are the most notable shared amino acid interactions at site 2 (Figure 3.8). PM3S has a very low binding affinity and binding mode in all three sites tested as suggested in Table 3-5.
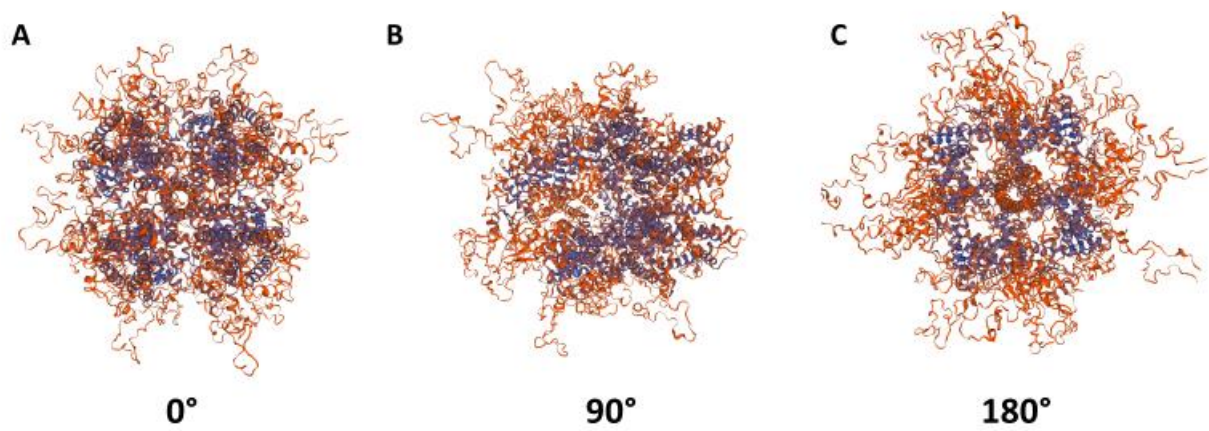
Figure 3.4: Computational structure of TRPM3.

Homology model of TRPM3 generated using TRPM7 (PDB code 5ZX5) as a template. TRPM3 is represented at different horizontal rotations: **(A)** 0° **(B)** 90° **(C)** 180°**.**
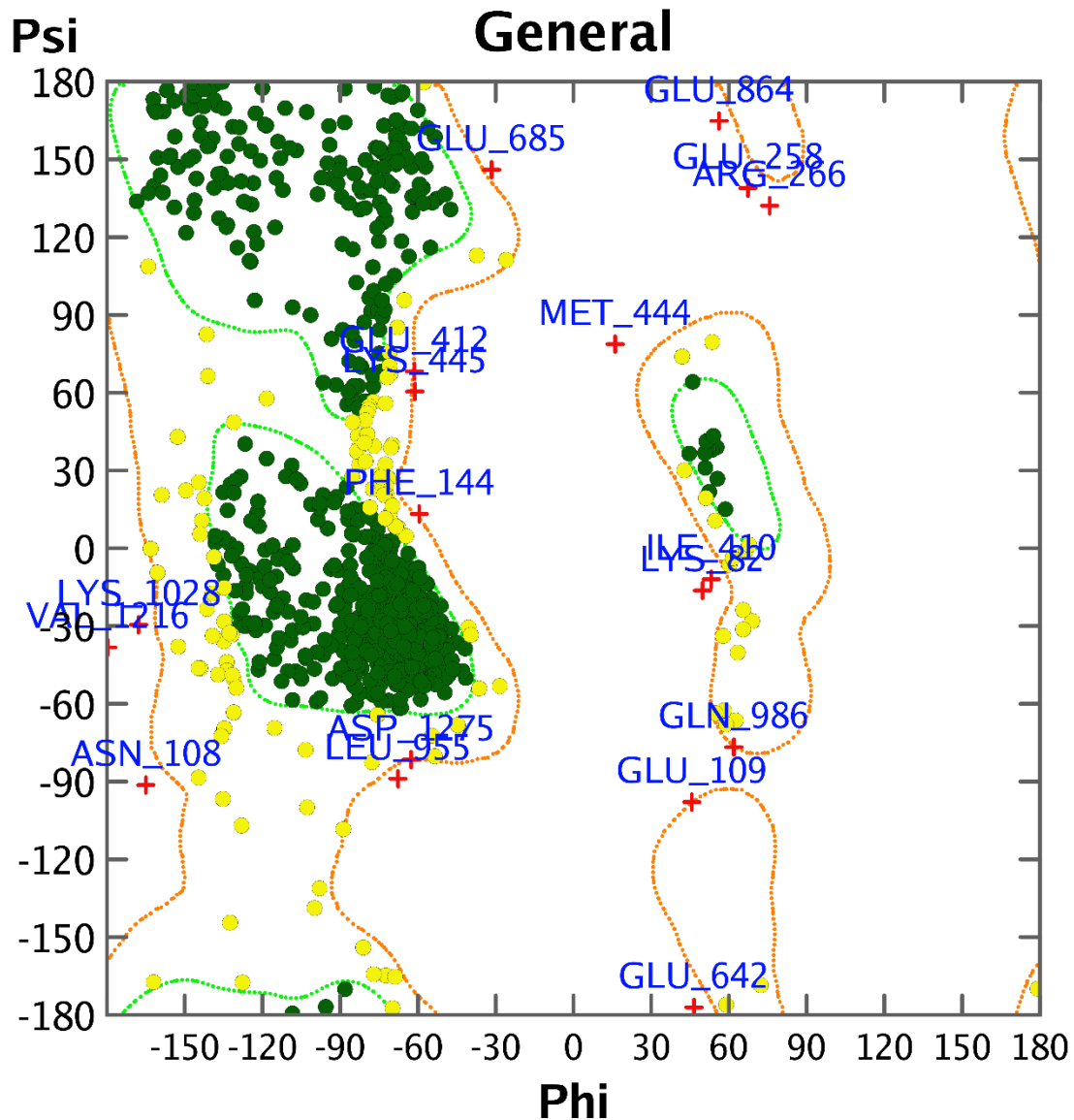
Figure 3.5: Ramachandran plot of human TRPM3.

Plot shows the torsion angles of Phi (φ) and Psi (ψ) for all the amino acid residues in TRPM3. The plot demonstrates which combination of torsional angles are permitted in the amino acid structure, determining the conformation of the residues and gives insight into the structure of TRPM3. The core regions, encased within the green lines and marked by the green circles, contain the most favourable combinations of Phi and Psi and contain most of the residues. The regions encased within the orange lines and marked by the yellow circles, surrounding the core region, show the allowed angles of some residues.
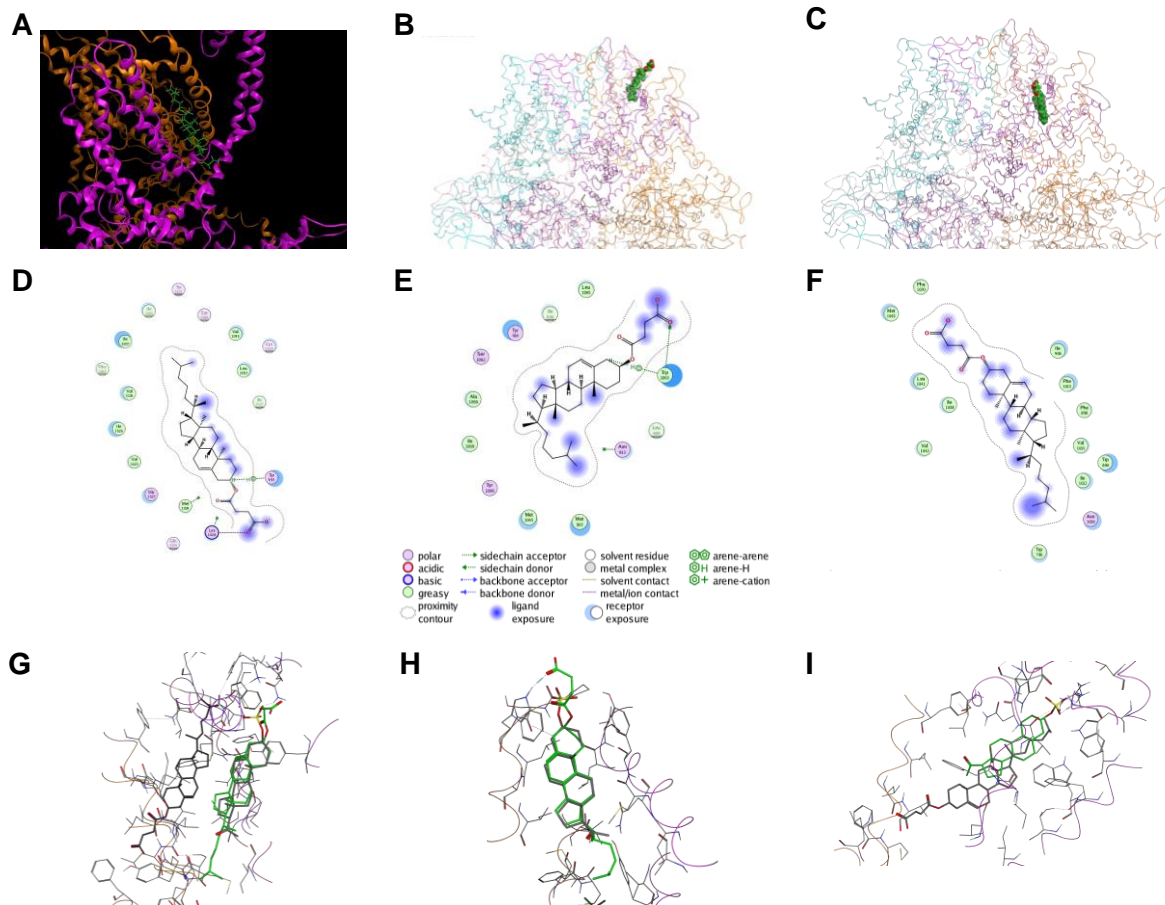
Figure 3.6: Cholesterol hemisuccinate binding.

Cholesterol hemisuccinate binding to three sites on TRPM3. The 3D structure of cholesterol binding at the **(A)** first site, **(B)** binding at the second site and **(C)** the third site. Cholesterol hemisuccinate interaction with binding pocket at **(D)** site 1, **(E)** site 2 and **(F)** site 3. PM5S (shown in grey) showing good superimposition in **(G)** and **(H)** on top of the cholesterol hemisuccinate ligand (shown in green), showing binding at sites 1 and 2 respectively. **(I)** shows cholesterol hemisuccinate (in grey) with no suitable pose for superimposition of PM5S (in green) at site 3.
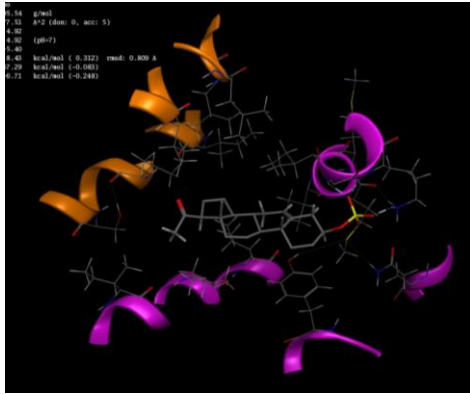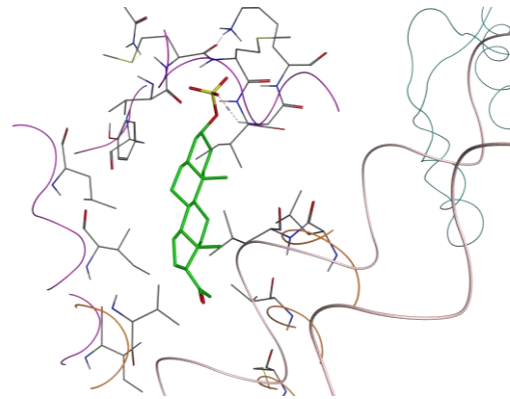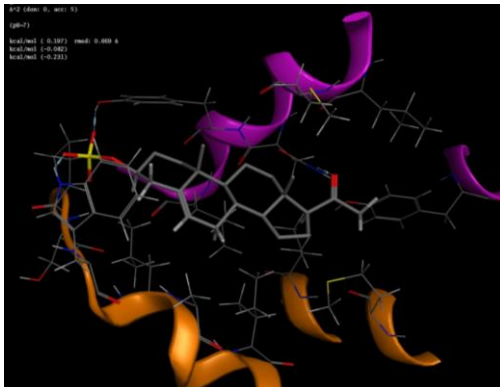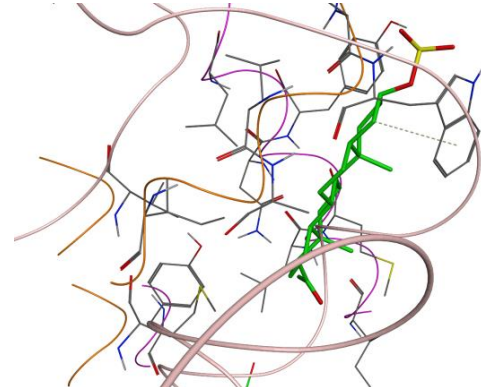
Figure 3.7: 3D images of PMΔ5S and PM5S binding to first 2 sites.

Progesterone sulphate binding at both sites. **(A)** and **(B)** shows the 3D structure of PMΔ5S and PM5S, respectively, binding at site 1. Similarly, 3D structures of PMΔ5S and PM5S, binding at site 2 as shown in **(C)** and **(D)** respectively.
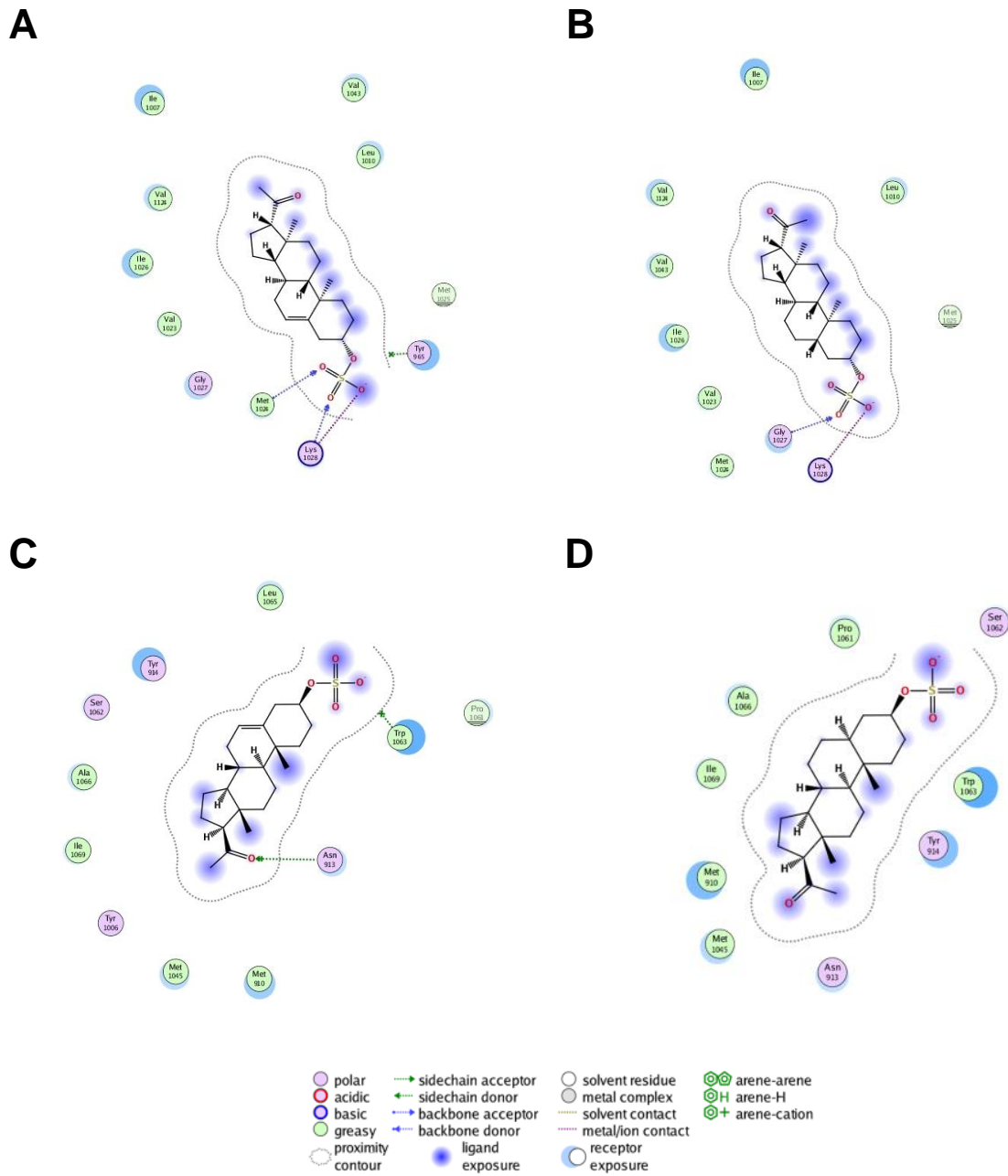
Figure 3.8: Ligand interactions with PMΔ5S and PM5S in sites 1 and 2.

(A and B) Ligand interaction at site 1 and **(C and D)** site 2 for PMΔ5S **(A and C)** and PM5S **(B and D)**.

Table 3-5: Table detailing the affinity of the poses the progesterone sulphates form at each docking site.

A

Energy (kcal/mol)	RMSD	Ligand
-6.8199997	1.1125467	PM5S
-6.6702867	1.4927102	PMΔ5S
-6.5948405	2.1555789	PMΔ5S
-6.5424743	1.2312251	PM4S
-6.439683	1.8100771	PM5S
-6.4153433	2.1325195	PMΔ5S
-6.4101419	1.6385957	PM3S
-6.4068756	1.9603847	PM5S
-6.3827085	1.5345517	PMΔ5S
-6.3573208	1.8047764	PMΔ5S
-6.3540144	2.3486347	PM3S
-6.3538809	2.0648024	PM5S
-6.3411345	2.4843247	PM5S
-6.0677705	3.4442627	PM3S
-6.063601	1.3541986	PM4S
-5.8733807	1.5544147	PM4S
-5.8704696	2.3242292	PM3S
-5.8615518	1.7133332	PM4S
-5.8240767	2.1893353	PM3S
-5.7720551	1.5481328	PM4S

B

Energy (kcal/mol)	RMSD	Ligand
-6.8524556	1.4652321	PMΔ5S
-6.7510605	1.3558685	PM5S
-6.6705761	0.96520036	PMΔ5S
-6.6407328	3.300529	PM5S
-6.5805449	1.3545821	PMΔ5S
-6.5199375	2.2168357	PMΔ5S
-6.4493017	1.5938253	PMΔ5S
-6.3897424	3.51401	PM3S
-6.3854003	2.3206003	PM5S
-6.3802776	2.2265522	PM3S
-6.322556	1.9600726	PM3S
-6.3022604	2.1742473	PM5S
-6.2860308	1.9575782	PM5S
-6.2518678	2.4348776	PM3S
-6.2481518	0.88207519	PM4S
-6.1969061	4.4030252	PM3S
-6.0141978	1.0526633	PM4S
-6.0022035	2.6718736	PM4S
-5.987834	0.97104383	PM4S
-5.9865279	2.0926847	PM4S

C

Energy (kcal/mol)	RMSD	Ligand
-6.0231051	2.7763674	PM5S
-5.9996939	1.6458317	PM5S
-5.9769263	1.0221686	PM5S
-5.9701791	2.7118475	PMΔ5S
-5.9436126	3.4987359	PMΔ5S
-5.9239669	1.8993984	PMΔ5S
-5.9137206	3.8773561	PM3S
-5.9122968	2.45784	PM5S
-5.8920221	1.3614609	PM4S
-5.8661146	2.0471373	PM5S
-5.8643146	1.8455839	PMΔ5S
-5.8574042	1.4660836	PM4S
-5.8362899	4.5342412	PM3S
-5.754652	1.5124441	PMΔ5S
-5.6872506	2.2099655	PM4S
-5.6868806	3.8968232	PM3S
-5.5618906	1.6737609	PM4S
-5.5287905	3.2175353	PM3S
-5.5134492	3.1202154	PM3S
-5.4297891	1.4013846	PM4S

(A) and **(B)** shows the positions tested in sites 1 and 2 respectively, the highlighted row indicates the best position PM5S binds in both sites. **(C)** shows the positions tested in sites 3. PMΔ5S, PM3S, PM4S and PM5S were all tested at these docking sites. The energy represents the affinity for that dock pose in the active site. RMSD, root-mean-square deviation of atomic positions.

3.4. Discussion

In normal circumstances, progesterone sulphates are significantly elevated in pregnancy (Abu-Hayyeh et al., 2013). However, our results show that serum concentrations of progesterone sulphates PMΔ5S and PM5S are lower in the third trimester of pregnancy in women with GDM, (Cohort 2), and in women with high fasting plasma glucose concentrations, which are consistent with a diagnosis of GDM (Cohort 1). PM5S was also decreased in women from a BMI ≥35 kg/m^2 from the first trimester who then went on to be diagnosed as having GDM (Cohort 3). Since PM5S was consistently lowered in each cohort, the impact of progesterone sulphates on secretion of glucose from islets was studied. PM5S significantly augmented GSIS in both mouse and human islets at high glucose concentrations. This augmentation was not abolished in Fxr$^{-/-}$ and Tgr5$^{-/-}$ mouse islets but was prevented by a well-established TRPM3 antagonist, ISO (Straub et al., 2013). This suggests that PM5S mediates the increased GSIS through TRPM3. Both PMΔ5S, a known agonist of TRPM3 (Wagner et al., 2008), and PM5S caused concentration-dependent increases in intracellular calcium concentration in TRPM3-transfected HEK cells.

To date, no research had been conducted on serum concentrations of progesterone sulphates in GDM pregnancies. Of note, in the first trimester, only women with a BMI ≥35 kg/m^2 that subsequently developed GDM had lower serum concentrations of PM5S. Currently, no suitable first trimester serum markers for predicting GDM development exist, and prediction of GDM before 28 weeks' gestation is likely important to enable interventions with the aim of reducing adverse pregnancy outcomes and improve clinical management. BMI has been used as a predictor for GDM. Higher BMI is associated with increased susceptibility to developing GDM (Artzi et al., 2020; Takmaz et al., 2019; Zheng et al., 2019). Recently, several studies have sought to identify early serum biomarkers using hormones and metabolites to find positive associations in early pregnancy and GDM risk. Positive association between prolactin levels and early pregnancy and a higher risk of developing GDM has been reported (Li et al., 2020). Other first trimester markers such as adiponectin, leptin and IL-6 have also been suggested predictors of GDM (Abell et al., 2017; Lorenzo-Almorós et al., 2019). These biomarkers alone are not strong predictors of GDM and have been studied with limited power to detect moderate associations. Most studies have a larger proportion of participants with a high BMI in each of their groups (Abell et al., 2017; Li et al., 2020) and it will be necessary to consider whether maternal BMI influences biomarker concentration. Whilst our study demonstrates that PM5S may be reduced in women of high BMI before diagnosis of GDM, logistic regression suggests that it is not a strong predictor when used in isolation. However, it will be interesting to establish whether progesterone sulphates could be used in conjunction with other biochemical markers to predict GDM.

The use of progesterone sulphates to target TRPM3 for insulin signalling also remains a possibility as a therapeutic option. The channel has been suggested to be an essential component of ionotropic steroid receptor enabling crosstalk between steroidal and insulin-signalling endocrine systems (Wagner et al., 2008). Though minor roles have only been suggested between TRPM3 and insulin signalling due to TRPM3 KO mice displaying no effects in FPG, nor did they have signs of developmental or metabolic defects (Vriens et al., 2011). There is a possibility of TRPM3 influencing and modulating insulin release at times of altered physiology such as pregnancy, or under pathophysiological conditions such as GDM (Wagner et al., 2010). It is hoped that further research will establish the relationship between TRPM3 and GDM.

We next investigated how progesterone sulphates interact with islets to modulate glucose homeostasis. PM5S augmented GSIS in mouse islets at high glucose concentrations, with the same effects observed in human islets, exhibiting translational evidence. Our results suggest PM5S may play a role in the increased GSIS in islets, this may contribute to the altered GSIS occurring during pregnancy. Lower levels of PM5S in GDM pregnancies might contribute to the pathophysiology of GDM if this results in reduced insulin secretion observed in affected pregnancies (McIntyre et al., 2019). Reduced PM5S levels in the first trimester may similarly promote GDM development by reduced insulin secretion.

Previous studies have indicated that islets express TRPM3 and it is well known that activation by PMΔ5S causes insulin secretion (Thiel et al., 2013; Wagner et al., 2010). Interestingly, Wagner *et al.* similarly demonstrated no significant increase in GSIS with PMΔ5S at 3 mM glucose, which was their lowest glucose concentration, but changes in GSIS began to appear as low as 6 mM glucose (Wagner et al., 2008). This observation of increased GSIS has been extended by demonstrating that the sulphate group situated in the β-position at carbon 3, and the hydrogen in the α position on carbon 5 in progesterone sulphates contribute to the ability to activate TRPM3, and can best augment GSIS in islets. Structurally, PM5S and PMΔ5S are similar. PM5S meets most of the reported structural requirements to activate TRPM3 as previously established (Drews et al., 2014; Majeed et al., 2010). ISO prevented the PM5S-induced increase in GSIS, consistent with PM5S acting via TRPM3; this was further supported by the increase in intracellular calcium in TRPM3-expressing HEK293 cells when incubated with PM5S. Similarly, single concentration (50 μM) of PM5S or PMΔ5S has been shown to also activate TRPM3-transfected HEK293 cells to a similar extent in other studies (Drews et al., 2014), which are consistent with our findings. Furthermore, a study using INS-1, a insulin releasing cell line, showed that PMΔ5S (100 μM) activating TRPM3, increasing GSIS and allows Ca^{2+} entry into the cell. Trpm3-deficient INS-1 cells also lacked PMΔ5S-induced Ca^{2+} signals and had reduced PMΔ5S-induced GSIS (Becker et al., 2020). We observed some effects with PM4S due to it sharing

some structural similarities to PMΔ5S and PM5S; the orientation of carbon 5 is the same as PM5S, though the sulphate group is in the α orientation which is the opposite to both PMΔ5S and PM5S. Likewise, PM3S has different orientations to both PMΔ5S and PM5S. PM3S has the sulphate group orientated in the opposite direction and has carbon 5 in the β orientation, which is the opposite direction to PMΔ5S, PM5S and PM4S. This further supports the importance in the orientation of the carbon 5 and sulphate group for determining which progesterone sulphates can bind to and activate TRPM3.

Currently, no high-resolution 3D crystal structure of TRPM3 has been solved. The model of TRPM3 was computed to help predict how and where progesterone sulphate derivatives bind to the channel. This further reveals the potential TRPM3 regulation mechanisms that would occur at the cell membrane and gives a molecular representation of how progesterone sulphates could recognise TRPM3.

The existing ligand binding models of cholesterol hemisuccinate to TRPM7, which our homology TRPM3 model is based on, helped predict how progesterone sulphates would bind to the active site of human TRPM3. TRPM7 and TRPM3 share similarities, for example they are both highly permeable to calcium ions (Huang et al., 2020). Biophysically, their properties also share similarities, and their primary structures show high similarity levels in addition to well conserved secondary structure features (Harteneck, 2005). To further understand the function of TRPM3, it is important to decipher the ligand binding mode. Few studies have investigated TRPM3-ligand binding complexes. A recent study constructed crystal structures of Gβγ-proteins complexed with TRPM3 and confirming the Gβγ-protein binding with the specific exon 17-encoded peptide in TRPM3 through mutagenesis experiments (Behrendt et al., 2020). Our results suggest that among the tested ligands, similar amino acid interactions are involved in two of the three investigated binding sites and therefore likely to operate this target in a similar way. Our structural bioinformatics approach can help predict the affinity and binding modes when ligands are complexed to their receptor. Knowing how progesterone sulphates can specifically interact with TRPM3 is key to understanding progesterone biological activities.

Our studies have some limitations. Our first two cohorts examined had mixed BMI and ethnicities. Furthermore, different criteria were used to diagnose GDM in Cohorts 2 and 3, making comparisons difficult. Thus, it is possible that ethnic and BMI-specific changes in progesterone sulphates were not detected in our cohort studies. Indeed, changes in progesterone sulphates in different ethnicities could also help explain the changes in GDM prevalence in different ethnicities (McIntyre et al., 2019). Future studies should consider using the same diagnostic criteria for GDM and separating the

populations studied according to BMI and ethnicity. The islet experiments only demonstrate how much insulin is released over a set period of time, and future experiments could use perifusion experiments to observe the changes in insulin release over longer periods of time. There could also be some level of FXR interaction with progesterone sulphates (Abu-Hayyeh et al., 2013), and this may have an effect on the islets. We only incubated the islets for 1 hour with progesterone sulphates, which would not be enough time to observe any effect on transcription caused by binding to nuclear receptors like FXR, and future studies should consider longer periods of incubation with progesterone sulphates. Previous studies have shown that FXR agonists can cause insulin release (Düfer, Hörth, Wagner, et al., 2012). Therefore, there is a possibility of longer-term effects of progesterone sulphates on islet function. We were not able to use TRPM3 KO mice for our islet studies to confirm the mechanism of action associated with progesterone sulphates, and ideally future studies should incorporate this. Future studies should also evaluate the toxicity of progesterone sulphates to islets. High concentrations may be harmful to cells and could result in apoptosis or altered morphology of the islets. The calcium experiments clearly showed the effects of the structural differences of the progesterone sulphates, though further studies using combinations of progesterone sulphates at the physiological concentrations may give further clues on their effects on TRPM3 and calcium. Testing at these concentrations may give an indication on how this may impact pregnancy and GDM when they are increased or decreased at these physiological/pathological states.

The TRPM3 modelling was theoretical and based on models from previously built TRPM structures. Therefore, the figures we have shown are not fully conclusive structures. Future experiments involving site-directed mutagenesis, based on key residues in the binding sites of our ligand binding models, would help confirm the structures and binding sites. These experiments will further validate and help with the understanding of TRPM3 binding to progesterone sulphates and how we can utilise these structures for future studies involving receptor physiology and therapeutics.

In conclusion, we have shown reduced serum progesterone sulphates in pregnancies complicated by GDM. PM5S has been shown to be already reduced in the serum from the first trimester of pregnancies of women that were subsequently diagnosed with GDM and had a high BMI. PM5S was also shown to be a strong activator of TRPM3, augmenting GSIS in both mouse and human islets. Lower serum concentrations of progesterone sulphates in GDM pregnancies could reduce the level of insulin release in response to high serum glucose, thereby contributing to glucose intolerance during pregnancy. These results indicate a link between progesterone sulphates and the development of GDM.

Chapter 4: Investigation of bile acids in GDM

4.1 Introduction

During pregnancy, metabolic signalling changes to accommodate the demands of the fetus. For a subgroup of pregnant women, the changes can be sufficiently marked to be considered pathological as observed in diseases such as gestational diabetes mellitus (GDM) and intrahepatic cholestasis or pregnancy (ICP). The metabolic signals that cause hyperglycaemia in GDM have not been fully elucidated. In chapter 3 a potential role for progesterone sulphates in GDM is discussed, and there is a demonstration of a potential mechanism by which progesterone sulphates may influence glucose homeostasis. Only a small number of studies evaluating the serum concentrations of bile acids in GDM exist. In normal pregnancy, bile acids are elevated and changes in the bile acid pool occur (Carter, 1991; Dai et al., 2018; Fulton et al., 1983; Larsson et al., 2008; B. Zhu et al., 2019). These changes can become pathological if bile acids are elevated beyond the normal range for pregnancy, resulting in a diagnosis of ICP (McIlvride et al., 2017). Women with ICP have a higher risk of developing GDM (Arafa & Dong, 2020; Liu et al., 2020; Majewska et al., 2019; Marathe et al., 2017; Martineau et al., 2014; Wikström Shemer et al., 2013). The link between ICP and GDM highlights the importance bile acids may have in influencing metabolism during pregnancy. GDM pregnancies have been reported to have differences in serum bile acids. Some studies have reported elevations in the third and first trimester and associations of high bile acids with the risk of developing GDM (Gagnon et al., 2021; Gao et al., 2016; Hou et al., 2016, 2018; Kong et al., 2020). However, others found reductions in bile acids in the third trimester (Dudzik et al., 2014; Li et al., 2018; B. Zhu et al., 2021). The discrepancy between studies may be explained by the use of different techniques to assay bile acids and a focus on populations with different ethnic origin or BMI. Further work is needed to address the inconsistencies found in current studies looking at bile acids and GDM.

There are currently limited data about variation in bile acid concentrations in pregnant women of different BMI or ethnic origin. As mentioned in the previous chapter, BMI has been used as a diagnostic tool and predictor of GDM, with higher BMI associated with increased probability of developing GDM (Artzi et al., 2020; Takmaz et al., 2019; Zheng et al., 2019). Bile acid concentrations and profiles have also been suggested to be dependent upon BMI. Plasma bile acid concentrations show positive correlations with BMI, with higher circulating levels and increased bile acid synthesis in obese patients. Specifically noted was increased 12α-hydroxylated bile acids, CA and DCA, being higher in obese subjects when compared to lean individuals (Prinz et al., 2015; Vincent et al., 2013; Xie et al., 2015). Indeed, diet-induced weight loss and obesity has been found to alter bile acid

concentrations and gene expression in murine studies (La Frano et al., 2017). Ethnicity may also be used to identify and screen individuals susceptible to GDM. Differences in metabolites, such as insulin, lipids, HbA1C and amino acids, have been observed in people with T2DM of different ethnicities (Alderete et al., 2014; Herman et al., 2007; Van Valkengoed et al., 2017). Indeed, ethnicity-associated differences in metabolite concentrations are also reported in uncomplicated pregnancy, with Europeans having higher circulating lipids and lower levels of glucose compared to South Asian women (Taylor et al., 2019). Serum bile acid concentrations have also been found to vary between ethnicities (Luo et al., 2018; Mitchell et al., 2021); ethnic differences in polymorphisms of enzymes involved in bile acid metabolism and bile acid transporters also exist (Dias & Ribeiro, 2011; Ho et al., 2011; Pan et al., 2011). It is plausible that bile acid changes could be affected by both BMI and ethnicity, as well as being associated with GDM status. Very few studies examining differences in metabolites between ethnicities in GDM pregnancies exist (Sachse et al., 2012). Identifying how bile acids differ between GDM women of different ethnicities and BMI may help further the understanding of the racial/weight disparities in GDM prevalence, risk and pathophysiology.

Bile acids bind to the receptors FXR and TGR5, which both influence glucose and lipid homeostasis. Both receptors are expressed in multiple organs of interest and often have organ specific roles when activated. FXR has shown beneficial effects in lipid metabolism when activated in the liver, with its activation stimulating β-oxidation and decreasing lipid levels in serum and the liver (Savkur et al., 2005). FXR also has significant impacts in glucose metabolism as evident from Fxr$^{-/-}$ mice having impaired GSIS and glucose intolerance (Bellafante et al., 2020; Ma et al., 2006; Popescu et al., 2010; Zhang et al., 2006). TGR5 stimulation induces β-oxidation and increases energy expenditure (Velazquez-Villegas et al., 2018; Zietak & Kozak, 2016). Furthermore, binding of ligands to TGR5 in the enteroendocrine cells of the gut causes GLP-1 release which increases insulin secretion and changes glucose homeostasis (Christiansen et al., 2019; Kuhre et al., 2018; Pathak et al., 2018). It is plausible that both FXR and TGR5 could have reduced activity during GDM pregnancy, thereby influencing metabolism. Understanding how both glucose and lipid metabolism is altered by bile acids in GDM pregnancies could be key for understanding the pathophysiology and expanding the therapeutic areas of GDM research.

We hypothesise that bile acid profiles change in pregnancies diagnosed with GDM and may vary in women of different BMI or ethnicity.

The aims of this chapter are to:

- Investigate the serum bile acid concentrations in women with GDM compared to controls.
- Explore how bile acids influence insulin secretion in islets isolated from mouse and humans.

4.2 Materials and Methods

4.2.1 Human samples assayed

Bile acids were quantified, by UPLC-MS/MS, in women of south Asian or European ancestry, of either normal BMI (<23 and <25 kg/m^2 respectively) or with obese BMI (>27 and >30 kg/m^2 respectively) (cohort 4) and were diagnosed with GDM or without GDM. We also quantified bile acids from women at 11-13 weeks' gestation (cohort 3) by UPLC-MS/MS, which were also divided by normal BMI ($\leq$25 kg/m^2) and obese BMI ($\geq$35 kg/m^2). These women were then further divided based on whether they developed GDM or have uncomplicated pregnancies. Further details on each cohort and UPLC-MS/MS can be found in chapter 2, pp.62, 65-69.

4.2.2 Islet studies

Islets from WT C57BL/6 mice and human islets donated from King's College Hospital were isolated for static incubations. Insulin content was determined using an in-house radioimmunoassay. Methods previously described in chapter 2, pp.63, 70-73.

4.2.3 Statistical analysis

Data presented as mean ± SEM in Chapter 4 for the human and islet studies. Statistical analysis was performed using GraphPad Prism 8. Data was assessed for normality using the Shapiro-Wilk normality test. When only two groups were compared, student's *t*-test or Mann-Whitney U test was used if data was not normally distributed. If there were multiple groups to compare, one-way ANOVA, followed by Tukey's post hoc test, the parametric multiple comparisons test, was carried out. If data was not normally distributed, Kruskal-Wallis test was used followed by a Dunn's multiple comparisons test, the non-parametric multiple comparisons test. Where there were two independent variables, data was transformed into logarithms if not normally distributed and a two-way ANOVA was used followed by a Sidak's post hoc analysis for multiple comparisons. A P<0.05 was considered significant. The post hoc multiple comparisons tests compare every mean with every other mean, this determines which of the specific means were significant from the others.

4.3 Results

4.3.1 Fasting plasma individual bile acid profiles

Analysis of individual fasting bile acid profiles were performed in women of European or South Asian ethnicity and having either normal (<25 and <23 kg/m^2) or obese (>30 and >27 kg/m^2) BMI. Total bile acid levels were also examined.

4.3.1.1 Differences in individual bile acids in GDM women of European ethnicity

In women of European ancestry with normal BMI (BMI <25 kg/m^2), GDCA was increased 1.3-fold in those with GDM (P=0.0374) (Table 4-1). Obese European women with GDM (BMI >30 kg/m^2) had increased total bile acids (P=0.0006), by 1.6-fold, compared to those with uncomplicated pregnancy. Increases were also seen in DCA (P=0.0022) and its conjugated versions GDCA (P=0.0043) and TDCA (P=0.0194), increasing by 1.6, 1.7 and 1.7-fold respectively. The conjugated primary bile acids were also raised; GCA (P=0.0127), GCDCA (P=0.0026) and TCDCA (P=0.0348) increased by 1.4, 1.55, 1.4-fold respectively. UDCA (P=0.0076) and its glycine conjugate GUDCA (P=0.0014) also increased by 1.3, 1.6-fold respectively (Table 4-2).

4.3.1.2 Differences in individual bile acids in GDM women of South Asian ethnicity

South Asian women with normal BMI (BMI <23 kg/m^2) and GDM had decreased serum LCA (P=0.0401) concentrations (2-fold) compared to those with uncomplicated pregnancy (Table 4-3). The obese South Asians (BMI >27 kg/m^2) with GDM also had significant decreases in DCA (P=0.0070) and LCA (P<0.0001) by 1.5 and 4-fold respectively (Table 4-4).

Table 4-1: Individual and total bile acids in European women with BMI <25 kg/m^2.

Bile acid	Cohort 4: PRiDE study, European BMI <25 kg/m^2						Significance
	Non-GDM			GDM			
	Median	25%	75%	Median	25%	75%	
TBA	1.498	1.062	2.404	1.582	1.143	2.558	NS
UDCA	0.015	0.005	0.030	0.013	0.005	0.031	NS
CDCA	0.046	0.021	0.091	0.036	0.010	0.094	NS
DCA	0.214	0.092	0.339	0.260	0.128	0.346	NS
CA	0.044	0.015	0.289	0.027	0.010	0.243	NS
LCA	0.005	0.003	0.011	0.005	0.000	0.011	NS
GUDCA	0.022	0.013	0.046	0.027	0.012	0.052	NS
GCDCA	0.232	0.149	0.425	0.234	0.152	0.548	NS
GDCA	0.173	0.094	0.313	0.232	0.125	0.352	*
GCA	0.151	0.076	0.288	0.153	0.077	0.293	NS
GLCA	0.005	0.002	0.010	0.005	0.004	0.010	NS
TUDCA	0.002	0.000	0.004	0.000	0.000	0.004	NS
TCDCA	0.106	0.053	0.237	0.096	0.041	0.152	NS
TDCA	0.064	0.033	0.148	0.084	0.043	0.170	NS
TCA	0.050	0.016	0.115	0.043	0.020	0.093	NS
TLCA	0.000	0.000	0.004	0.000	0.000	0.003	NS

Fasting serum samples from European, BMI <25 kg/m^2 were assayed for bile acids (μmol/L) in non-GDM (n=72) and GDM (n=34) women. Significant differences indicated by: * P < 0.05, as determined by Mann-Whitney U test. Data expressed as median and interquartile range. NS; no significance, TBA; Total bile acids.

Table 4-2: Individual and total bile acids in European women with BMI >30 kg/m^2.

Bile acid	Cohort 4: PRiDE study, European BMI >30 kg/m^2						Significance
	Non-GDM			GDM			
	Median	25%	75%	Median	25%	75%	
TBA	1.358	0.991	2.280	2.114	1.287	3.651	***
UDCA	0.015	0.005	0.029	0.020	0.010	0.042	**
CDCA	0.064	0.025	0.147	0.098	0.033	0.244	NS
DCA	0.175	0.081	0.286	0.281	0.131	0.416	**
CA	0.051	0.027	0.279	0.132	0.030	0.479	NS
LCA	0.005	0.002	0.011	0.005	0.003	0.011	NS
GUDCA	0.022	0.013	0.040	0.036	0.022	0.071	**
GCDCA	0.246	0.152	0.487	0.384	0.225	0.672	**
GDCA	0.123	0.078	0.227	0.203	0.111	0.422	**
GCA	0.174	0.108	0.311	0.241	0.143	0.451	*
GLCA	0.005	0.001	0.010	0.006	0.004	0.010	NS
TUDCA	0.003	<0.001	0.004	0.002	0.000	0.004	NS
TCDCA	0.096	0.056	0.169	0.135	0.076	0.248	*
TDCA	0.042	0.023	0.097	0.072	0.038	0.116	*
TCA	0.051	0.035	0.100	0.071	0.043	0.140	NS
TLCA	0.000	0.000	0.001	0.000	0.000	0.002	NS

Fasting serum samples from European, BMI >30 kg/m^2 were assayed for bile acids (μmol/L) in non-GDM (n=80) and GDM (n=80) women. Significant differences indicated by: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, as determined by Mann-Whitney U test. Data expressed as median and interquartile range. NS; no significance, TBA; Total bile acids.

Table 4-3: Individual and total bile acids in South Asian with BMI <23 kg/m^2.

Bile acid	Cohort 4: PRiDE study, South Asian BMI <23 kg/m^2						Significance
	Non-GDM			GDM			
	Median	25%	75%	Median	25%	75%	
TBA	1.616	0.953	2.970	1.586	0.859	2.987	NS
UDCA	0.015	0.005	0.038	0.005	0.000	0.019	NS
CDCA	0.048	0.026	0.122	0.046	0.020	0.110	NS
DCA	0.207	0.125	0.370	0.153	0.071	0.216	NS
CA	0.064	0.027	0.221	0.069	0.034	0.172	NS
LCA	0.008	0.005	0.011	0.003	0.000	0.005	*
GUDCA	0.027	0.018	0.062	0.025	0.007	0.040	NS
GCDCA	0.325	0.176	0.561	0.303	0.135	0.641	NS
GDCA	0.200	0.111	0.350	0.134	0.071	0.312	NS
GCA	0.179	0.083	0.381	0.237	0.148	0.437	NS
GLCA	0.005	0.004	0.009	0.005	0.000	0.006	NS
TUDCA	0.002	0.000	0.004	0.000	0.000	0.004	NS
TCDCA	0.096	0.052	0.208	0.096	0.046	0.186	NS
TDCA	0.068	0.030	0.164	0.038	0.026	0.112	NS
TCA	0.050	0.023	0.132	0.066	0.035	0.117	NS
TLCA	0.000	0.000	0.002	0.000	0.000	0.000	NS

Fasting serum samples from South Asian, BMI <23 kg/m^2 were assayed for bile acids (µmol/L) in non-GDM (n=66) and GDM (n=12) women. Significant differences indicated by: * $P < 0.05$, as determined by Mann-Whitney U test. Data expressed as median and interquartile range. NS; no significance, TBA; Total bile acids.

Table 4-4: Individual and total bile acids in South Asian with BMI >27 kg/m^2.

Bile acid	Cohort 4: PRiDE study, South Asian BMI >27 kg/m^2						Significance
	Non-GDM			GDM			
	Median	25%	75%	Median	25%	75%	
TBA	2.102	0.936	4.058	2.093	1.306	3.036	NS
UDCA	0.013	0.005	0.027	0.015	0.008	0.026	NS
CDCA	0.077	0.029	0.181	0.097	0.043	0.188	NS
DCA	0.204	0.120	0.403	0.168	0.099	0.221	**
CA	0.086	0.027	0.249	0.123	0.039	0.460	NS
LCA	0.011	0.005	0.016	0.000	0.000	0.005	***
GUDCA	0.022	0.013	0.060	0.020	0.010	0.041	NS
GCDCA	0.352	0.160	0.707	0.352	0.213	0.658	NS
GDCA	0.178	0.071	0.384	0.160	0.084	0.248	NS
GCA	0.202	0.123	0.495	0.290	0.158	0.685	NS
GLCA	0.004	0.000	0.009	0.003	0.000	0.005	NS
TUDCA	0.002	0.000	0.004	0.002	0.000	0.004	NS
TCDCA	0.116	0.054	0.232	0.172	0.061	0.303	NS
TDCA	0.052	0.024	0.155	0.060	0.032	0.086	NS
TCA	0.072	0.031	0.156	0.105	0.053	0.229	NS
TLCA	0.000	0.000	0.000	0.000	0.000	0.000	NS

Fasting serum samples from South Asian, BMI >27 kg/m^2 were assayed for bile acids (µmol/L) in non-GDM (n=80) and GDM (n=55) women. Significant differences indicated by: ** $P < 0.01$, *** $P < 0.001$, as determined by Mann-Whitney U test. Data expressed as median and interquartile range. NS; no significance, TBA; Total bile acids.

4.3.2. Analysing differences in fasting bile acid concentrations

The unconjugated and conjugated forms of the bile acids, CA, CDCA, UCDA, DCA and LCA were combined to generate a final concentration that comprised the sum of each bile acid.

4.3.2.1 Differences in total unconjugated and conjugated bile acids in women of European ethnicity

There were no differences in total concentrations of each specific serum bile acid (i.e. when considering the unconjugated and conjugated forms together) when comparing women with or without GDM in Europeans with BMI <25 kg/m^2 (Figure 4.1A). However, obese European women with GDM had significantly higher total serum concentrations of CA (P=0.0467), CDCA (P=0.0284) and DCA (P=0.0006), increasing by 1.4, 1.4 and 1.5-fold respectively. UDCA (P=0.0007) also increased by 1.5-fold (Figure 4.1B).

4.3.2.2 Differences in total unconjugated and conjugated forms of bile acids in women of South Asian ethnicity

South Asian women with GDM and BMI <23 kg/m^2 had no significant differences in total serum bile acid concentrations compared to those without GDM (Figure 4.2A). South Asian women with GDM and >27 kg/m^2 had significantly lower total serum concentrations of DCA (P=0.0413), decreasing by 1.75-fold (Figure 4.2B).
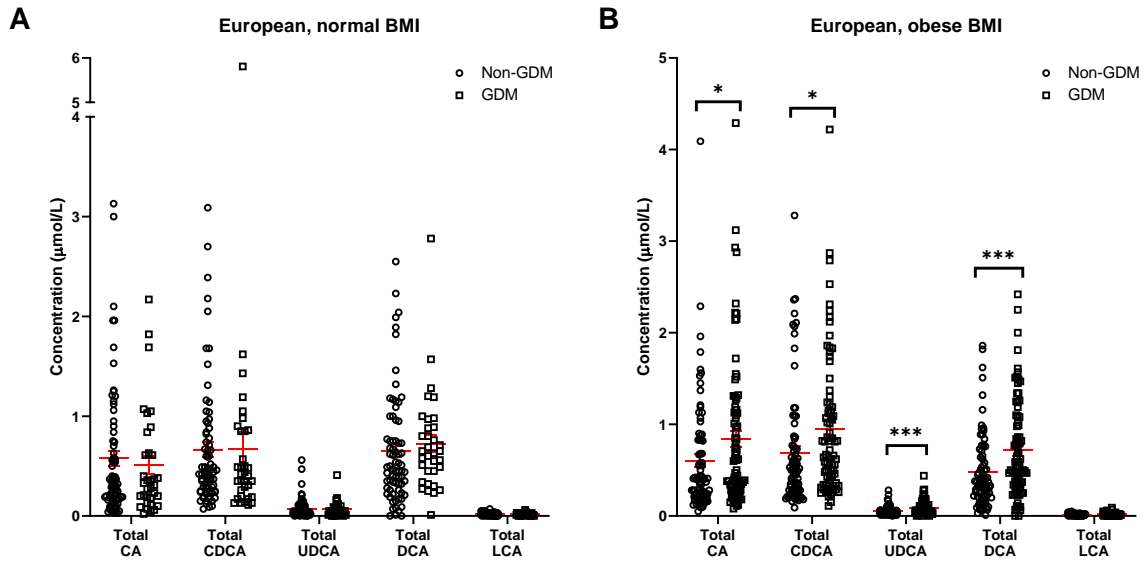
Figure 4.1: Total conjugated and unconjugated forms of each bile acid in European women.

The unconjugated and conjugated forms of each bile acid were combined and analysed for differences. The groups were: **(A)** normal BMI <25 kg/m^2 European, with either having non-GDM (n=72) or GDM (n=34). **(B)** Obese BMI >30 kg/m^2 European, either being non-GDM (n=80) or GDM (n=80). Significant differences are indicated by: * P <0.05, *** P <0.001 as determined two-way ANOVA, followed by Sidak multiple comparisons test, after data was transformed by logarithms. Data expressed as mean ± SEM.
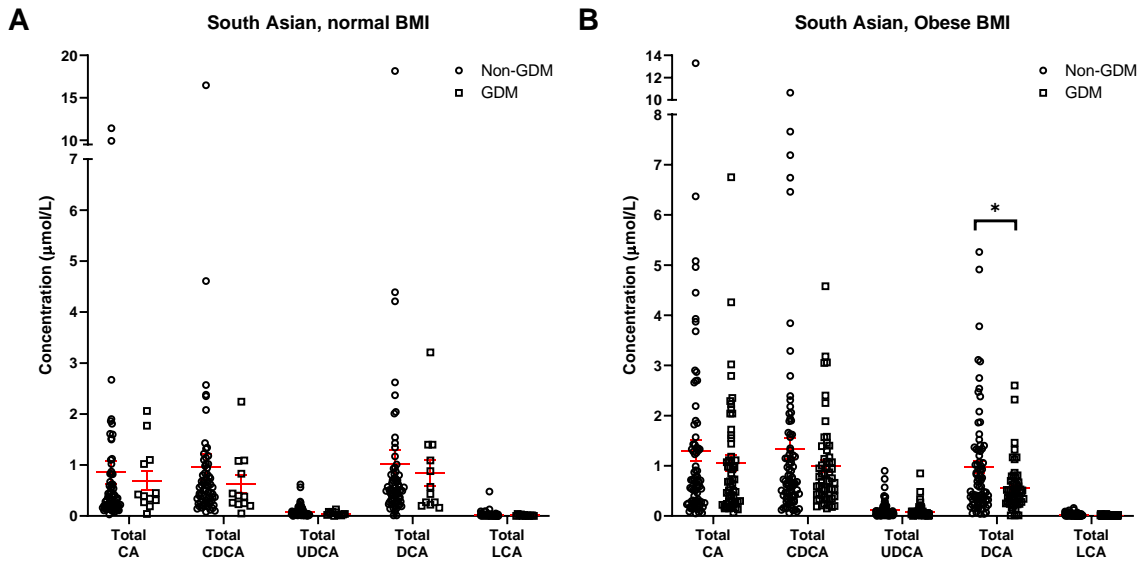
Figure 4.2: Total conjugated and unconjugated forms of each bile acid in South Asian women.

The unconjugated and conjugated forms of each bile acid were combined and analysed for differences. The groups were: **(A)** normal BMI <23 kg/m^2 South Asians, being non-GDM (n=66) or GDM (n=12). **(B)** South Asians with an obese BMI >27 kg/m^2, having either non-GDM (n=80) or GDM (n=55). Significant differences are indicated by: * P <0.05 as determined two-way ANOVA, followed by Sidak multiple comparisons test, after data was transformed by logarithms. Data expressed as mean ± SEM.

4.3.3. Analysing differences in fasting bile acid concentrations in pregnant women with normal or high BMI

The cohort was separated by BMI to assess the impact these factors may have on total bile acid concentrations. European women without GDM did not have any significant differences in bile acid concentrations between women of normal and raised BMI <25 kg/m^2 and >30 kg/m^2 (Figure 4.3A). Obese South Asian women without GDM had significant higher concentrations of CA (P=0.0111), compared to normal BMI <25 kg/m^2 South Asians, increasing by 1.5-fold (Figure 4.3B). European women with BMI ≥30 kg/m^2 and GDM had increased total CA (P=0.0013) and CDCA (P=0.0136) by 1.7 and 1.4-fold respectively, compared to European women with BMI <25 kg/m^2 and GDM (Figure 4.3C). Obese GDM South Asians had no significant changes compared to non-obese south Asians with GDM (Figure 4.3D).
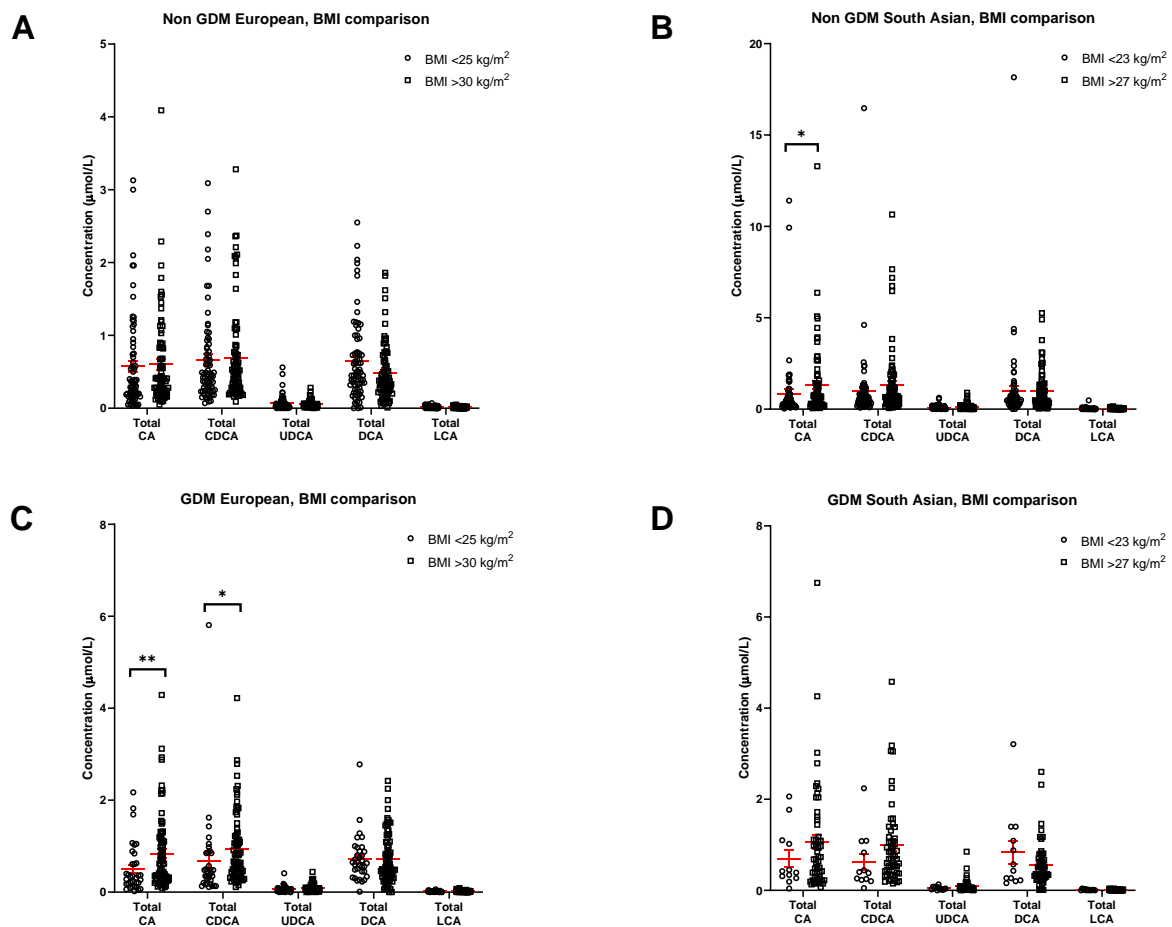
Figure 4.3: Analysing changes in BMI in total conjugated and unconjugated forms of each bile acid.

Total conjugated and unconjugated bile acids were summed, then analysing for changes between BMI in the third trimester. **(A)** Non GDM Europeans at BMI >30 kg/m² (n=80) and <25 kg/m² (n=72), **(B)** Non GDM South Asians at BMI >27 kg/m² (n=80) and <23 kg/m² (n=66). Then the GDM only groups were analysed: **(C)** GDM Europeans at BMI >30 kg/m² (n=80) and <25 kg/m² (n=34), **(D)** GDM South Asians at BMI >27 kg/m² (n=55) and <23 kg/m² (n=12). Significant differences are indicated by: * $P < 0.05$, ** $P < 0.01$ as determined two-way ANOVA, followed by Sidak multiple comparisons test, after data was transformed by logarithms. Data expressed as mean ± SEM.

4.3.4. Analysing differences in ethnicity in fasting bile acid concentrations

Cohorts were further separated by ethnicity to assess how they affect total bile acid concentrations. The normal BMI group showed no significant differences between ethnicity in the non-GDM group. (Figure 4.4A). The non GDM obese (>27 kg/m^2) South Asians had higher concentrations of CA (P=0.0047), CDCA (P=0.0445), UDCA (P=0.0129) and DCA (P=0.0009) increasing by 2.2, 1.95, 2 and 2-fold, respectively, compared to non GDM obese (>30 kg/m^2) Europeans (Figure 4.4B). The normal BMI (<23 kg/m^2) GDM South Asians and (<25 kg/m^2) Europeans also showed no significant difference (Figure 4.4C). However, the GDM Europeans in the obese (>30 kg/m^2) BMI group showed higher concentrations of total unconjugated and conjugated forms of DCA (P=0.0381) compared with obese (>27 kg/m^2) South Asians, increasing by 1.3-fold respectively (Figure 4.4D).
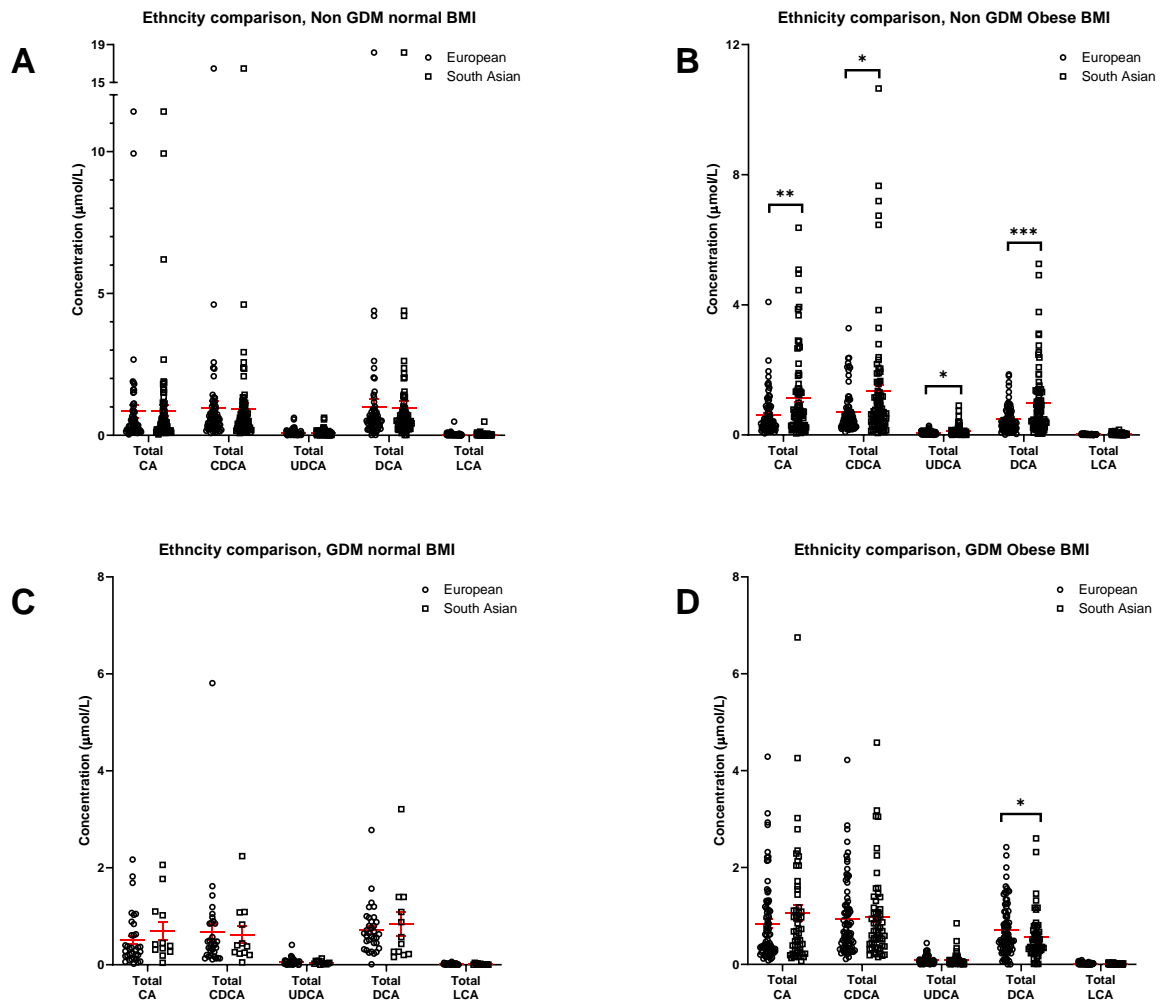
Figure 4.4: Analysing changes in ethnicity in total conjugated and unconjugated forms of each bile acid.

Ethnic differences in total bile acids were examined and were BMI matched, the groups were: **(A)** Non GDM European (n=72) and South Asians (n=66) at BMI <25 kg/m^2 and <23 kg/m^2 respectively **(B)** Non GDM European (n=80) and South Asians (n=80) at BMI >30 kg/m^2 and >27 kg/m^2 respectively **(C)** normal BMI <25 kg/m^2 GDM European (n=34) and BMI <23 kg/m^2 South Asians (n=12). **(D)** Obese BMI >30 kg/m^2 GDM European (n=80) and BMI >27 kg/m^2 South Asians (n=55), Significant differences are indicated by: * P <0.05, ** P <0.01, *** P <0.001, as determined two-way ANOVA, followed by Sidak multiple comparisons test, after data was transformed by logarithms. Data expressed as mean ± SEM.

4.3.5. Women with GDM have different concentrations of specific bile acid species

4.3.5.1 Examining changes in total conjugated and total unconjugated bile acids

Bile acids were further grouped according to their structures and differences were observed between groups. We first analysed the differences in the total concentrations of conjugated only and unconjugated only bile acids in non-GDM vs GDM women. European women with BMI >30 kg/m^2 and GDM had increased conjugated (P=0.0491) and unconjugated (P=0.0389) bile acids, increasing by 1.4 and 1.5-fold respectively, compared to those without GDM (Figure 4.5B). Limited changes were observed in European women with BMI <25 kg/m^2, and South Asian women with either BMI <23 kg/m^2 or >27 kg/m^2 had no significant differences in concentrations of these bile acid between non-GDM and GDM women (Figure 4.5A, C, D).

4.3.5.2 Investigating changes in total primary and total secondary bile acids

We next examined the changes in total primary and total secondary bile acids in women with GDM vs non-GDM. The obese GDM European women displayed a 1.4 and 1.5-fold increase in primary (P=0.0342) and secondary (P=0.0037) bile acids, respectively, compared to non-GDM (Figure 4.6B). Women with GDM from the other groups (i.e. normal BMI Europeans and South Asians, and obese South Asians) did not have any significant changes in primary or secondary bile acids compared to non-GDM (Figure 4.6A, C, D).

4.3.5.3 Assessing changes in total 12α-hydroxylated bile acid concentrations

Finally, we calculated the total 12α-hydroxylated bile acids to see if a GDM phenotype was associated with altered concentrations. Compared to non-GDM, the GDM obese Europeans had increased 12α-hydroxylated bile acids, increasing by 1.45-fold (P=0.0004) (Figure 4.7B). The other groups did not have any discernible differences in concentrations of 12α-hydroxylated bile acids between the non-GDM and GDM (Figure 4.7A, C, D).
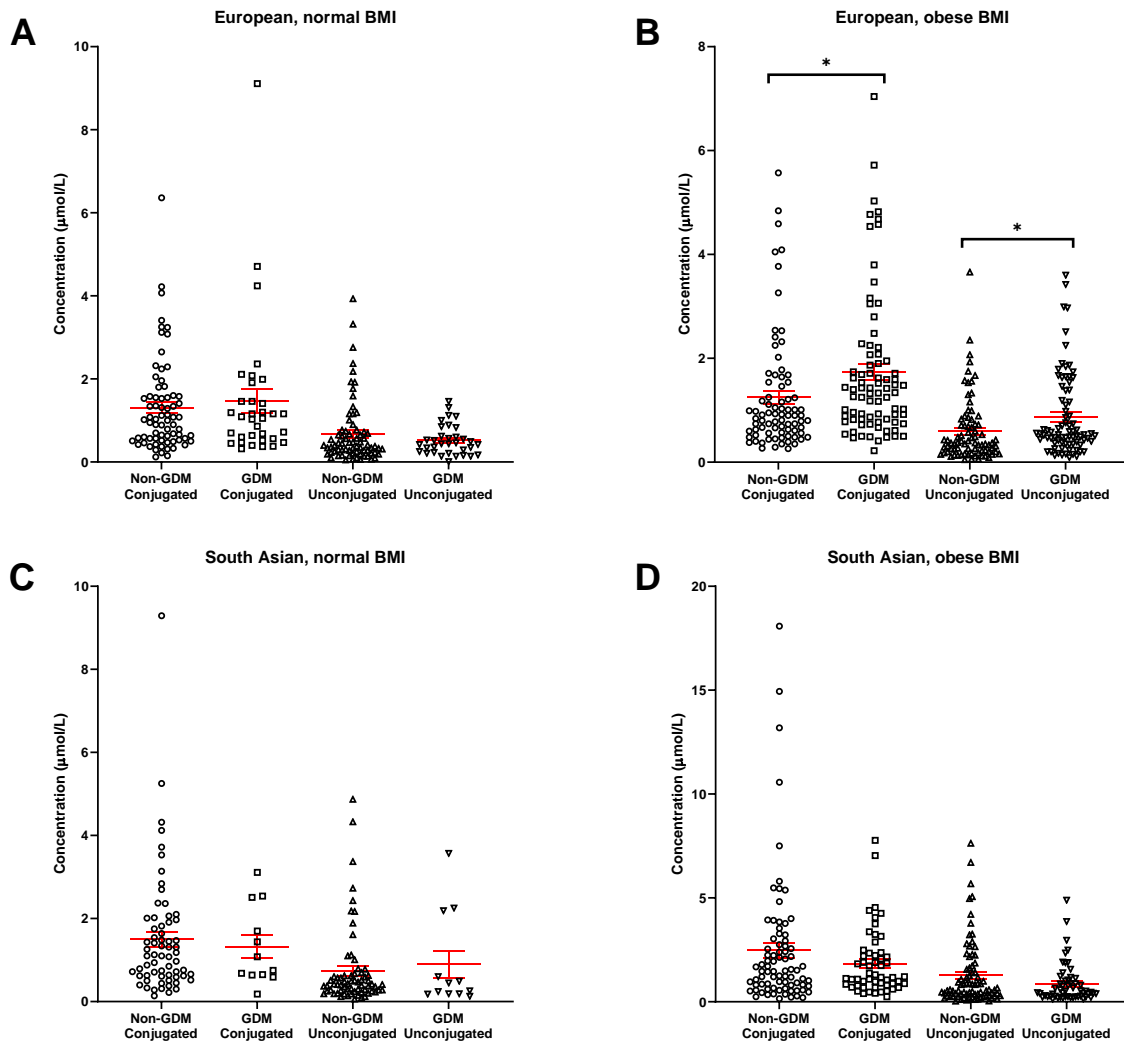
Figure 4.5: Differences in concentrations of conjugated and unconjugated bile acids.

Groups studied were: **(A)** normal BMI Europeans, non-GDM (n=72) or GDM (n=34). **(B)** Obese BMI Europeans, non-GDM (n=80) or GDM (n=80). **(C)** normal BMI South Asians, non-GDM (n=66) or GDM (n=12). **(D)** Obese south Asians, non-GDM (n=80) or GDM (n=55). Significant differences are indicated by: * P <0.05, as determined by one-way ANOVA followed by Tukey's post hoc test. If data was not normally distributed, Kruskal-Wallis test was used followed by a Dunn's multiple comparisons test. Data expressed as mean ± SEM.
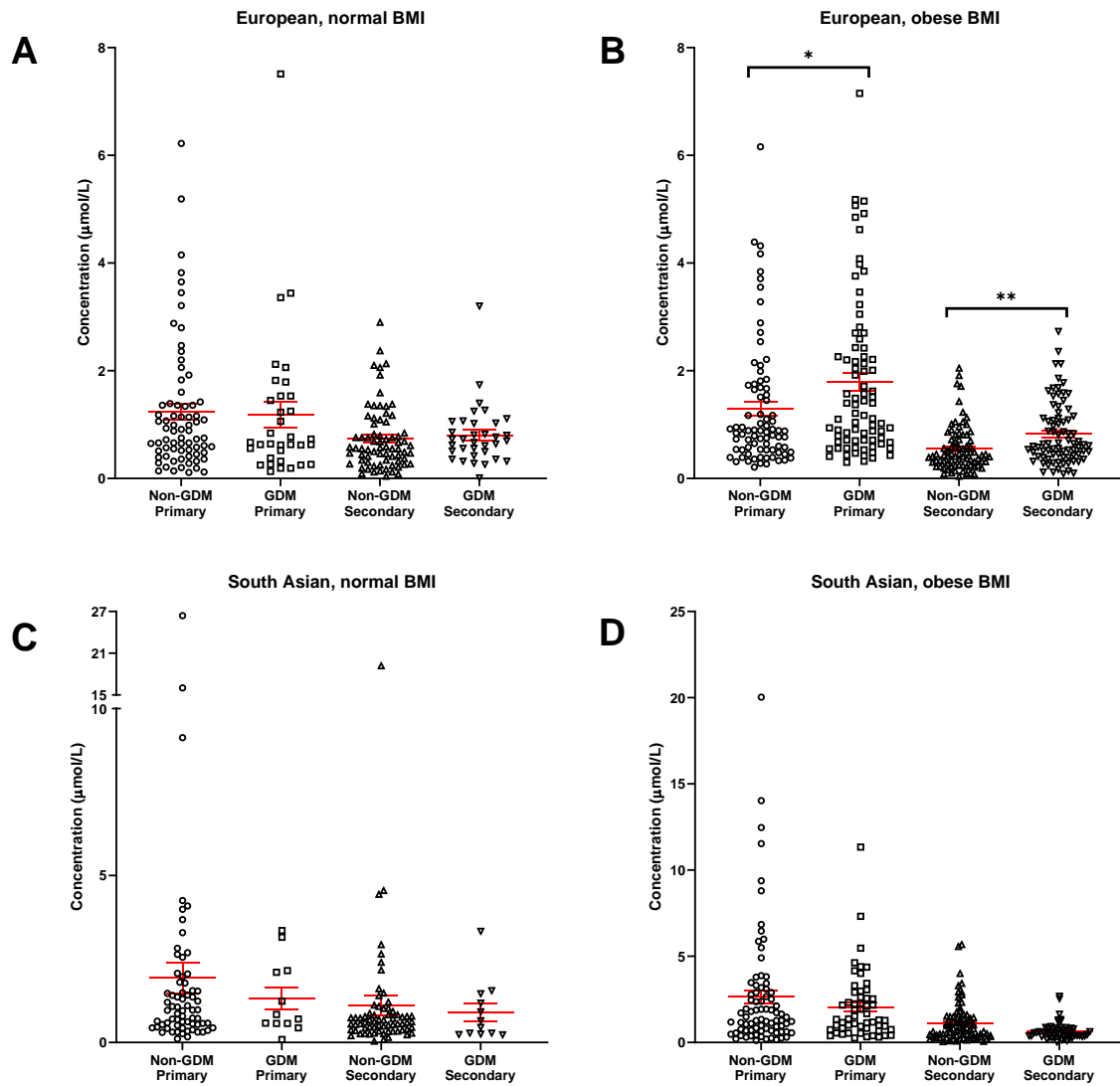
Figure 4.6: Differences in concentrations of primary and secondary bile acids.

The groups split according to BMI and ethnicity were: **(A)** normal BMI Europeans, non-GDM (n=72) or GDM (n=34). **(B)** Obese BMI Europeans, non-GDM (n=80) or GDM (n=80). **(C)** normal BMI South Asians, non-GDM (n=66) or GDM (n=12). **(D)** Obese BMI south Asians non-GDM (n=80) or GDM (n=55). Significant differences are indicated by: * P <0.05, ** P <0.01, as determined by one-way ANOVA followed by Tukey's post hoc test. If data was not normally distributed, Kruskal-Wallis test was used followed by a Dunn's multiple comparisons test. Data expressed as mean ± SEM.
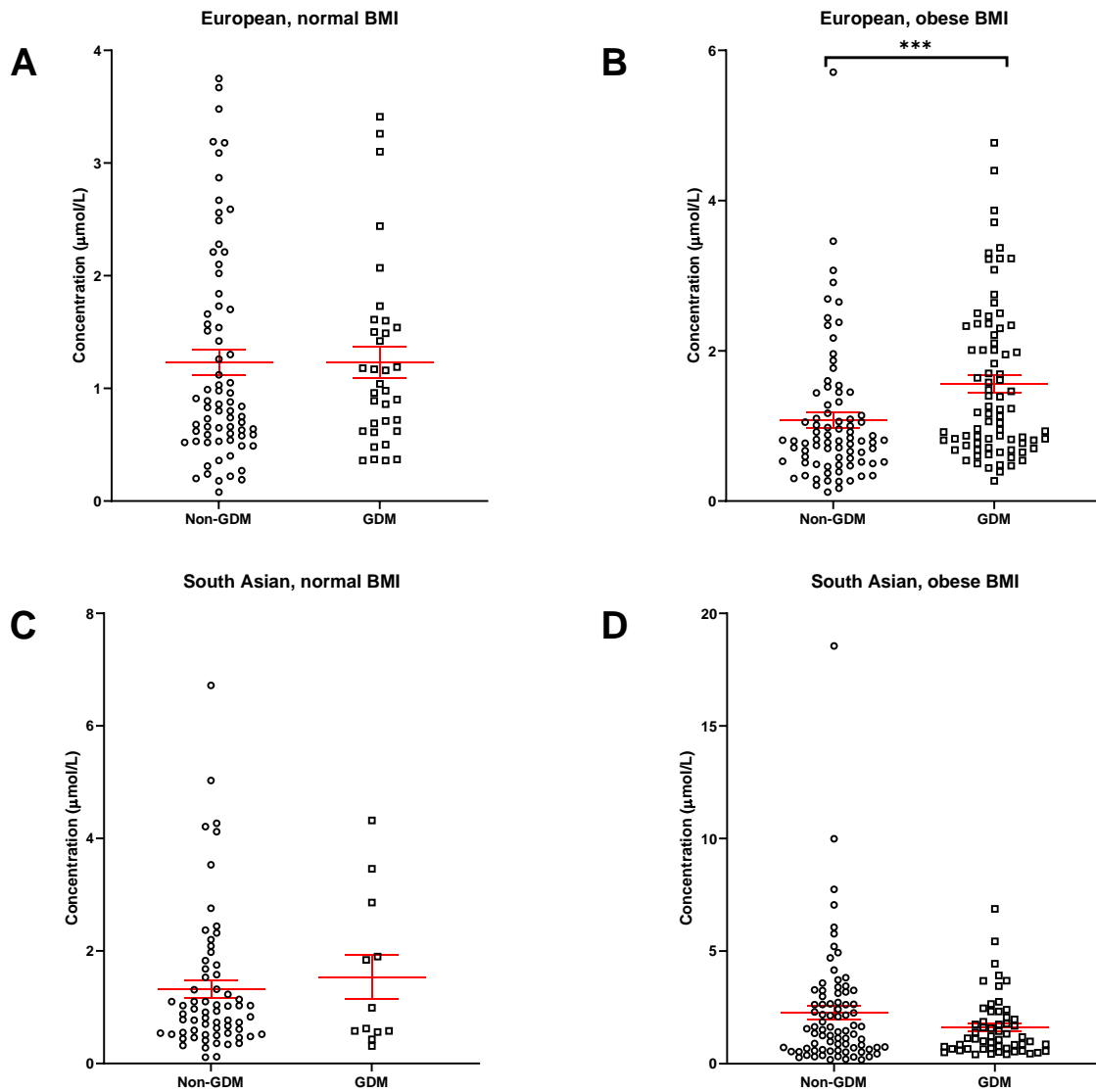
Figure 4.7: Differences in 12α-hydroxylated bile acids.

Total 12α-hydroxylated bile acids were calculated and analysed for differences according to BMI and ethnicity. These groups were: **(A)** normal BMI Europeans, non-GDM (n=72) or GDM (n=34). **(B)** Obese BMI Europeans, non-GDM (n=80) or GDM (n=80). **(C)** normal BMI South Asians, non-GDM (n=66) or GDM (n=12). **(D)** Obese south Asians, non-GDM (n=80) or GDM (n=55). Significant differences are indicated by: *** $P < 0.001$, as determined by T-tests, if data was not normally distributed, Mann-Whitney U test was used. Data expressed as mean ± SEM.

4.3.6. Analysis of serum bile acids in first trimester women

4.3.6.1 No changes in bile acid concentrations in first trimester women who went onto develop GDM

There were no significant differences in individual and total bile acids (i.e. including the conjugated and unconjugated forms) between women who developed GDM and those who went on to have uncomplicated pregnancies. The data for these results have been included in the appendix 2 chapter which also includes data from cohorts 1 and 2. Changes in conjugated and unconjugated bile acids were also examined, along with primary and secondary and if 12α-hydroxylated bile acids differed in this cohort, and no significant differences were observed (Figure 4.8).

4.3.6.2 Examination of differences in bile acids in different BMI of first trimester women

We then investigated whether maternal BMI influences the concentrations of specific bile acids in women with, or without GDM. No changes in bile acid concentrations were observed in non-GDM women between BMI ≤25 kg/m^2 and ≥35 kg/m^2 (Figure 4.9A). Obese women who went onto develop GDM had lower total LCA concentrations (P=0.0053) compared to normal BMI women that subsequently developed GDM decreasing by 1.8-fold (Figure 4.9B), but this bile acid is present at very low concentrations so this change may not be of physiological relevance.

BMI ≤ 25 kg/m² (A, C, E)

BMI ≥ 35 kg/m² (B, D, F)
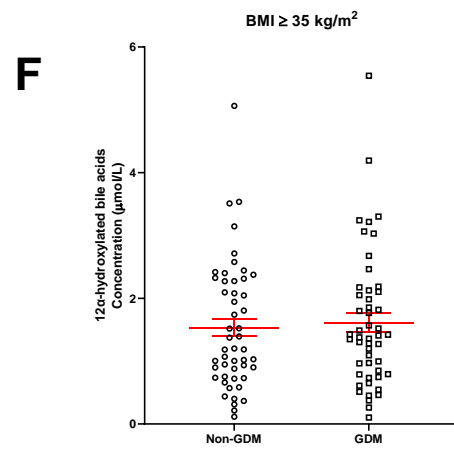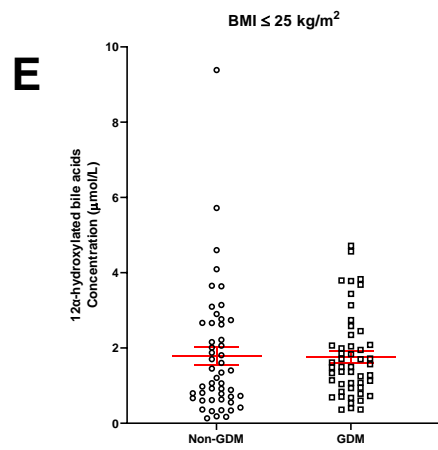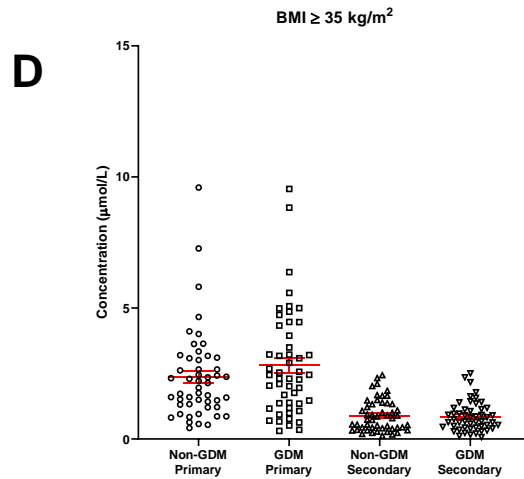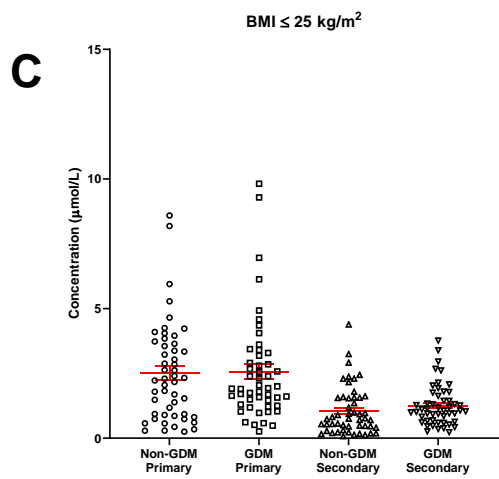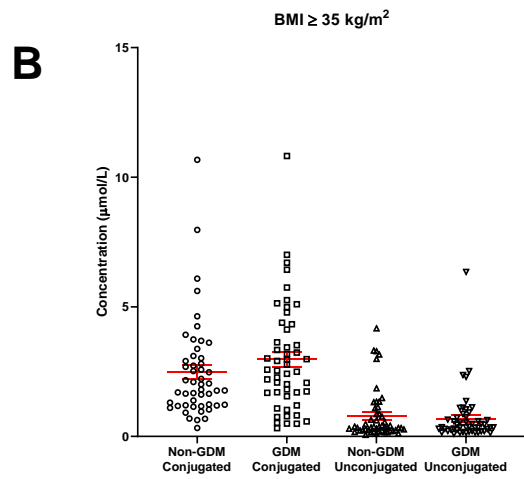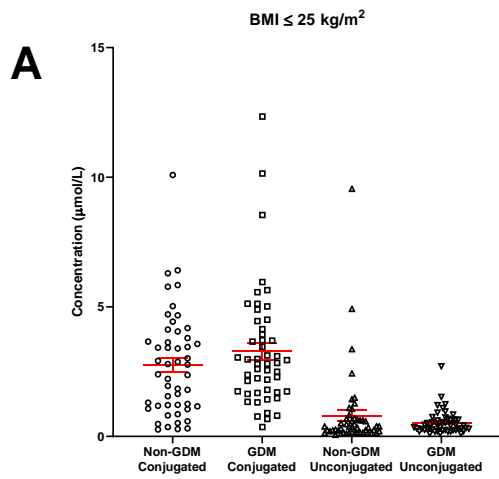
Figure 4.8: Bile acid concentrations in blood samples from women at 11-13 weeks' gestation.

Bile acids were grouped into total conjugated, unconjugated, primary, secondary, and 12α-hydroxylated bile acid concentrations for analysis. These samples originated from cohort 3 which were separated based on BMI and whether they developed GDM or not. Total conjugated and total unconjugated bile acids were examined for differences between non GDM and GDM in BMI ≤25 kg/m^2 **(A)** and ≥35 kg/m^2 **(B)**. Total primary and total secondary bile acids were investigated between non GDM and GDM in BMI ≤25 kg/m^2 **(C)** and ≥35 kg/m^2 **(D)**. The 12α-hydroxylated bile acids, which consist of both CA and DCA, were evaluated for differences in women of BMI ≤25 kg/m^2 **(E)** and ≥35 kg/m^2 **(F)**. n=50 per group. Data expressed as mean ± SEM.
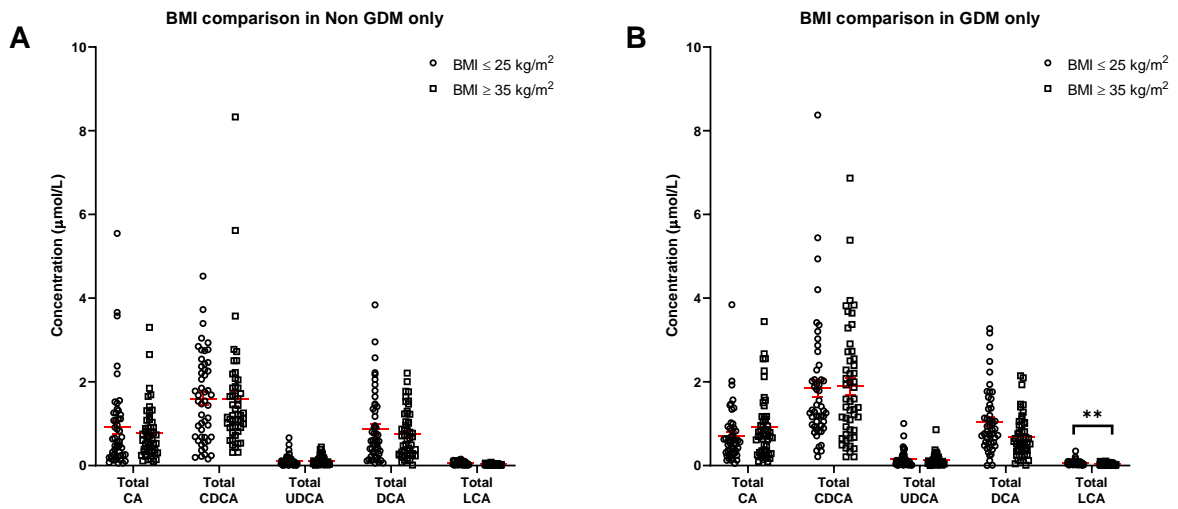
Figure 4.9: Analysing changes in total conjugated and unconjugated forms of each bile acid between different BMI in the first trimester.

Total conjugated and unconjugated bile acids were analysed for changes between BMI. The first trimester samples were separated based on BMI and whether they developed GDM or not. The groups were: women with BMI ≤25 kg/m^2 and women with BMI ≥35 kg/m^2, n=50 per group. **(A)** examined the differences in BMI in women without GDM. **(B)** examined the differences in BMI in women who developed GDM.

4.3.7. Effect of bile acids in islets

WT mouse islets challenged with 20 mM glucose had significantly increased GSIS by 3.7-fold (Figure 4.10A). The bile acids TCA and TCDCA at 50 μM alone were investigated at both high and low glucose concentrations. At 2 mM glucose, TCA and TCDCA caused an increase in GSIS, increasing by 3.7 and 2.5-fold respectively (Figure 4.10A). Similarly, at 20 mM glucose, TCA enhanced GSIS by 1.9-fold (Figure 4.10A). TCDCA also appeared to increase GSIS at high glucose concentrations though this was not statistically significant (Figure 4.10A).

Further evaluation using potent TGR5 and FXR receptor agonists was carried out to see if activating the bile acid receptors individually could cause GSIS. Normal GSIS was observed at 20 mM glucose, increasing GSIS by 2.7 and 2.4-fold (Figure 4.10B, C). INT-777, the TGR5 specific agonist, was used at increasing concentrations of 1, 5, 10 and 25 μM to determine if enhancement of GSIS was concentration dependent. Similarly, GW4064, the FXR specific agonist, was also used at increasing concentration of 0.5, 1 and 50 μM at low and high glucose concentrations. However, neither INT-777 nor GW4064 at any concentration significantly enhanced GSIS at low or high glucose concentrations in WT islets (Figure 4.10B, C).

In human islets, normal GSIS was observed in the presence of 20 mM glucose. Like the murine data, at 2 mM glucose, TCA and TCDCA alone increased GSIS by 2.5 and 1.8-fold respectively (Figure 4.10D). At 20 mM glucose, both TCA and TCDCA also increased GSIS by 1.5 and 1.6-fold respectively (Figure 4.10D).
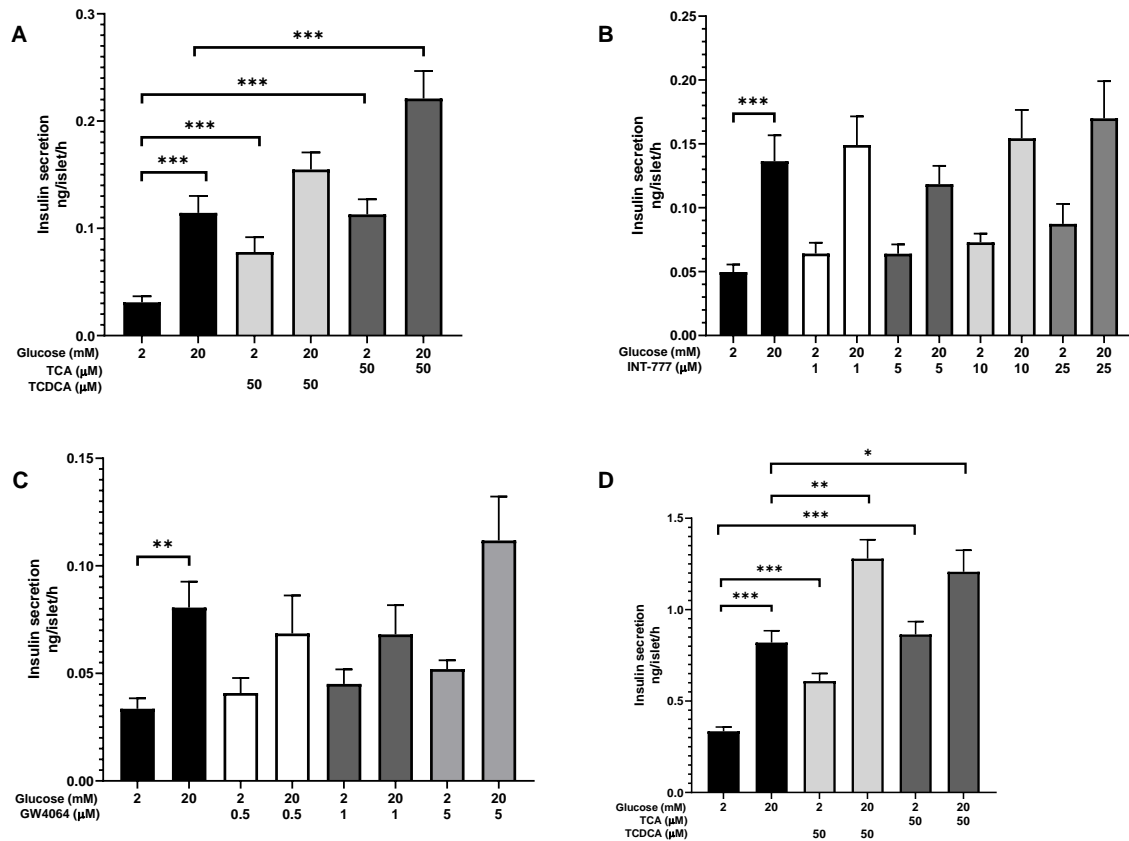
Figure 4.10: Bile acids alter GSIS in murine and human islets.

Islets isolated from mice or humans were isolated and incubated with bile acids at low or high concentrations of glucose. **(A)** Insulin secretion in response to the bile acids TCA and TCDCA at 50 μM were assessed in WT mouse islets. WT mouse islets were then incubated with potent TGR5 and FXR agonists, **(B)** INT-777 and **(C)** GW4064 respectively at increasing concentrations. **(D)** In human islets, TCA and TCDCA, both at 50 μM, were similarly incubated at low and high glucose concentrations. Significant differences are indicated by * P<0.05, ** P<0.01, *** P <0.001 as determined by one-way ANOVA followed by Tukey's multiple comparisons test or Kruskal-Wallis test followed by Dunn's multiple comparisons test if data is not normally distributed. Data expressed as mean ± SEM, each graph n=3-6 independent experiments.

4.3.8. Effect of UDCA in islets

WT mouse islets challenged with 20 mM glucose displayed normal GSIS (Figure 4.11A). The bile acid UDCA and in combination with TCA and TCDCA at 50 µM was investigated at both high and low glucose concentrations. UDCA alone did not cause any changes in GSIS in the presence of low or high glucose concentrations. At 2 mM glucose, TCA or TCDCA, combined with UDCA, increased GSIS, increasing by 5.1 and 3.1-fold respectively (Figure 4.11A). At 20 mM glucose, TCA + UDCA enhanced GSIS by 2.1-fold (Figure 4.11A). TCDCA + UDCA also appeared to increase GSIS at high glucose concentrations though this was not statistically significant (Figure 4.11A).

In human islets, normal GSIS was observed in the presence of 20 mM glucose. As with mouse islets, UDCA alone did not change GSIS. At 2 mM glucose, TCA and TCDCA in combination with UDCA significantly increased GSIS by 2.9 and 2.65-fold respectively (Figure 4.11B). At 20 mM glucose, both TCA and TCDCA in combination with UDCA increased GSIS by 1.7 and 1.8-fold respectively (Figure 4.11B).
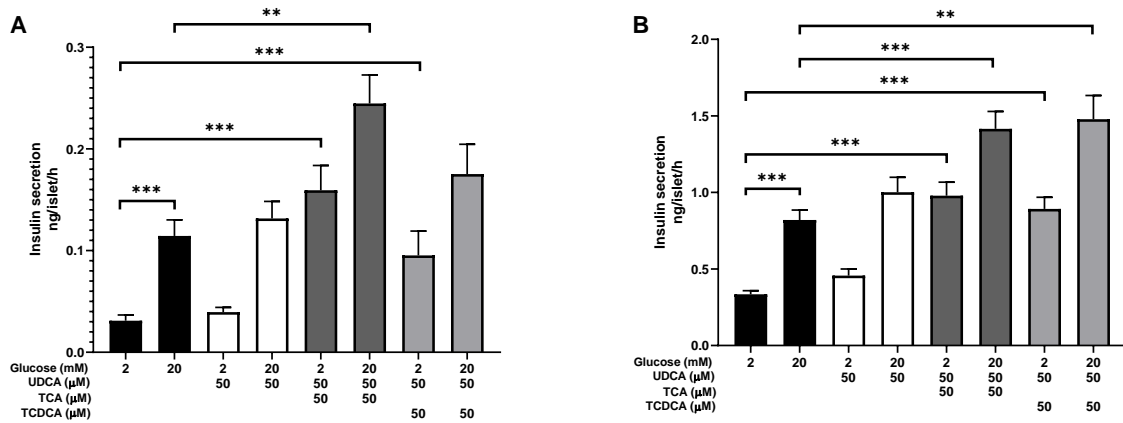
Figure 4.11: UDCA does not alter GSIS in murine or human islets.

Islets isolated from mice or humans were isolated and incubated with bile acids at low or high concentrations of glucose. **(A)** Insulin secretion in response to the bile acid UDCA and in combination with TCA or TCDCA at 50 µM were assessed in WT mouse islets. **(B)** In human islets, UDCA and in combination with TCA or TCDCA, all at 50 µM, were similarly incubated at low and high glucose concentrations. Significant differences are indicated by ** $P<0.01$, *** $P <0.001$ as determined by one-way ANOVA followed by Tukey's multiple comparisons test or Kruskal-Wallis test followed by Dunn's multiple comparisons test if data is not normally distributed. Data expressed as mean ± SEM, each graph n=3-6 independent experiments.

4.4 Discussion

GDM is a growing problem, with current figures ranging from 1 to >30% of pregnancies affected, with one in ten pregnant women by age 30 affected by some form of diabetes (Feig et al., 2014; McIntyre et al., 2019). Improved insights into the aetiology and pathophysiology of GDM may help with earlier diagnosis, thereby enabling earlier intervention and reducing severity of symptoms. Bile acid research in GDM is limited, and existing studies have discrepancies. Correlations between bile acids and insulin, glucose and lipid metabolites in T2DM have been found (Bennion & Grundy, 1977; Brufau, Stellaard, et al., 2010; Cariou et al., 2011; Haeusler et al., 2016; Mantovani et al., 2021; Vincent et al., 2013; Wang et al., 2020; Wewalka et al., 2014; T. Wu et al., 2015; Zhao et al., 2020; Zhu et al., 2020) This indicates that bile acid signalling has a bigger role in glucose and lipid metabolism and diabetes development, than previously thought. Bile acids bind to and interact with the receptors FXR and TGR5, with subsequent alterations in glucose and lipid metabolism. As previously mentioned, $Fxr^{-/-}$ and $Tgr5^{-/-}$ mice have impaired glucose tolerance in pregnancy (Bellafante et al., 2020), implicating these receptors in the development of GDM. The data implicating bile acid signalling in T2DM could also be of relevance to GDM, and it is plausible to propose that bile acids play a role in the pathophysiology of GDM.

We studied bile acid concentrations in a patient cohort that was accurately phenotyped in terms of ethnicity and BMI, as well as having the same GDM diagnostic criteria used in all groups for the study. GDM was diagnosed using the modified NICE 2015 criteria, where a fasting plasma glucose ≥5.6 mmol/l and/or, following an OGTT, 2 hour plasma glucose ≥7.8 mmol/l were diagnosed as GDM. This enabled comparison between women of different ethnicities and BMI. The work in this chapter did not focus on the other third trimester GDM cohorts (described in chapter 2 and 3) as they were of mixed ethnicity and/or BMI. Most of the significant changes in bile acids described were seen in obese women of European ancestry. Total conjugated and unconjugated bile acids CA, CDCA and DCA were increased in GDM women in this group. Significant increases were also observed in total conjugated, unconjugated, primary, secondary and 12α-hydroxylated bile acids in the GDM women of European ancestry. This suggests changes in bile acids in GDM may be specific this sub-group of women. It would be of interest for future studies to compare bile acid species in serum samples from women with and without GDM from a wider range of ethnic groups.

Other studies that examined bile acids in GDM and T2DM focussed on fasting bile acids concentrations, enabling easier comparisons with the data presented in this chapter. Post prandial levels of bile acids will largely differ within individuals and depends on the type of meal ingested, as different fats help stimulate the release of bile acids at varying degrees (Costarelli & Sanders, 2001). Therefore, studying post-prandial bile acids without use of a standardised meal makes comparisons

difficult between existing and future studies. It would be of interest to investigate the impact of a standardised meal on bile acid concentrations in post-prandial samples from pregnant women with and without GDM.

Most studies of bile acids in people with T2DM and GDM used similar quantitative methods to calculate the differing concentrations of bile acids to our study, i.e. liquid chromatography- mass spectrometry, with nearly all reporting bile acids in their unconjugated and conjugated forms (Cariou et al., 2011; Gagnon et al., 2021; Jieying Gao et al., 2016; Mantovani et al., 2021; Vincent et al., 2013; Wewalka et al., 2014; T. Wu et al., 2015; Zhao et al., 2020; B. Zhu et al., 2021). When considering altered concentrations of specific bile acids, it is important to consider the concentration of the bile acid compared to others, as bile acids present in very low concentrations may not be of physiological or pathophysiological. For example, serum concentrations of GHDCA and THDCA were reported to be altered in one GDM study (Gao et al., 2016), but this was only present at very low concentrations, increasing to 0.014 µmol/L and 0.008 µmol/L respectively in GDM patients. Other GDM studies focused on certain groups of bile acids like the 12α-hydroxylated bile acids, which are present in higher concentrations averaging around 0.8-1 µmol/L (B. Zhu et al., 2021). Our studies focused on CA, CDCA and DCA as these bile acids are present in higher circulating concentrations in maternal serum, and therefore changes in these bile acids are more likely to be of relevance to susceptibility to GDM and impact physiology.

As previously mentioned, bile acid levels gradually increase as gestation progresses (McIlvride et al., 2017). A majority of studies examining the relationship between T2DM and bile acids report that bile acids positively correlate with insulin resistance and FPG (Bennion & Grundy, 1977; Cariou et al., 2011; Mantovani et al., 2021; Vincent et al., 2013; Wang et al., 2020; Wewalka et al., 2014; T. Wu et al., 2015; Zhao et al., 2020; Zhu et al., 2020). The inconsistent results of studies report of change in individual bile acid species in women with GDM, with different investigators reporting increased or decreased concentrations of specific bile acids, may be explained in part by differences in ethnicity, and the results reported in this chapter support this. We have shown increased bile acids in European women with GDM, while the South Asian GDM population showed reductions. The increases in bile acids in the third trimester in GDM supports the conclusions found from other studies (Gagnon et al., 2021; Gao et al., 2016; Kong et al., 2020). Though Gao *et al* found increases in bile acids in the third trimester of GDM women, none of the individual bile acids which significantly increased were found to be increased in any of our groups (Gao et al., 2016). The differences in their study may be attributed to having much smaller numbers than our cohorts, differences in BMI and their study consisted of an East Asian population while we studied Europeans and south Asian populations. They also diagnosed their women using the IADPSG criteria, whereas our cohort was

diagnosed using modified NICE criteria, another factor that might explain the different results. The decrease in some of the bile acids in obese South Asians, in cohort 4, replicates some of the findings found by Zhu *et al* 2021, GDCA was found to be reduced in both cases. Zhu *et al* also demonstrated that GDCA negatively correlated with insulin sensitivity (B. Zhu et al., 2021). There were differences in the specific bile acids that were significantly reduced in our cohort compared to their study and these differences may again be due to differences in ethnicity as they had a predominately East Asian cohort diagnosed with GDM using IADPSG. It was interesting that both studies reported bile acids being influenced in different ways by GDM status, but had similar ethnicities, BMIs and assay to detect bile acids, UHPLC-MS/MS, suggesting other factors could be in play to determine how bile acids are changed in GDM. Interestingly increases and decreases of bile acid concentrations have also been observed in T2DM. One study observed increases in DCA, and their conjugated versions, and TCDCA in T2DM patients compared to patients without T2DM. Though the same group also saw decreases in CA and TCA within their study (Mantovani et al., 2021) and therefore the inconsistencies may not be limited to just GDM.

The total concentrations, consisting of their conjugated and unconjugated forms, of each of the bile acids CA, CDCA and DCA did not appear to significantly change in the European GDM group with normal BMI from the third trimester samples. The same bile acids had significant increases in obese Europeans. However, this was not seen in obese South Asians where total concentrations of DCA significantly decreased and the other bile acids had a non-significant trend, reducing in both obese and normal BMI groups. The increased bile acids are consistent with previous reports of alterations in the bile acid pool in T2DM (Zhao et al., 2020), though their ethnicity was not reported, and BMI was mixed between non-diabetic and diabetic groups. The increase demand of insulin during pregnancy could be driving the increase in bile acid concentrations. Particularly as an increased activation of TGR5 cause an increased release of insulin (Bala et al., 2014; Christiansen et al., 2019; Kuhre et al., 2018; Pathak et al., 2018).

The increase in the concentration of primary bile acids in obese Europeans with GDM may be caused by altered hepatic and intestinal FXR signalling, resulting in reduced SHP and FGF19 secretion respectively. This would result in loss of suppression of transcription of CYP7A1, thereby increasing bile acid synthesis (Ahmad & Haeusler, 2019). As already mentioned, ethnic differences in the genetic polymorphisms in CYP enzymes and bile acid transporters such, as NTCP and ASBT, could explain these differences (Dias & Ribeiro, 2011; Ho et al., 2011; Pan et al., 2011). Further work in clarifying the ethnic differences in genetic polymorphisms in bile acid metabolism is needed and could help to guide strategies for early intervention of metabolic diseases in pregnancy.

Looking specifically at conjugated only and unconjugated bile acids, both are also increased in obese European women with GDM. Conjugated bile acids increase through each trimester, reaching their highest in the third (Gagnon et al., 2021; B. Zhu et al., 2019). It is interesting to note that conjugated bile acids are weak agonists towards TGR5 compared with their unconjugated forms (Ibrahim et al., 2018). If women with higher serum conjugated bile acids have reduced secretion of GLP-1, this could explain the changes in glucose and lipid metabolism seen in normal pregnancy. It would be of interest in future studies to measure GLP-1 secretion in pregnant women with GDM alongside serum bile acid concentrations. A change in the gut microbiota could be responsible for the increased conjugation. As previously mentioned, the gut microbiome alters during pregnancy (Collado et al., 2008; Koren et al., 2012; Ovadia, Perdones-Montero, et al., 2019), and it is plausible that gut microbiota could differ in a subgroup of women with GDM, with fewer microbes expressing bile salt hydrolase, resulting in less de-conjugation of bile acids. This could explain the increase of conjugated bile acids in obese European women and possibly reduce GLP-1 secretion. However, in this study we also observed an increase in unconjugated bile acids in the obese European women with GDM, and this is not consistent with this hypothesis. To consider the potential relevance of increased unconjugated bile acids, some of these bile acid species, such as DCA, enhance GLP-1 secretion in the distal gut, thereby increasing insulin secretion. This could be a response to the increased insulin resistance associated with GDM. More research is needed using *in vitro* and *in vivo* studies to establish the clinical relevance of the observed changes in conjugated and unconjugated bile acids.

We found that 12α-hydroxylated bile acids were increased in serum from obese European women with GDM. Serum concentrations of 12α-hydroxylated bile acids, which consist of both CA and DCA and their conjugated forms, are of interest in women with GDM as higher levels are associated with insulin resistance and T2DM (Brufau, Stellaard, et al., 2010; Haeusler et al., 2016). Increased levels of 12α-hydroxylated bile acids are also observed in mice lacking hepatic insulin receptors and in rodent models of diabetes (Biddinger et al., 2008; Li et al., 2012; Uchida et al., 1996). One study similarly found increases in 12α-hydroxylated bile acids in obese patients with T2DM compared to obese patients with normal glucose tolerance (Ferrannini et al., 2015), indicating a relationship between these bile acids and glucose homeostasis. As there is a positive correlation between 12α-hydroxylated bile acids and insulin secretion (Yoshitsugu et al., 2020), these bile acids may be increased as a mechanism to compensate for the increased insulin resistance in a diabetic state. The increase of 12α-hydroxylated bile acids in GDM could also be a cause of impaired glucose tolerance, and they may increase as consequence of raised reproductive hormones. Elevated levels of glucose and hyperglycaemia have been shown to increase CYP7A1 expression (Li et al., 2012), and this may be an explanation for the increase in CA and also the overall elevation of primary bile acids seen in

the obese GDM European group. Evaluating the total 12α-hydroxylated bile acids using timed serum samples taken throughout gestation may provide an insight into how bile acid and glucose metabolism change during pregnancy. Our data support the conclusions of previous studies, but only in the subgroup of obese European women with GDM that have a significant increase in 12α-hydroxylated bile acids. The pattern of serum 12α-hydroxylated bile acids in South Asians in either BMI category does not change significantly, and there is a significant reduction in DCA. This is consistent with another study with a predominant East Asian cohort where they also found decreased 12α-hydroxylated bile acids and no changes in the non-12α-hydroxylated bile acids in GDM women (B. Zhu et al., 2021). The increase in 12α-hydroxylated bile acids may therefore be specific to ethnicity and BMI, though further studies would be needed to confirm this. The CYP8B1 enzyme, responsible for the synthesis of CA, could also be key to the increased concentration of 12α-hydroxylated bile acids. Regulation of CYP8B1 expression is primarily through FXR and TGR5, both of which inhibit CYP8B1 expression when activated (Zaborska & Cummings, 2018). Thus, meaning that reduced activity of either receptor in GDM could cause the CYP8B1 to stay active longer and increase synthesis of CA, which could be a plausible explanation for the increase in concentration of these bile acids. These findings may also translate into helping explain the pathophysiology of GDM and could give further clues into how specifically modulating 12α-hydroxylated bile acids could influence glucose homeostasis in a specific population of women.

The differences in bile acid concentrations in women of different ethnicity were notable. Indeed, serum bile acid profiles have been noted to differ between ethnicities in non-pregnant humans (Luo et al., 2018), with Asians having higher concentrations in CA and CDCA, and no changes to DCA compared with Caucasians. These differences from our data could be due to the fact this study looked at non-pregnant individuals, had significantly smaller numbers of Asians compared to Caucasians, and had no separation of different type of Asian, grouping the continent together in one group to compare with Caucasians. The results of their study however did corroborate with our obese non-GDM data, which showed South Asians having higher concentrations of CA, CDCA and DCA compared to Europeans. South Asians are generally more at risk of diabetes with higher incidence seen in both T2DM and GDM when compared to Caucasians (Gujral, Pradeepa, Weber, Narayan, & Mohan, 2013; Sanchalika & Teresa, 2015; Yuen, 2015). The incidence of GDM differs between ethnicities across the world and these variation in bile acids may help explain these differences.

Total conjugated and unconjugated bile acids were compared in BMI matched GDM Europeans, versus South Asians. The GDM South Asians in the obese BMI group had significantly lower DCA concentrations, compared to Europeans. Ethnic differences between the CYP enzymes, CYP7A1 and

CYP27A1, that synthesise the primary bile acids has been found between Europeans, East Africans and Latinos (Dias & Ribeiro, 2011), so it is plausible that there are differences between Europeans and South Asians. There are also ethnic differences in the diversity of gut microbiota (Brooks, Priya, Blekhman, & Bordenstein, 2018), and this may influence microbial bile acid transformation in the gut, resulting in lower concentrations of secondary bile acids in the South Asian population and maybe increased in European. Studies of faecal bile acids in women with GDM of different ethnic origin would be needed to clarify and support this suggestion. If this hypothesis is correct, there would be less TGR5 activation by the more potent secondary bile acids in South Asians. This would result in a reduction in GLP-1 secretion, and also less insulin secretion (Christiansen et al., 2019; Kuhre et al., 2018; Pathak et al., 2018), leading to increased susceptibility to GDM. This may provide one explanation for the higher prevalence of GDM in south Asians compared to Europeans (Hedderson et al., 2012).

Studies looking at the relationship between BMI and serum bile acid concentrations are scarce. Most studies show total bile acids positively correlate with BMI in non-diabetic patients (Prinz et al., 2015; Vincent et al., 2013; Xie et al., 2015). In our studies, bile acid changes were observed in pregnant women with different BMI, and when compared in samples from women in the first trimester, those in the obese BMI group who went on to develop GDM had significantly lower concentrations of LCA. As mentioned previously, it should be noted that LCA is present at relatively low concentrations, so the physiological significance of this change is not clear. However, a lack of secondary bile acids in the obese group may contribute to less insulin secretion due to reduced activation of intestinal TGR5 by secondary bile acids, and therefore less GLP-1 secretion.

The primary bile acids CA and CDCA, the most potent ligand for FXR, were both significantly lower in third trimester women with normal BMI, compared to the obese women of European origin. This may result in reduced FXR activity, affecting genes which regulate glucose and lipid metabolism (Herman et al., 2012; Iizuka et al., 2004; Noordeen et al., 2010). Other studies have reported similar results, with CDCA showing a positive correlations with BMI (Cariou et al., 2011), though this study did not report ethnic origin of the participants. Another study of a population primarily consisting of healthy East Asians, reported positive correlations between the primary bile acids, particularly the glycine-conjugated and unconjugated versions, and BMI (Xie et al., 2015). These differences in bile acids between people of normal and obese BMI indicate a need for future studies to match study groups based on BMI, as bile acids appear to be influenced by this parameter. Different approaches to the treatment of GDM used for different BMI groups may be necessary for future therapeutics.

The first trimester samples were split according to BMI ≤25 kg/m^2 and ≥35 kg/m^2, similar to the third trimester samples, and whether the women subsequently developed GDM in their third trimester. This group was also of mixed ethnicity, though the BMI ≤25 kg/m^2 group was primarily of European origin, whilst the BMI ≥35 kg/m^2 group included similar numbers of women of European and black ethnic origin. Bile acid changes in the first trimester may also give an insight into potential biomarkers or metabolic changes which may occur before onset of GDM, giving insight to the progression in pathophysiology and how the bile acids may change through each trimester. We observed no significant changes, suggesting that changes in bile acid changes associated with GDM are likely to develop at a later stage of pregnancy. However, it would be valuable to obtain larger numbers of first trimester samples from pregnant women to enable a focussed study of bile acid concentrations in women of different ethnic origins.

In the first trimester samples from normal BMI women who went onto develop GDM, there was an increase in TLCA, though this bile acid is at very low concentrations and may not be of physiological relevance compared to the other bile acids. The rest of the cohort did not show any significant differences between women who went onto develop GDM or had uncomplicated pregnancies in individual bile acids or when bile acids were grouped into total conjugated and unconjugated forms. This contrasts to other studies that found increases (Hou et al., 2018, 2016) and decreases (Li et al., 2018) in first trimester bile acids. However, many of the reported changes involve minor bile acids found in small concentrations, and therefore may not be of relevance to altered glucose metabolism through bile acid receptor signalling. Differences in ethnicity and criteria used to diagnose GDM may also explain the differences between these published studies (Hou et al., 2016, 2018; Li et al., 2018) and the results from our first trimester samples. Few studies have examined bile acids in the first trimester of women who went onto develop GDM and some of the differences were minor. This indicates that bile acid related metabolic changes, which could potentially contribute to the pathophysiology of GDM, may occur more prominently in the second/third trimesters of pregnancy where changes in bile acid concentrations are much greater.

We next wanted to evaluate how islets are influenced by bile acids. Increased fasting total serum bile acids are associated with impaired islet β-cell function in response to glucose challenge in T2DM (Wang et al., 2020). Both FXR and TGR5 are expressed in murine and human pancreatic islets (Düfer, Hörth, Krippeit-Drews, & Drews, 2012; Kumar et al., 2016) and studies have shown bile acid interaction with these receptors in islets increases GSIS in both *in vitro* and *in vivo* models (Düfer, Hörth, Wagner, et al., 2012; Hoffmeister, Kaiser, Lüdtke, Drews, & Düfer, 2020; Kumar et al., 2012; Schittenhelm et al., 2015; Vettorazzi et al., 2016). Our results show that TCA and TCDCA increase GSIS in both human and mouse islets. Interestingly, both also increased insulin secretion in low

glucose conditions, an effect observed in other studies (Schittenhelm et al., 2015), suggesting bile acids may cause insulin secretion through non-glucose mediated effects in the islets. The other studies demonstrated TCDCA causing increases in GSIS at concentrations as low as 500 nM (Düfer, Hörth, Wagner, et al., 2012; Schittenhelm et al., 2015). Insulin secretion in low glucose conditions is also considered unusual as islets typically would not release high concentrations of insulin in the human body if blood glucose levels are low. We aimed to use the TGR5 and FXR specific agonists, INT-777 and GW4064 respectively, to investigate whether signalling via either receptor was responsible for the TCA and TCDCA effects we observed on insulin secretion. Both of these agonists have shown to increase insulin release in mouse and human islets in other studies (Düfer, Hörth, Wagner, et al., 2012; Kumar et al., 2012; Schittenhelm et al., 2015; Vettorazzi et al., 2016). However, we did not observe an effect with either agonist. Kumar *et* al used 25 µM INT-777 in human islets, incubating for 30 minutes, and saw increased GSIS at both 3 and 25 mmol/L (Kumar et al., 2012), suggesting INT-777 could increase GSIS in human islets. However, another study also used 25 µM INT-777 in mouse islets and observed increased GSIS in 11.1 mmol/L glucose, though they had an n=10-12 (Vettorazzi et al., 2016), which suggests the number of experiments we conducted could be too low to observe an effect. Düfer *et al* observed an increase in GSIS with 0.5 and 5 µM GW4064 in 10 mmol/L glucose, incubating the agonist for 60 minutes, similar to ours. However, they had an n=10, differing to our n=3 (Düfer, Hörth, Wagner, et al., 2012). Schittenhelm *et al* similarly saw increased GSIS with 0.5 µM GW4064 in 15 mmol/L glucose, incubating for 60 minutes, though this depended on diet, with islets originating from lean mice displaying increased GSIS and those with a high fat diet having no change with GW4064 (Schittenhelm et al., 2015). This suggests diet should be taken into consideration as an explanation for the differences seen in our experiment and those studies. Our studies could perhaps incubate the islets with FXR agonists longer to observe GSIS. FXR is a nuclear receptor and would need longer incubation times to distinguish any significant effect due to the time it would take for agonists to enter cells, interact with the receptor, and produce a change in insulin secretion via altered gene expression.

We also assessed the impact of UDCA on insulin secretion in islets, alone and in combination with TCA or TCDCA. UDCA alone did not cause an increase in GSIS in the presence of low or high glucose concentrations. In combination with either TCA or TCDCA, UDCA did not impact GSIS in either mouse or human islets. Interestingly others found that TUDCA dose-dependently increased GSIS in islets from male mice (Vettorazzi et al., 2016). Others found similar results to ours, using UDCA at 10 µM found no change in GSIS (Düfer, Hörth, Wagner, et al., 2012; Schittenhelm et al., 2015). However, 48 hour preincubation with UDCA was found to increase GSIS (Schittenhelm et al., 2015).

The increased GSIS we have demonstrated in both human and mouse islets at low glucose concentration could be due to the interaction of bile acids with the potassium channel in islet β-cells. Düfer *et al.* demonstrated that TCDCA stimulated insulin secretion in mouse islets through the stimulation of FXR, which in turn increases intracellular calcium concentrations to cause insulin release. They also demonstrate that TCDCA also leads to the closure of K_{ATP} channel and inhibits the potassium channel which coincides with calcium influx to induce membrane depolarisation (Düfer, Hörth, Wagner, et al., 2012). It is possible that TCA could also be interacting with the potassium channel the same way as it has been demonstrated that other bile acids act on K_{ATP} channels (Jun et al., 2005; Lavoie et al., 2010; Miragoli et al., 2011). However, Vettorazzi *et al.* used TUDCA in mouse islets to show that the increased insulin secretion was not mediated by K_{ATP} channel or change in calcium signals, but through the TGR5 receptor through the cAMP/PKA pathway (Vettorazzi et al., 2016). Further studies are required to clarify these inconsistencies, though both TGR5 and FXR could be involved in the enhancement of GSIS. TGR5 could provide a short-term boost of insulin release, whilst FXR, as shown by Düfer *et al.*, could enhance the calcium influx and inhibit potassium channels in the long term.

Other cells in the islets could also enhance insulin release. For example, when the TGR5 receptors are activated by INT-777 in α-cells, there is increased GLP-1 release (Kumar et al., 2016). However, upon activation of TGR5 receptors in islets, using INT-777, we would also expect to see an increase in insulin secretion through the interaction of GLP-1 to β-cells, in which they would be released from α-cells, which we did not observe in our experiments. Bile acids may also have other beneficial effects to the islets that do not directly stimulate the release of insulin, including reduced apoptosis of islets and β-cells, and increased islet mass and β-cell proliferation (Bronczek et al., 2019; Kumar et al., 2016; Lee et al., 2010; Lukivskaya et al., 2004; Zhu et al., 2013). Thus, increased serum bile acids in GDM could provide beneficial effects in not only enhancing insulin release, but also protecting the islets from cytotoxicity or stress. Further studies looking at bile acids in islets from animal models of GDM are needed as current studies remain lacking.

Limitations of this study include the availability of fewer south Asian GDM samples than European samples, thereby reducing the power of the study. One of the key issues with studies examining cohorts of women is the consistency of diagnostic criteria. Many of the studies discussed had differing diagnostic criteria for GDM. Future studies should aim to use consistent diagnostic criteria, although this is difficult due to the differing guidance from hospital trusts, countries, and senior clinicians. Ethnicity and BMI are key variables in dictating how bile acids are changed during pregnancy and GDM, and it is therefore important for future studies to consider the population to study and clearly state the ethnicities involved in the study. Controls should be matched to GDM

with the same BMI groups, to allow easier comparison and form stronger conclusions. Combining bile acids into groups such as total primary, secondary, 12α-hydroxylated, or conjugated or unconjugated forms may give further useful information and clues on the overall changes in bile acid metabolism in GDM, ethnicity and BMI. Though we did not observe any individual bile acid changes in the first trimester serum samples from women who subsequently developed GDM, compared to those who did not, others have reported alterations in bile acid concentrations (Hou et al., 2016, 2018; Li et al., 2018) in early pregnancy samples. Therefore, future bile acid studies using first trimester serum of women, differentiated by both BMI and ethnicity, who went onto develop GDM, compared to those who did not, could clarify the inconsistencies discussed. Future studies into other ethnicities should be performed. Particularly in Afro-Caribbean, Latin American and Southeast Asian populations where no bile acid GDM studies have been conducted to date. It would be of interest to investigate bile acid concentrations in women with GDM compared to controls in these populations, and to further detail the pathophysiology of GDM and possible biomarkers. Examining the faeces of the GDM women would also clarify differences in the microbiome and faecal bile acid concentrations in women from varying ethnic origins, and may further explain the bile acid changes observed in our studies. Whilst we observed many significant differences in serum bile acid concentrations between non-GDM and GDM patients, some of the differences were <1μmol/L. Such differences may have questionable impact on FXR activation, and further mechanistic studies are required to determine if this would have any change to GDM physiology. Unlike FXR, the EC_{50} for LCA and DCA for TGR5 activation is much lower, being 0.5 μM and 1 μM respectively (for CDCA and CA, EC_{50} - 4.4μM and 7.7μM respectively), as mentioned in Chapter 1. Therefore, such small changes could influence TGR5 receptor activation in GDM women.

One of the limitations of the islet studies was a short incubation time, as this was too short to allow alterations in gene expression to be evaluated. Future studies should carry out longer incubation/preincubation periods with bile acids to observe differences in insulin release and also time-based studies to evaluate the changes of insulin over time. Additional islet experiments to assess insulin release may also be more beneficial and future studies using perifusion experiments could generate more meaningful data, particularly with the human islets. Following the insulin secretion experiments, it may also be useful to measure apoptotic markers and evaluate the toxicity of the bile acids to the islets. Histology staining would enable observation of morphological changes caused by bile acids in the islets, as observed by other studies (Bellafante et al., 2020; Bronczek et al., 2019; Kumar et al., 2016; Lee et al., 2010; Lukivskaya et al., 2004; Zhu et al., 2013). Using cell lines with combinations of bile acids or inhibitors that block cell signalling pathways in TGR5 or FXR may indicate how the bile acids act on islets to release insulin. For example, using PKA inhibitors or

K_{ATP} channel blockers would indicate the involvement of TGR5 receptors in insulin release. Other hormones like glucagon, somatostatin and amylin could also be evaluated to see if bile acids have other metabolic effects on islets.

In conclusion, this study shows that bile acid concentrations differ in women with GDM compared to women who have uncomplicated pregnancies. These differences are also dictated by ethnicity and BMI. For example, obese GDM European women have increased primary and secondary bile acids, whilst obese GDM South Asians had reduced DCA levels. Further studies are required to confirm if any changes occur in the first trimester and if these changes could be considered early biomarkers in detecting GDM before onset of symptoms. We also confirm that TCA and TCDCA can induce increased GSIS, though the mechanism of action remains inconclusive. The bile acid receptors, TGR5 and FXR, when activated, have been known to influence lipid and glucose metabolism as previously mentioned in chapter 1. Reduced activity of the bile acid receptors, possibly due to partial repression secondary to elevated concentrations of oestrogen, progesterone, or their metabolites, could be part of the aetiology of GDM. Our data support bile acids having a role in the development of GDM. Further work is required to elucidate the mechanisms involved and how we can use bile acids to influence glucose homeostasis for therapeutic uses.

Chapter 5: Investigation of bile acids and progesterone sulphates in the gut

5.1 Introduction

Gestational diabetes mellitus (GDM) has a complex aetiology and pathophysiology. The rise in pregnancy hormones affects multiple organs, including the gut, and this in turn may contribute to the aetiology of GDM. Alterations in the gut during pregnancy include changes in the gut microbiome, bile acid metabolism and ileal FXR activity (Koren et al., 2012; Ovadia, Perdones-Montero, et al., 2019). As explained in the previous chapters, one of the major bile acid receptors, TGR5, is highly expressed in the gut, particularly in the distal ileum and colon (Roberts et al., 2019). Upon TGR5 activation by bile acids, GLP-1 release is stimulated, and this can alter glucose metabolism (Christiansen et al., 2019; Kuhre et al., 2018; Pathak et al., 2018). The most potent TGR5 ligands are secondary bile acids (LCA>DCA>CDCA>CA) (Kawamata et al., 2003). GLP-1 is key not only to enhancement of insulin secretion, but also for pancreatic β-cell adaptations by mediating β-cell mass expansion, as seen in mice (Moffett et al., 2014). Also, important to note is GLP-1 is found in two forms, its active and inactive form. Initially released GLP-1 (1-37) is cleaved to its active form, GLP-1 (7-36). This is then further cleaved by dipeptidyl peptidase-4 into an inactive form, GLP-1 (9-36) This results in the inactivation of GLP-1 and the hormone having a very low affinity for the GLP-1 receptor, rendering it incapable of stimulating insulin secretion (Knudsen & Pridal, 1996; Sandoval & D'Alessio, 2015). A reduction in GLP-1 secretion is seen during pregnancy (Mosavat et al., 2020; Sukumar et al., 2018), a plausible explanation for islets failing to adapt during some pregnancies, leading to the diagnosis of GDM. Enhancing GLP-1 secretion could be key to alleviating the symptoms of GDM.

During GDM, it is possible that TGR5 signalling is altered through changes in bile acid metabolism. Bacterial transformation of primary bile acids to secondary bile acids is key to the activation of TGR5 and to release of GLP-1. Changes in the composition of the gut microbiome during pregnancy, such as the *Firmicutes/Bacteroidetes* ratio positively correlating with gestational age (Collado et al., 2008; Koren et al., 2012), could alter this transformation, thereby reducing GLP-1 secretion. It should be noted that the changes in the gut microbiome in the third trimester have some resemblances to the gut microbiome associated with obesity (Koren et al., 2012). This dysbiosis is likely to contribute to some of the metabolic alterations seen in normal pregnancy, promoting energy fat storage and

greater insulin resistance, and these alterations have the potential to increase the risk of developing diabetes. Targeting TGR5 signalling could therefore improve GDM outcomes.

Bile acid composition and concentration changes during pregnancy and in gestational disease states. As gestation progresses bile acids gradually increase and CA and CDCA concentrations are reported to change as described in Chapter 1 (1.2.4) (McIlvride et al., 2017). The increase in bile acids is further amplified in ICP with the CA/CDCA ratio increasing (Brites et al., 1998; Heikkinen, 1983). Alterations of serum bile acids in GDM are discussed in the previous chapter and further studies are needed to establish the underlying aetiology of the reported alterations. Understanding bile acid metabolism during normal and GDM pregnancy would enable better understanding of how TGR5 signalling is altered. Progesterone sulphates have also been shown to bind to TGR5, with PM3S being shown to activate the receptor (Abu-Hayyeh et al., 2016). Given the nature of the structural similarities between the different progesterone sulphates, it is plausible that other progesterone sulphates could also activate TGR5 in the gut to release GLP-1. Normally, progesterone sulphates are increased 80-fold in pregnancy (Abu-Hayyeh et al., 2010). However, as reported in chapter 3, some progesterone sulphates, PM5S and PMΔ5S, are reduced in pregnancies diagnosed with GDM. A significant reduction in progesterone sulphate concentrations during pregnancy could therefore reduce activation of TGR5, which contributes to the pathophysiology of GDM.

We hypothesise that bile acids and progesterone sulphates alter enteroendocrine signalling and GLP-1 secretion through TGR5 signalling in the gut. Two models will be used to test this: isolated and cultured murine crypts and human colonoids cultured and differentiated from patient biopsies.

The aims of this chapter are to:

- Assess GLP-1 secretion in cultured WT and Tgr5$^{-/-}$ murine crypts using bile acids and progesterone sulphates.
- Further investigate GLP-1 secretion in human colonoids using bile acids and progesterone sulphates.

5.2 Materials and Methods

5.2.1. Isolation and cell culture of murine crypts

Colons from WT and Tgr5$^{-/-}$ mice at 12 weeks of age were dissected out and cleaned with PBS to remove faecal matter. The colons were then cut into 1 mm^2 squares before collating together and washed again, with PBS, until clean. The colon pieces were then digested with collagenase (C9407) for 15 minutes in 37°C. The supernatant was then collected and centrifuged at 300 g for 5 minutes, then supernatant discarded and DMEM (D6546, 10% FCS, 1% penicillin/streptomycin) added. Fresh collagenase was added into the remaining chopped colon and this process was repeated 3 more times, until no colon remained to be digested. All 4 digested colon mixtures were spun again at 300 g for 5 minutes, the supernatant discarded and the pellets collated together and resuspended in DMEM. The suspension was then filtered through a net curtain (containing 250 µm holes) and the mixture plated into pre-prepared coated wells containing Matrigel basement membrane matrix (Corning, USA; 356234). The crypt cultures were incubated for at least 24 hours at 37°C, 5% CO_2 before use.

5.2.2. Cell culture and differentiation of human colonoids

Human colonic crypts were isolated from serial colonic biopsies (x2 ascending, x2 transverse, x2 descending, x2 rectosigmoid colon) taken from a 50-year-old male donor without past medical history or regular medication who attended for routine colonoscopy. Informed consent was given and approved by the ethics committee of King's College Hospital (REC number: 15/LO/1998).

The differentiation and maintenance of the human colonoids were carried out by Dr Patricia Fronseca Pedro and Dr Anastasia Tsakmaki. Human colonoids were seeded into matrigel and kept in WENRAS medium for 3 days, size and weight of colonoids were dependent on plate size and volume. Following this, the medium was changed to differentiation medium for 9 days with the medium being changed every 2/3 days. Two days into the differentiation medium, 10 µM of DAPT, the notch inhibitor, was added for 48 hours and removed. Samples were collected after day 9 for secretion experiments. Table 5-1 shows the components of WENRAS and differentiation medium.

Table 5-1: Components of WENRAS and differentiation medium.

Component	Volume (final concentration)	
	WENRAS medium	**Differentiation medium**
Advanced DMEM/F12 with GlutaMAX, HEPES and Penicillin/streptomycin	3.622 ml	7.135 ml
50% Wnt3a conditioned medium	5 ml	1.5 ml
10% R-spondin-1 conditioned medium	1 ml	1 ml
Noggin	10 µl (100 ng/ml)	10 µl (100 ng/ml)
B27 supplement	200 µl	200 µl
N2 supplement	100 µl	100 µl
N-Acetylcysteine	25 µl (1.25 mM)	25 µl (1.25 µM)
EGF	10 µl (50 ng/ml)	10 µl (50 ng/ml)
Gastrin	10 µl (10 nM)	10 µl (10 nM)
Nicotinamide	10 µl (10 mM)	-
A83-01	10 µl (500 nM)	10 µl (500 nM)
SB202190	3.35 µl (10 µM)	-
Total	**10 ml**	**10 ml**

50% Wnt3a conditioned medium and 10% R-spondin-1 conditioned medium were made in house as previously described (Fujii et al., 2015).

5.2.3. Murine crypt GLP-1 secretion experiments

Crypt cultures were incubated overnight, and media removed. Crypts were washed twice with secretion buffer (Table 5-2) before treatments were randomly added into the wells of the plate. Each experimental replicate would have 8 wells per treatment. Reagents used were DCA, LCA, TDCA, TLCA, UDCA, PM3S and PM5S alone and in combination. Bile acids had a final concentration of 100 µM and progesterone sulphates a final concentration of 50 µM. Forskolin/IBMX (F/I) at 10 µM was used as a positive control. Crypts were incubated with reagents for 2 hours at 37°C, 5% CO_2. Supernatant was collected and centrifuged at 100 g for 3 minutes and supernatant collected for GLP-

1 analysis before remaining material is discarded. 250 µL of Lysis buffer (Table 5-3) was added to each well. Cells were then scraped and collected. Wells were further washed with 250 µL secretion buffer and collected into the lysed cells. Supernatant containing the lysed cells were analysed for GLP-1.

5.2.4. Human colonoid GLP-1 secretion experiments

Once the differentiation of colonoids was complete, matrigel cell recovery solution was added to each well on the plate and incubated for 1 hour on ice. Colonoid samples were then collected and washed three times in cold PBS. After, colonoids were centrifuged at 300 g for 5 minutes at 4°C. Supernatant was discarded and organoids were incubated for 2 hours in secretion buffer (Table 5-2) at 37°C. After, colonoids were centrifuged at 300 g for 5 minutes at room temperature. Supernatant was removed, reagents added into the colonoids and incubated for 2 hours at 37°C, 5% CO_2. Each experimental replicate had 8 colonoid sample per treatment. Reagents tested on colonoids were TDCA, UDCA, PM3S and PM5S. 10 µM F/I was used as the positive control. After incubation, samples were centrifuged at 200 g for 3 minutes and supernatants collected for GLP-1 analysis. The organoids were then mixed in with a mixture of 1x PBS containing 1 tablet of complete EDTA-free protease inhibitor cocktail. Organoids were then sonicated for 30 seconds (amplitude 10-14) on ice. Homogenates were centrifuged at 5000 g for 5 minutes, supernatants removed and used for GLP-1 analysis.

Table 5-2: Components of secretion buffer.

Salt	Final concentration mM	Amount added to 1L water g
KCl	4.5	0.335
NaCl	138	8.065
$NaHCO_3$	4.2	0.353
$NaH_2PO_4 \bullet 2H_2O$	1.2	0.187
$CaCl_2$	2.6	2.6 ml of 1M stock
$MgCl_2$	1.2	1.2 ml of 1M stock
HEPES	10	2.383
pH to 7.4 using NaOH		

On the day of use, 0.1% BSA was added.

Table 5-3: Components of lysis buffer.

Reagent	Volume/weight
Water	40 ml
Sodium deoxycholic acid	0.25 g
Igepal	0.5 ml
NaCl (5M)	0.88 g
Tris HCL (1M, pH 8)	2.5 ml

Once lysis buffer is made up, it is then placed in 37°C water bath for 15 minutes, then 1 tablet of complete EDTA-free protease inhibitor cocktail was added, then kept on ice.

5.2.5. Quantification of GLP-1

Plasma GLP-1 was measured from supernatant collected from the murine crypt and human colonoid experiments using the Merck GLP-1 ELISA kit (EGLP-35K) according to manufacturer's instructions.

GLP-1 was calculated as a percentage total, whereby the concentration of GLP-1 was quantified in the secretion buffer and in the lysed/homogenated cells. GLP-1 secretion in each well was expressed as a fraction of the total GLP-1 measured in the same well.

5.2.6. Statistical analysis

Data presented as mean ± SEM for crypt culture and human colonoid experiments in chapter 5. Statistical analysis was performed using GraphPad Prism 8. Data was assessed for normality using the Shapiro-Wilk normality test. One-way ANOVA was used followed by Tukey's post-hoc analysis, the parametric multiple comparisons test. If the data was not normally distributed, Kruskal-Wallis test followed by a Dunn's post-hoc test, the non-parametric multiple comparisons test, was used for multiple comparisons. Where there were two independent variables, a two-way ANOVA was used followed by a Tukey's post hoc analysis for multiple comparisons. $P < 0.05$ was considered significant.

5.3 Results

5.3.1. Effect of bile acids and progesterone sulphates in crypt cultures

The previous chapter demonstrated that serum bile acids increase in European women with high BMI and GDM and are not increased in South Asian women with GDM vs normal pregnancy. Secondary bile acids are of particular interest due to having stronger potency towards TGR5. Bile acids were assessed for GLP-1 secretion in WT vs Tgr5$^{-/-}$ murine crypt cultures. In the WT crypts, all bile acids (DCA, LCA, TDCA, TLCA and UDCA), at 100 μM, significantly increased GLP-1 secretion to levels close to the positive control, F/I (Figure 5.1). Comparing the GLP-1 secreted in WT vs Tgr5$^{-/-}$, there was significantly lower GLP-1 secreted when Tgr5$^{-/-}$crypts were incubated with any of the bile acids, with approximate 2.2, 3.1, 4.4, 4.2 and 3.1-fold reductions seen in DCA, LCA, TDCA, TLCA and UDCA respectively (Figure 5.1).

As there are reductions in some serum progesterone sulphates in GDM, such as PM5S and PMΔ5S, the influence of these progesterone sulphates on secretion of GLP-1 from crypt cultures was investigated. TGR5 is of interest due to progesterone sulphates having the ability to bind to this receptor. The progesterone sulphates, PM3S and PM5S at 50 μM, were incubated with murine crypts, and increased GLP-1 secretion by 5.4 and 6-fold respectively, i.e. comparable levels to F/I (Figure 5.1). Similar to incubation with bile acids, when Tgr5$^{-/-}$ crypts were incubated with the progesterone sulphates PM3S or PM5S, there was lower GLP-1 secretion compared to WT crypts by 2.1 and 2.2-fold, respectively (Figure 5.1).

Both bile acids and progesterone sulphates were incubated with murine crypt cultures to assess if a combination further potentiated GLP-1 secretion. Combining both DCA and LCA with either PM3S or PM5S significantly increased GLP-1 secretion compared to baseline. However, combining progesterone sulphates with bile acids did not increase GLP-1 secretion compared to either bile acid alone. Combining LCA with either PM3S or PM5S enhanced GLP-1 secretion significantly by 1.45 and 1.4-fold respectively compared to PM3S or PM5S alone(Figure 5.1).

Though Tgr5$^{-/-}$ crypts showed lower GLP-1 secretion compared with WT crypts with bile acids or progesterone sulphates alone, or in combination, some treatments still demonstrated a small significant increase compared to KO CTL crypts. DCA, PM3S, PM5S and the combination of DCA with PM3S increased GLP-1 secretion by 2.8, 2.7, 2.9 and 3.7-fold respectively(Figure 5.1).
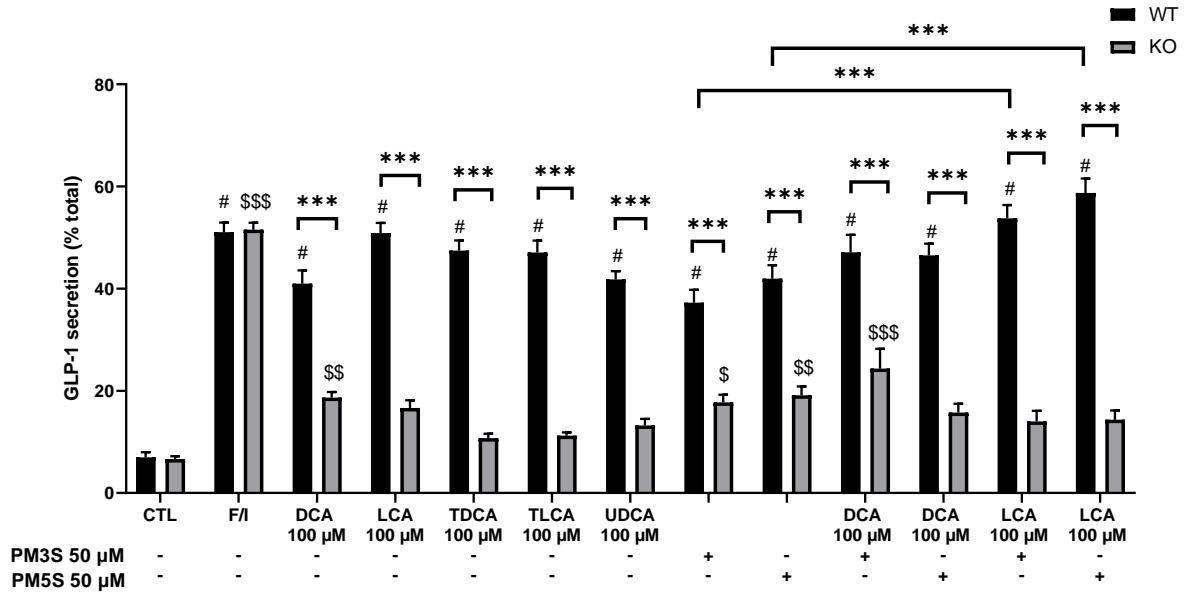
Figure 5.1: Bile acids and progesterone sulphates increase GLP-1 secretion in murine crypt cultures via TGR5.

WT and Tgr5^{-/-} murine colonic crypts were isolated and cultured. GLP-1 secretion as a percentage total was assessed in response to bile acids and progesterone sulphates. WT and Tgr5^{-/-} crypts were incubated with bile acids, PM3S and PM5S for assessment of GLP-1 secretion. Differences between WT and Tgr5^{-/-} crypts were evaluated. Furthermore, combinations of bile acids and progesterone sulphates were incubated with WT and Tgr5^{-/-} crypts and GLP-1 secretion assessed. Significant differences indicated by: *** $P < 0.001$, # $P < 0.001$ for comparing WT treatments to WT CTL, $ $P < 0.05$, $$ $P < 0.01$ and $$$ $P < 0.001$ for comparison KO treatments to KO control, as determined by two-way ANOVA with multiple comparisons. Data expressed as mean ± SEM, n=3. CTL; control, F/I; forskolin/IBMX.

5.3.2. Effect of progesterone sulphates and bile acids in human colonoids

We have demonstrated that both bile acids and progesterone sulphates act on the TGR5 receptor to cause GLP-1 release in murine crypts. We next tested whether these effects could be replicated in human colonoids. The colonoids were first differentiated, using DAPT notch inhibition, to give a phenotype that gave high secretory GLP-1 response in order to maximise the potential of the bile acids or progesterone sulphates to cause GLP-1 release. Inhibition of notch shifts differentiation towards the secretory lineage, which includes enteroendocrine cells, and requires a pulse of DAPT at the correct time during the differentiation period. Incubation of human colonoids with either 50 µM PM3S and PM5S produced significant increases in GLP-1 secretion at 11.1 and 10.7-fold respectively, compared to control (Figure 5.2A). Similarly, 100 µM of TDCA and UDCA were incubated with human colonoids and increased GLP-1 secretion by 3.5 and 2.9-fold respectively, compared to control (Figure 5.2B). Thus, PM3S and PM5S, and the secondary bile acids TDCA and UDCA, are able to stimulate GLP-1 secretion from enteroendocrine L-cells in both mouse and human models.
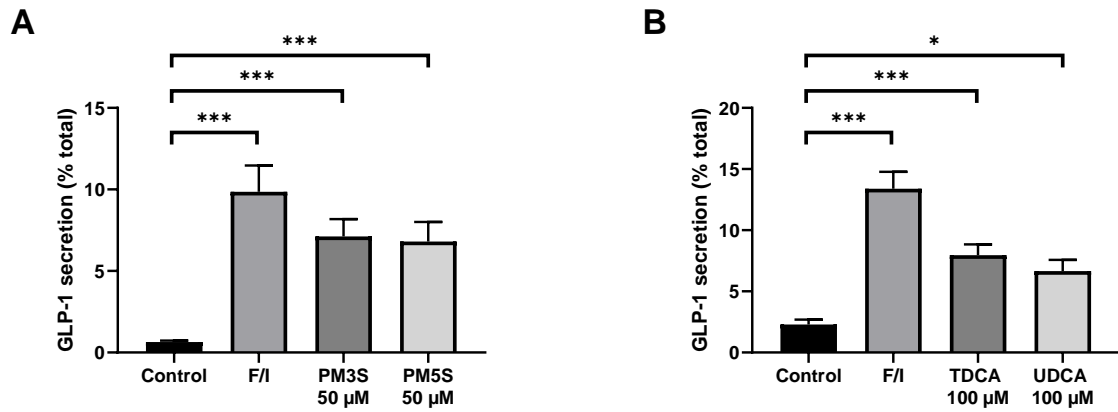
Figure 5.2: Bile acids and progesterone sulphates increase GLP-1 secretion in human colonoids.

Human colonoids were differentiated and cultured with progesterone sulphates and bile acids to assess GLP-1 secretion. **(A)** Progesterone sulphates were incubated with human colonoids. **(B)** GLP-1 secretion in colonoids was examined after incubation with bile acids TDCA and UDCA. Significant differences indicated by: * $P < 0.05$, *** $P < 0.001$, as determined by Kruskal-Wallis test followed by Dunn's multiple comparisons test. Data expressed as mean ± SEM, each graph n=3 independent experiments. CTL; control, F/I; forskolin/IBMX.

5.4 Discussion

Both bile acids and progesterone sulphates are altered in GDM. Our previous chapters demonstrate progesterone sulphates decreasing, whilst bile acids increase or decrease depending on ethnicity and BMI. Both types of metabolite are of interest in terms of the pathophysiology of GDM. Our results show that in both murine colonic crypts and human colonoids, bile acids and progesterone sulphates stimulate GLP-1 secretion via activation of the TGR5 receptor. Though we do not know if this process is TGR5 dependent in humans. Most bile acids did not increase GLP-1 secretion in Tgr5$^{-/-}$ crypts, though DCA and both PM3S and PM5S demonstrated a small significant increase in GLP-1 secretion. However, all bile acids and progesterone sulphates were significantly lowered in Tgr5$^{-/-}$ crypts compared to WT, indicating that the GLP-1 secretion was primarily achieved through activation of TGR5. Other investigators have used TCA, TDCA and TLCA at the same concentrations in small intestinal and colonic cultures, and observed an increased GLP-1 secretion caused by the bile acids, similar to our results. However this was completely eliminated from small intestinal cultures derived from Tgr5$^{-/-}$ mice, differing slightly to the small increases we saw with DCA (Brighton et al., 2015). The increase in GLP-1 in our Tgr5$^{-/-}$ crypts suggests other receptors and mechanisms may also be involved in the interaction of bile acids and progesterone sulphates with enteroendocrine cells. They could possibly be directly interacting with the closure of potassium or opening of calcium channels to cause an increase in calcium ions in the cell, causing GLP-1 release. Overall, our results indicate that bile acids and progesterone sulphates can trigger the TGR5 receptor signalling in the gut to cause GLP-1 release.

Our results are unlikely to relate to signalling by the other bile acid receptor, FXR, due to the bile acid and progesterone sulphate incubation times being relatively short. As explained in chapter 1, FXR is a nuclear receptor that affects transcriptional activity upon activation, and therefore would take longer than cell surface receptors such as TGR5 to cause physiological responses, e.g. alterations in hormone secretion. Therefore, the small increase in GLP-1 release with DCA, PM3S and PM5S we observed in Tgr5$^{-/-}$ crypts is unlikely to be due to FXR interaction. Interestingly, it has been found that when FXR is activated in L-cells, GLP-1 secretion decreases and Fxr$^{-/-}$ mice had increased GLP-1 levels and improved glycaemia (Trabelsi et al., 2015). Intestinal specific FXR antagonism has also resulted in improved and favourable metabolic outcomes. Intestinal specific Fxr$^{-/-}$ mice resulted in increased lipid oxidation, lower insulin resistance and resistance to obesity. Furthermore, the same study used Tempol to antagonise FXR and demonstrate similar complimentary metabolic outcomes compared to the intestinal specific FXR null mice (Li et al., 2013). Thus, it is likely that TGR5 produces an immediate response to ingestion of food (and bile acids release) to cause an incretin effect, whilst activation of FXR will produce a more delayed response and change the initial

postprandial response to influence glucose metabolism hours after a meal. Activation of FXR post-prandially may change energy metabolism to induce browning of WAT and enhance energy expenditure in BAT (Fang et al., 2015). Though controversially, agonism of FXR has been reported to also increase GLP-1 secretion (Fang et al., 2015; Pathak et al., 2017, 2018). FXR and TGR5 crosstalk could occur in the control of glucose homeostasis. A study has found using a dual FXR and TGR5 agonist, INT-767, altered bile acids composition, in mice, decreasing TCA and CA, whilst increasing TβMCA and TωMCA. IN-767 also increased GLP-1 secretion and improved glucose and lipid metabolism more than FXR- or TGR5-selective agonists. Furthermore, INT-767 activation of FXR induces TGR5 gene expression, suggesting bile acid receptor interplay occurs to promote improved glucose metabolism (Pathak et al., 2017). Dual action of intestinal TGR5 agonism and either FXR agonism or antagonism could provide a promising approach for diabetic treatments, though further research is needed to clarify intestinal FXR role in GLP-1 secretion.

Our results indicate that progesterone sulphates are of increasing interest as potential mediators of altered glucose homeostasis, and we have shown in previous chapters that serum levels are reduced in GDM pregnancies. Reduced serum levels therefore would result in lower activation of TGR5 in the gut and would likely cause lower GLP-1 secretion during pregnancy. This could contribute to the pathophysiology of GDM if it causes affected women to fail to meet the increased requirements for insulin during pregnancy. PM3S stimulation of cAMP production in HEK293T cells expressing TGR5 has been shown (Abu-Hayyeh et al., 2016), however few studies exist exploring the relationship between progesterone sulphate and TGR5. To date, no one has evaluated the effects of progesterone sulphates in the gut and these experiments are the first to demonstrate how they stimulate GLP-1 release. These results also provide insights into potential strategies to target TGR5 with progesterone sulphates. As TGR5 is expressed in many different tissues, organ-specific effects would occur upon activation making TGR5 a difficult choice for a therapeutic target due to the possible systemic side effects (Duboc, Taché, & Hofmann, 2014).

Unlike the progesterone sulphates, the relationship between bile acids and release of GLP-1 has been explored in detail. Similar observations have been seen when bile acids were delivered *in vivo* in mice or *ex vivo* in rat/mouse perfused colons, stimulating GLP-1 secretion by a similar amount to our experiments (Christiansen et al., 2019; Rafferty et al., 2011). It is important to consider which bile acids are altered in GDM vs normal pregnancy to determine if the changes in GDM alter the activation of TGR5 in the gut. However important considerations are needed for which bile acid is best to stimulate GLP-1 secretion in humans. Secondary bile acids, particularly LCA, though most potent to TGR5, are more toxic especially at high levels. Whilst DCA is less harmful than LCA, DCA has been shown to be more toxic than CA (Kurdi, Kawanishi, Mizutani, & Yokota, 2006). It is also

important to note that very little LCA crosses to the basolateral side of the gut, and concentrations in serum are very low, so the LCA that reaches TGR5 will be of much lower serum concentrations compared to the concentrations in the gut (Šarenac & Mikov, 2018). Therefore, delivering high levels of the secondary bile acid LCA to act as a TGR5 agonist in the gut may not be feasible. Combinations of bile acids and progesterone sulphates could yield interesting results. We saw no significant changes or further potentiation when compared to bile acids alone, however LCA with either PM3S or PM5S did increase GLP-1 secretion compared to either progesterone sulphates alone. It is possible that FXR interplay does occur, and this might have been demonstrated if the progesterone sulphates and bile acids in combination had been studied for longer incubation times with the crypt cultures. It would be of interest to study this in future experiments. PM5S has partial agonism for FXR (Abu-Hayyeh et al., 2013), meaning progesterone sulphates at the intestinal level could lower FXR activity, potentiating GLP-1 secretion as suggested by others (Li et al., 2013; Trabelsi et al., 2015).

Due to the increasing evidence of the involvement of FXR and TGR5 in energy metabolism, manipulation of these receptors could be key in improving metabolic disease such as T2DM and GDM. As a result of the cytotoxic nature of hydrophobic bile acids, concerns have been raised as to the safety of using bile acids as pharmaceuticals. To avoid this issue, much research has evaluated semi-synthetic analogues or synthetic TGR5 or FXR agonists as potential therapeutics for metabolic diseases. Studies targeting FXR-mediated regulation of metabolism appear to be contradictory. The FXR agonist obeticholic acid (OCA) has delivered promising results in clinical trials for liver-based metabolic diseases and T2DM in non-pregnant adults, including improving insulin sensitivity (Mudaliar et al., 2013). OCA improved histological features in the liver after 72 weeks treatment with 25mg daily in patients with non-alcoholic fatty liver disease, a disorder linked with T2DM and previous GDM (Neuschwander-Tetri et al., 2015). However, in mouse models of GDM, OCA did not produce the full effects seen in other studies, and whilst treatment reduced plasma cholesterol, glucose tolerance was not improved (McIlvride et al., 2019). Other studies have also shown that CDCA treatment influences lipid metabolism in humans and increases plasma LDL cholesterol as a result of reduced plasma LDL clearance (Ghosh Laskar, Eriksson, Rudling, & Angelin, 2017). Research into FXR-specific therapeutics for gestational disorders such as ICP or GDM is also currently lacking, however, FXR agonism provides some benefits in mouse models of hypercholanaemia, with improved fetal bile acid profiles (Pataia et al., 2020; W. B. Wu et al., 2015).

Research into TGR5 agonists as a therapeutic has been explored, as manipulation of the release of gut hormones could be key for alleviating symptoms of diabetes. TGR5 agonists in diabetic mice has shown to improve glucose homeostasis (Huang et al., 2019). A clinical study examined the effects of

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ileo-colonic delivered conjugated bile acids in patients with obesity and T2DM. When compared to placebo, there was increased postprandial GLP-1 secretion (Calderon et al., 2020). To date, no research has looked specifically at the impact of TGR5 receptor activation on biochemical features of GDM. Several novel TGR5 agonists exist and many show encouraging effects when used in animal models of diabetes (Agarwal et al., 2018; Finn et al., 2019; Phillips et al., 2014; Rizzo et al., 2010; Zambad et al., 2013; Zheng et al., 2015). One recent example used a novel, orally-administered TGR5 agonist, RDC8940. This induced incretin secretion and improved insulin sensitivity with minimal side effects in western diet-fed mice (Finn et al., 2019). Another study used another novel TGR5 agonist, WB403, on a model of T2DM mice, improving glucose tolerance and decreased fasting blood glucose. WB403 administration also caused changes at the islet level, preserving pancreatic β-cells in diabetic treated mice compared to untreated diabetic mice (Zheng et al., 2015). Clinical trials using TGR5-selective agonists have also been carried out with promising results (Hodge et al., 2013). Side effects are a major concern for novel TGR5 agonists, due to TGR5 having broad multi-organ expression. However, using organ-restricted agonists would avoid these side effects and would consequently likely have acceptable safety profiles. Currently, TGR5 agonists with low intestinal absorption rates have been designed. Studies have shown their ability to cross cell membranes and that they show specificity for TGR5 receptors without contributing to systemic absorption (Chen et al., 2018; Lasalle et al., 2017). Lasalle *et al.* 2017 designed a gut-restricted TGR5 agonist named compound 24. When used in diet-induced obese and insulin resistant mice, sustained GLP-1 release and decreased fasted plasma insulin levels were observed, with low systemic levels of compound 24 detected. This compound has low intestinal permeability to prevent high systemic circulation and therefore unwanted side effects. Much of the compound reaches and remains in the ileum and colon at 8 hours, where TGR5 expression is maximal, and a significant fraction of the compound would cross the cell membrane, activate TGR5 on the basolateral side, without contributing to systemic absorption (Lasalle et al., 2017). Many of these agonists could have the potential to lower glucose levels and therefore result in better management of GDM, though further studies are required to explore the relationship between TGR5 and GDM.

Bile acids for the treatment of diabetes are also currently being investigated (McGlone et al., 2021; Meyer-Gerspach et al., 2013; Nielsen et al., 2017; Shima et al., 2018). Gut delivery of bile acids could be key to managing the symptoms of GDM. UDCA is commonly used to treat ICP, a disease linked to increased risk of developing GDM, so it could be plausible to use UDCA to treat GDM. Indeed, our studies have shown clear elevation of GLP-1 release when UDCA is incubated directly in our crypt and human colonoid experiments, indicating a direct effect in the gut. Other studies show that oral gavage of UDCA into alloxan-induced diabetic mice brought about reductions in elevated blood

glucose and altered the concentrations of CDCA and LCA, suggesting protective effects on the β-cells (Mooranian et al., 2020). Orally administered UDCA in diet-induced obese mice also reduced saturated fatty acid levels, reducing free fatty acid uptake and reducing triglyceride formation (Y. Zhang et al., 2019). In a small pilot study using UDCA in non-diabetic subjects, UDCA treatment resulted in higher GLP-1 secretion and lower blood glucose, though no significant changes were observed in insulin release (Murakami et al., 2013). A recent study of UDCA treatment in people with T2DM and chronic liver disease reported weight loss and reduced HbA1c over 12 weeks (Shima et al., 2018). Furthermore, a meta-analysis showed significant reductions in fasting plasma glucose, HbA1c and plasma insulin concentrations in UDCA-treated participants with non-alcoholic fatty liver disease (Foghsgaard et al., 2017; Sánchez-García, Sahebkar, Simental-Mendía, & Simental-Mendía, 2018). Similarly, other studies have shown other bile acids to have beneficial potential for diabetes. Oral delivery of CDCA to healthy volunteers has shown to enhance GLP-1 secretion through TGR5 activation. Though in contrast, they found UDCA did not bring any changes in GLP-1 secretion (McGlone et al., 2021; Meyer-Gerspach et al., 2013; Nielsen et al., 2017). Further studies are needed to address these discrepancies and which bile acid is best for stimulating GLP-1 secretion, and is suitable for GDM treatment. The improvements seen in UDCA or bile acid treatment for T2DM could translate as a plausible treatment for those who have GDM and future studies could look to see if these improvements in T2DM could be replicated in GDM patients.

Future studies evaluating FXR role in GLP-1 secretion would clarify some of the inconsistencies in current studies. This would require either incubating progesterone sulphates and bile acids for longer incubation times or pre-treating *in vitro* models. Future *in vivo* experiments by delivering bile acids into the gut of mice and measuring hormonal markers like GLP-1 will also help reinforce the results reported in murine crypt culture and human colonoids. Taking this further, it would be of interest to test this in GDM models and observe if this may have any effects in reducing diabetic symptoms. The toxicity of bile acids in our biological models should also be tested, with apoptotic markers measured in the gut. The more potent secondary bile acids are also more cytotoxic (Pavlović et al., 2018) and careful consideration is needed about the use of bile acids and progesterone sulphates to influence glucose metabolism and as a therapeutic for GDM. Clinical studies using faecal samples and analysis of the gut microbiota, bile acids and progesterone sulphates may also give a further indication on how the physiology differs in normal vs GDM pregnancies. Analysing the gut microbiota phenotype may also highlight whether primary bile acids being microbially transformed into secondary bile acids differs in GDM vs normal pregnancies.

In conclusion, I have shown the secondary bile acids TDCA and UDCA, as well as PM3S and PM5S activate TGR5 to cause GLP-1 secretion in murine crypt cultures and human colonoids. However, we

do not know if the mechanism is TGR5 specific in humans. Enhanced GLP-1 secretion is likely to be important for reduction of glucose intolerance and may improve GDM outcomes by normalising glucose homeostasis. Increasing GLP-1 release by targeting TGR5 with bile acids/progesterone sulphates or synthetic agonists could be key to preventing severe outcomes in GDM.

Chapter 6: Overall summary and conclusions

The maternal metabolic changes that occur during pregnancy are necessary to meet the demands of the baby. Altered glucose and lipid metabolism, alongside enhanced insulin sensitivity, are typically seen in normal gestation. These changes become more prominent with progression into the third trimester, and insulin resistance, lipid mobilisation and demand for plasma glucose increases (McIntyre et al., 2019). These metabolic adaptations are complex, involving multiple tissues and hormones that are secreted during gestation, and careful regulation of these process is required to ensure minimal adverse pregnancy outcomes. Failure to cope with these adaptations and metabolic demands often result in metabolic diseases occurring in susceptible women. GDM typically occurs if the islets cannot meet heightened insulin demand, and hyperglycaemia can result from this. Intervention is required to minimise pregnancy complications, and it is hoped improved glycaemic control will also reduce the long-term risks associated with GDM. Early intervention and identification of biomarkers to use for timely diagnosis is a current research focus, as is improved understanding of the pathophysiology of GDM, as this will also give ideas for future treatment strategies.

The aim of the work presented in this thesis was to enhance understanding of the relationship between bile acids, progesterone sulphates and GDM, and how these metabolites could contribute to the pathophysiology of GDM. We first examined how progesterone sulphates differed in the serum of GDM women and how they influence insulin secretion when incubated with the islets of Langerhans. Then we examined the bile acids in a similar way to the progesterone sulphates, including the mechanistic studies using islets. Finally, we also examined how both bile acids and progesterone sulphates influence gut signals to change glucose metabolism.

It is well established that progesterone sulphates increase during pregnancy (Abu-Hayyeh et al., 2013). Interestingly, some progesterone sulphates, like PM5S, were found to be increased further in the third trimester of pregnancies complicated with ICP. Progesterone sulphates, like PM3S, PM2DiS and PM3DiS, were also found to be elevated in the first trimester pregnancies that went on to develop ICP, revealing them to be possible prognostic markers for ICP (Abu-Hayyeh et al., 2016). Furthermore, their interaction with FXR and TGR5 (Abu-Hayyeh et al., 2016, 2013), receptors known to influence glucose and lipid metabolism, make them an interesting, prospective hormones to study in GDM. Whilst serum progesterone sulphate concentrations are increased in pregnancies complicated by ICP, women with GDM appeared to have lower concentrations of progesterone sulphates in the third trimester. When analysed in the first trimester, PM5S was lower in high BMI women who went onto develop GDM, though logistic regression suggests this was not a strong

predictor for GDM. Further studies are required to examine how ethnicity and BMI affects progesterone sulphate concentrations and whether the aetiological factors of GDM similarly affects their levels. Our mechanistic studies reveal that PM5S increased GSIS, similarly seen by PMΔ5S in other studies (Thiel et al., 2013; Wagner et al., 2010), suggesting that lower levels of progesterone sulphates like PM5S, could contribute to the development of GDM though reduced insulin secretion. Further to this, we demonstrate that the mechanism by which PM5S, PMΔ5S and, to a lesser degree PM4S increase insulin secretion is through interaction with TRPM3. The work in this thesis demonstrates that progesterone sulphates require specific structural requirements to activate this ion channel and allow calcium entry into cells, a mechanism that had been previously reported for PMΔ5S, but not for the other progesterone sulphates (Drews et al., 2014; Majeed et al., 2010). Therefore, future studies looking at progesterone sulphates in GDM women should examine which specific progesterone sulphates are increased or decreased in GDM and how these changes affect certain sub-groups of GDM women. We were also able to construct a computational model of TRPM3. Using existing models of TRPM7 helped predict the binding site and further allowed us to model the ligand binding site of our homology TRPM3 model. It is hoped that these models can be used to further reveal potential regulatory mechanisms for insulin secretion and will facilitate further understanding of progesterone sulphate-TRPM3 complexes and therapeutics. In future, it will be possible for site-directed mutagenesis studies to confirm the ligand binding modelling structures and validate the active site of TRPM3. These results show a link between the progression of GDM and progesterone sulphates and should be further explored to elucidate the pathophysiology. Table 6-1 summaries the changes in progesterone sulphate concentrations in GDM pregnancy.

Table 6-1: Impact of progesterone sulphate changes in GDM pregnancy.

Progesterone Sulphate	Concentration changes in:		Function and Impact of Change
	Normal pregnancy	GDM pregnancy	
PM3S	Increase	No Change	TGR5 agonist, possible antagonist for TRPM3 No change in GSIS in normal or GDM pregnancy
PM4S	Increase	No Change	Partial agonist for TRPM3 No change in GSIS in normal or GDM pregnancy
PM5S	Increase	Decrease	Partial agonist for FXR, agonist for TRPM3 Increased GSIS in normal pregnancy, reduced GSIS in GDM pregnancy
PMΔ5S	Increase	Decrease	Agonist for TRPM3 Increased GSIS in normal pregnancy, reduced GSIS in GDM pregnancy

Bile acids are of particular interest due to their ability to bind to FXR and TGR5. Activation of both receptors have been reported to have beneficial effects in lowering glucose and lipid levels (Bellafante et al., 2020; Christiansen et al., 2019; Ma et al., 2006; Savkur et al., 2005; Velazquez-Villegas et al., 2018). It is plausible to further explore how the activity of these receptors could change during GDM and could in part be responsible for changes in metabolism and onset of GDM. A small number of studies exist that explore the relationship between bile acids and GDM, although not all studies show consistent bile acid changes in GDM, with studies reporting increases or decreases in the third trimester (Gagnon et al., 2021; Gao et al., 2016; Kong et al., 2020; B. Zhu et al., 2021). This study provided further insight and explored how BMI and ethnicity could affect bile acid concentrations. Interestingly, our studies demonstrated different changes in Europeans and South Asians, with the former having increased serum bile acid concentrations. The South Asians also notably had lower concentrations of the secondary bile acid DCA compared to Europeans. This may offer clues to explain ethnicity differences for onset of GDM. This observation could be extended to other ethnicities susceptible to GDM and future studies should consider analysing serum bile acid in

samples from women of different ethnic origins. Also important is BMI, and there were different trends serum bile acid concentrations in obese and the normal BMI groups. Studies of non-pregnant individuals have shown positive correlation between BMI and total bile acids (Prinz et al., 2015; Vincent et al., 2013; Xie et al., 2015). However, we observed lower concentrations of the primary bile acids in the normal BMI group compared to obese BMI in both South Asians and Europeans. These changes suggest that the reason for onset of GDM in the high and normal BMI groups could differ, further indicating the necessity of future studies using groups with matched BMI. Most changes in bile acid concentrations occur in GDM obese European women and a proposed schematic diagram summaries the proposed changes in these women (Figure 6.1) Further studies are needed to clarify how bile acids change in different sub-groups of women with GDM.
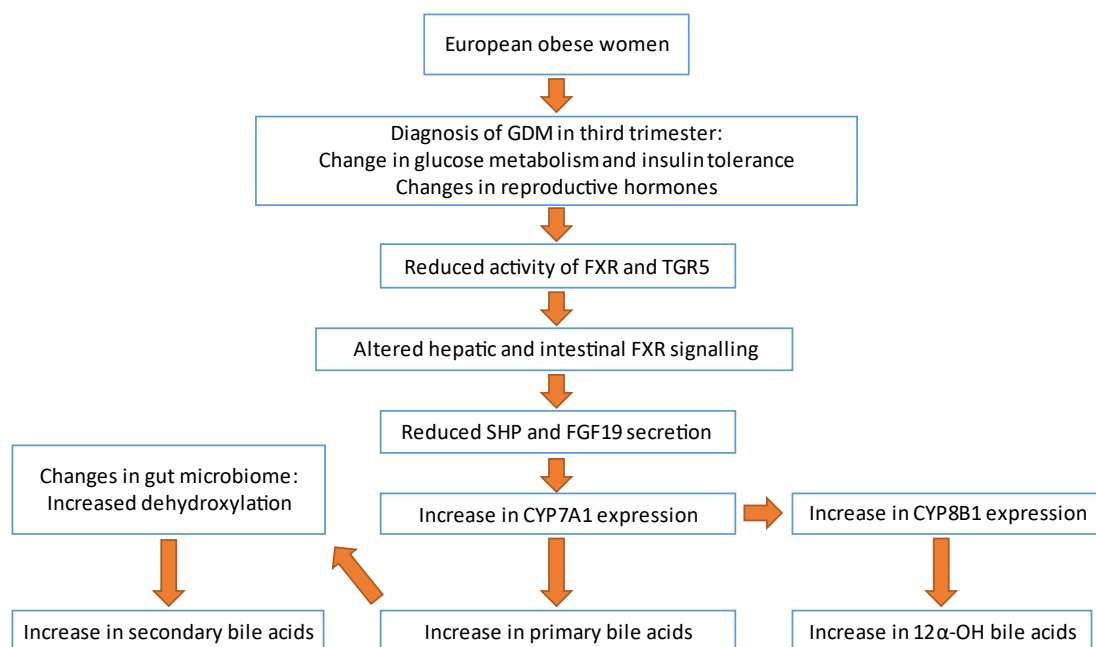
Figure 6.1: Schematic proposing how bile acids change in GDM pregnancy in obese European women.

We wanted to further our understanding of how bile acids may impact GDM mechanistically. Islet studies have generally shown that bile acids increase GSIS, although clarification on how bile acids do this is needed. Both FXR or TGR5 have been suggested as receptors that play a role in the increased GSIS (Düfer, Hörth, Wagner, et al., 2012; Hoffmeister et al., 2020; Kumar et al., 2012; Schittenhelm et al., 2015; Vettorazzi et al., 2016). We also observed increases in GSIS following exposure to TCA and TCDCA, though neither of the TGR5 or FXR specific agonists delivered similar effects. Due to the short incubation times, this is unlikely to be mediated by FXR, although this cannot be ruled out. Future studies should use longer incubation times or pre-incubation of islets with bile acids to allow time for FXR signalling to influence physiological responses. Further studies in how bile acids act in GDM animal models would also be necessary.

In addition to the islet studies, the work in this thesis aimed to characterise how bile acids and progesterone sulphates influence gut hormone signalling. The TGR5 receptor located on the basolateral side of enteroendocrine cells in the distal ileum and colon is becoming an increasingly therapeutic target of interest, particularly for metabolic diseases like diabetes, due to its ability to release GLP-1 and enhance insulin secretion. Changes in the gut during pregnancy include changes in bile acid metabolism and the gut microbiome which can alter bile acid activity (Koren et al., 2012; Ovadia, Perdones-Montero, et al., 2019). Bacterial transformation of bile acids from primary to secondary is key to activation of TGR5. The murine crypt cultures and human colonoid experiments show significantly increased GLP-1 secretion following exposure to secondary bile acids and the progesterone sulphates PM3S and PM5S. Moreover, Tgr5$^{-/-}$ mice demonstrated that all this was signalled through the TGR5 receptor as significant reductions in GLP-1 release occurred in animals deficient of the receptor. Our findings are in line with others who also observed increases of GLP-1 secretion following exposure to bile acids, and also progesterone sulphate activation of TGR5 receptor (Abu-Hayyeh et al., 2016; Brighton et al., 2015), however this presents novel findings of progesterone sulphate relationships with gut-activated TGR5. Both FXR and TGR5 are key targets in manipulating energy metabolism and research involving gut delivery of agonists or bile acids are continuous and seen as prime therapeutic areas in T2DM and GDM. Future studies evaluating the role of FXR is necessary due to current conflicting studies surround FXR ability to cause GLP-1 release. Faecal studies should also give an indication on which progesterone sulphates and bile acids could be impacting GDM vs normal pregnancies. Our studies show that both bile acids and progesterone sulphates activate TGR5 in the gut to cause GLP-1 release.

Overall, the work described in this thesis demonstrates a complex relationship between specific bile acids, progesterone sulphates and the pathophysiology of GDM. Our clinical studies demonstrate a correlation between onset of GDM and concentrations of progesterone sulphates and bile acids. Our

mechanistic studies also exhibit pathways on how these hormones affect our experimental models and influence metabolic homeostasis. The bile acid receptors FXR and TGR5 remain important receptors of interest in the regulation and determination of glucose and lipid metabolism and eventual onset of GDM. Targeting these receptors for therapeutic uses, or identifying biomarkers for early intervention of GDM, could reduce severity of outcomes for GDM, improving the health and well-being of pregnant women as well as the baby.

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Appendix 1

Metabolic impact of bile acids in gestation

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Abstract

Bile acids are lipid-solubilising molecules that also regulate metabolic processes. Farnesoid X receptor (FXR) and Takeda G-protein coupled receptor 5 (TGR5) are two bile acid receptors with key metabolic roles. FXR regulates bile acid synthesis in the liver and influences bile acid uptake in the intestine. TGR5 is mainly involved in regulation of signalling pathways in response to bile acid uptake in the gut and therefore prandial response. Both FXR and TGR5 have potential as therapeutic targets for disorders of glucose and/or lipid homeostasis. Gestation is also known to cause small increases in bile acid concentrations, but physiological hypercholanaemia of pregnancy is usually not sufficient to cause any clinically relevant effects. This review focuses on how gestation alters bile acid homeostasis, which can become pathological if the elevation of maternal serum bile acids is more marked than physiological hypercholanaemia, and on the influence of FXR and TGR5 function in pregnancy on glucose and lipid metabolism. This will be discussed with reference to two gestational disorders: intrahepatic cholestasis of pregnancy (ICP), a disease where bile acids are pathologically elevated, and gestational diabetes mellitus (GDM), characterised by hyperglycaemia during pregnancy.

Introduction

Bile acids are a group of cholesterol-derived steroids with an aliphatic side chain which are synthesised in the liver and exported into the bile. Before secretion, bile acids are conjugated with either glycine or taurine, increasing hydrophilicity and reducing cytotoxicity (1). The primary functions of bile acids is to solubilise lipids by forming micelles to aid emulsification and facilitate absorption of fat by the gut (2), however, recent research has demonstrated that bile acids also have hormonal and metabolic functions, particularly in glucose and lipid regulation. Bile acid signalling through the receptors farnesoid X receptor (FXR) and Takeda G-protein coupled receptor 5 (TGR5) (3) occurs in numerous cell types throughout the body to propagate metabolic processes.

Pregnancy is associated with a number of metabolic adaptations to facilitate fetal growth. There is a gradual increase in serum bile acids as gestation progresses, although for most women this remains within the normal reference range (4). However, for a small number of women, serum bile acids are elevated beyond this level, leading to intrahepatic cholestasis of pregnancy (ICP) which is associated with an increased risk of adverse pregnancy outcomes, including preterm birth, prolonged neonatal unit admission and stillbirth (4). Women with ICP also have an increased risk of developing gestational diabetes mellitus (GDM) (5), which is characterised by elevated plasma glucose levels and increased insulin resistance. Women with ICP have elevated serum triglyceride and LDL-cholesterol concentrations (6), similar to GDM, and it is thought bile acids and their receptors may also play a role in the development of impaired glucose tolerance in pregnancy.

In this review, bile acids and their receptors, FXR and TGR5, will be discussed in the context of regulation of glucose and lipid metabolism, human diseases, and recent research into their therapeutic potential. Focus will be on the gestational diseases ICP and GDM and the role bile acids play in their pathophysiology.

Bile acid homeostasis

Bile components, including bile salts are synthesised in the liver, exported into bile ducts and stored in the gallbladder until meal ingestion. High concentrations of bile acids are toxic and therefore production and excretion are tightly regulated. There are two main pathways for bile acid synthesis: the classical and alternative pathway.

In humans, the classical bile acid synthesis pathway results in the conversion of cholesterol into the primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA), and accounts for approximately 90% of bile acid synthesis. This hepatic-specific pathway involves at least 17 separate steps, and activity of the rate-limiting enzyme cholesterol 7α-hydroxylase (CYP7A1) determines the size of the bile acid pool (7), while sterol 12α-hydroxylase (CYP8B1) increases CA synthesis and the CA:CDCA ratio (7). The alternative bile acid synthesis pathway starts with hydroxylation of cholesterol by sterol 27-hydroxylase (CYP27A1) in extrahepatic sites to form 27-hydroxylcholesterol, which is then taken up by the liver and the majority converted to CDCA (3). Bile salts are formed by conjugation of bile acids with either taurine or glycine at a ratio of approximately 1:3 (7), and transported into the bile canaliculi through the bile salt export pump (BSEP; *ABCB11*)(8). Other membrane-residing transporters that influence bile components include multidrug resistance protein (MDR3; *ABCB4*), a phosphatidylcholine (PC) floppase that transports PC from the inner to the outer canalicular membrane (and into the bile), and ATP-binding cassette transporters G5/G8 heterodimer (*ABCG5/8*), which transport cholesterol into the bile canaliculi (9,10). From here, bile is transported to the gallbladder for storage. Figure 1 summarises the role of these hepatic pathways within the enterohepatic circulation of bile acids.

Ingestion of food stimulates release of bile from the gallbladder which facilitates the digestion and absorption of lipids and lipid-soluble vitamins. The gut microbiota in the ileum and colon deconjugate the primary bile acids, and further modify them through 7-dehydroxylation to produce secondary bile acids; lithocholic acid (LCA) is formed from CDCA whereas deoxycholic acid (DCA) is derived from CA (Figure 2). The gut microbiota can further modify bile acids by 7α/β-epimerisation

to make ursodeoxycholic acid (UDCA), and more rarely by 3α/β-epimerisation, 5α/β-epimerisation

or oxidation to produce iso-, allo-, or oxo-bile acids, respectively (11). The bile acid pool in the

terminal ileum comprises approximately 30% CA, 40% CDCA, 20-30% DCA and below 5% LCA (1),

although this varies between individuals as it is influenced by factors including nutrient availability

and gut microbiota composition. Approximately 95% of the bile salts are reabsorbed, either through

the apical sodium-dependent bile acid transporter (ASBT) at the distal ileum and colon, or through

passive absorption of deconjugated or protonated uncharged conjugated bile acids along the length

of the intestine (12). The remaining 5% is excreted in the faeces, and this loss is compensated by

approximately 500mg/day *de novo* bile acid synthesis (1). Reabsorbed bile acids are exported from

ileal enterocytes into the enterohepatic circulation by the heterodimeric organic solute transporter

α/β (OST α/β) on the basolateral membrane of the cells (1). The bile acids are transported via the

portal vein back to the hepatocytes through the sodium taurocholate co-transporting polypeptide

(NTCP) or organic anion transporting polypeptides (OATP) (1), reconjugated, and again exported into

the bile duct (Figure 1).

The role of bile acid receptors in glucose and lipid homeostasis

Daily synthesis of bile acids regulates the plasma cholesterol concentration, thereby ensuring this does not become too high. Catabolism of cholesterol to bile acids is regulated by CYP7A1 expression; high CYP7A1 expression leads to depletion of hepatic cholesterol and increased hepatic LDL receptor expression to replace the lost cholesterol by harvesting the circulatory cholesterol (13). The bile acid pool and composition are different in diabetic states, and there is evidence that the pool could increase in type 2 diabetes (T2DM). These changes could increase insulin resistance, affecting glucose metabolism and progress the pathogenesis of diabetes (2). Bile acid activation of FXR and TGR5 is well documented and both receptors have roles in bile acid, lipid and glucose metabolism. Understanding bile acid activation of FXR and TGR5 may provide key insights into the pathogenesis of metabolic disease states.

FXR

FXR is a nuclear receptor expressed mainly in the liver, intestine and kidneys, and is essential to regulating the metabolism and synthesis of bile acids. The primary bile acid CDCA is the most potent FXR ligand (CDCA>LCA>DCA>CA; Figure 3) (13). Hepatic FXR activation promotes transcription of small heterodimer protein (SHP), which represses transcription of CYP7A1, thereby reducing hepatic synthesis of bile acids (1) (Figure 1). FXR also upregulates the expression of MDR3 and BSEP, promoting efflux of bile acids to further prevent bile acid build-up within hepatocytes. Intestinal FXR activation, via transintestinal bile acid flux, induces the expression of fibroblast growth factor 19 (FGF19/FGF15 in mice) which is secreted by the intestinal epithelial cells. FGF19 is transported in the portal vein and binds hepatocyte fibroblast growth factor receptor 4 (FGFR4)/beta-Klotho to cause repression of CYP7A1 transcription, further downregulating bile acid synthesis (2) (Figure 1).

FXR influences lipid and glucose metabolism
Through transcriptional regulation, FXR activation also stimulates β-oxidation of fatty acids and decreases lipid levels in the serum and liver (14). Activation of hepatic FXR with agonists in diabetic/obese mice or rats fed a high-fat diet reduced serum and liver triglycerides and lipids.

Hepatic expression of genes involving fatty acid synthesis, lipogenesis and gluconeogenesis were also reduced (15,16). This demonstrates the importance of FXR in lipid metabolism and that use of FXR agonists has the potential to improve metabolic abnormalities.

Studies have also shown that activation of FXR has a beneficial effect on glucose metabolism, with FXR agonistic treatment or FXR overexpression lowering blood glucose levels in diabetic mice (17). Pathak and colleagues demonstrated that FXR agonists improve glycaemia and reduce diet-induced weight gain in mice (18), and another study demonstrated that bile acid activation of FXR in mice repressed gluconeogenic gene expression (19). Through FXR activation, mice fed a CA diet had reduced expression of phosphenolpyruvate carboxykinase, the rate-limiting enzyme in gluconeogenesis (20). FXR-null mice develop elevated serum free fatty acids, alongside impaired glucose and insulin tolerance, and elevated serum glucose levels. Activation of FXR with agonists in wildtype mice decreased serum glucose (17,19). Other studies, however, have shown beneficial effects of inhibition or deletion of FXR (21,22). Mice with intestine-specific FXR knockout had improved oral glucose tolerance and lower body weight (21,23). These contradictions could be explained by the differential effects of FXR activation in the liver versus the intestine.

It is also important to note that FXR expression is found in peripheral tissues including adipose tissue, islets of Langerhans and adrenal glands (24), and could contribute to glucose and lipid metabolism via actions in these tissues. *In vivo* and *in vitro* experiments involving animal islets demonstrated activation of FXR by bile acid stimulated insulin secretion (25,26). In adipocytes, FXR appears to play a role in differentiation and promotes adipogenesis (27,28).

TGR5

Bile acids also bind and activate TGR5, a cell surface G-protein-coupled receptor widely expressed in humans and animals. However, the most potent bile acid ligands for TGR5 differ to those that activate FXR (LCA>DCA>CDCA>CA; Figure 3) (29). When activated, TGR5 stimulates adenylyl cyclase to increase concentrations of cyclic adenosine monophosphate (cAMP), activating protein kinase A

(PKA) and exerting cytosolic effects including calcium mobilisation and activating cellular signalling cascades such as nuclear factor κB, extracellular signal-regulated kinases and Akt/protein kinase B pathways (30,31). Often, the signalling pathways of TGR5 are influenced by cell type and conditions. Most notable is its expression in the enteroendocrine L cells. TGR5 activation causes secretion of gut hormone glucagon-like-peptide 1 (GLP-1) in the small intestine and colon, which promotes insulin secretion (18,32,33)(Figure 1). Activation of TGR5 receptors at the pancreatic islets causes release of insulin and improves insulin sensitivity and glycaemic control (34,35)

TGR5 activation also plays a role in lipid metabolism and energy expenditure. White and brown adipose tissue (WAT and BAT, respectively) are the two major adipose tissues in the body. WAT is adapted for storage of surplus fatty acids derived from the diet in the form of triglycerides and for subsequent release under conditions of negative energy balance in the body. WAT is also known to contribute to the inflammatory response that occurs in obesity (36,37). In contrast, BAT is a highly vascularised, mitochondria-rich organ containing uncoupling protein 1 (UCP-1), which generates heat by uncoupling the mitochondrial proton gradient (38). TGR5 agonism causes remodelling of white adipocytes to give a more brown adipocyte-like phenotype, thus increasing β-oxidation and energy expenditure (39,40). Improved glucose metabolism and energy consumption are induced by the cAMP/PKA pathway in TGR5-activated skeletal muscle, alongside promoting muscle cell differentiation and hypertrophy to increase muscle strength and function (41,42). TGR5 expression is also found in several immune cells such as monocytes, macrophages and Kupffer cells; its activation exerts anti-inflammatory activities, including inhibition of the production of pro-inflammatory cytokines and induction of differentiation of anti-inflammatory immune cells (31,43,44). Many metabolic diseases have an inflammatory component, including diabetes; thus, TGR5-mediated regulation of immune cell function warrants further investigation.

Further bile acid receptors

Other receptors have been reported to have affinity towards bile acids. The primary function of the pregnane X receptor PXR is to detect foreign substances and protect the body by promoting transcription of genes involved in removing and metabolising toxic substances. PXR is highly expressed in the liver and intestine, with the most potent bile acid ligand being LCA (45). Strong evidence exists for ligand-activated PXR playing a role in lipid and glucose metabolism, though there are contradictory outcomes. Whilst some studies have shown PXR activation mediates lipogenesis, suppresses β-oxidation and induces hyperglycaemia (46,47), others have reported that PXR activation improves glucose homeostasis and insulin sensitivity (48). Species and gender-specific differences are thought to explain these variable results. However, research on metabolic regulation through bile acid bound PXR activation is limited, and further investigations may reveal new role for bile acids acting on PXR.

The vitamin D receptor (VDR), when bound to vitamin D, mediates calcium and bone metabolism, innate and adaptive immune system and cardiovascular function. LCA, can agonise VDR in the lower intestine, particularly in the ileum (49). However, the physiological relevance of LCA modulation of VDR function remains unclear. Recent research has pointed towards the ability of LCA to provide an immune protective effect at the epithelium via VDR activation (50). VDR has also been reported to have a role in maintaining glycaemia. A recent *in vitro* study demonstrated that an LCA derivative, LCA propionate, protects pancreatic β-cells from dedifferentiation (51). It remains to be seen whether LCA can regulate glucose metabolism via VDR.

The liver X receptor (LXR) is a nuclear receptor which has two isoforms: LXRα, which is highly expressed in tissues with high metabolic activity, including the liver, small intestine and adipocytes, and LXRβ, which is expressed ubiquitously (45). Unlike FXR, activation of LXR increases transcription and activity of CYP7A1, increasing bile acid formation and reverse cholesterol transport, thus decreasing plasma cholesterol levels (45). FXR and LXR activation thus finely tune lipid, glucose and bile acid metabolism (13). Though primary bile acids are not regarded as agonists for LXR, some

minor secondary bile acids have agonistic properties, such as hyocholic acid (HCA) and hyodeoxycholic acid (HDCA) (52,53). Both HCA and HDCA are found at low levels in the serum and the intestinal tract, which raises questions as to whether their activation of LXR is of physiological relevance.

Alongside TGR5, other G-protein coupled receptors have also been documented to be activated by bile acids. Bile acids are known to interact with the muscarinic receptors, M_1-M_5 (3). Muscarinic receptors are responsible for the physiological effects of acetylcholine, which acts as a neurotransmitter in the brain or neuromuscular junctions and also mediates the parasympathetic system (3). There is increasing evidence that positive allosteric modulation of M_3 muscarinic receptors improves glucose homeostasis and promotes insulin release (54,55), and mice lacking M_3 in pancreatic β-cells displayed impaired glucose control and reduced insulin release, producing a diabetic phenotype (54).

Sphingosine-1 phosphate receptor subtype 2 (S1PR2), expressed in a variety of tissues, is another G-protein coupled receptor (3) for which bile acids are also ligands. When activated, S1PR2 mediates numerous cell functions including increasing cell permeability, promoting immune cell function, muscle contraction and neuron migration (56). Research into the role of S1PR2 in metabolic function has demonstrated that its activation lowers glucose levels and upregulates lipid metabolism (56,57).

Gestational changes in bile acid metabolism

Serum bile acid concentrations are raised in pregnancy compared to non-pregnant adults, resulting in a mild gestational hypercholanaemia. The concentrations of CA and CDCA are also reported to change as gestation advances (58–62). The composition of the maternal gut microbiome may provide some answers to these alterations, with some studies reporting a gradual reduction of *Bacteroidetes* and increase in *Firmicutes* as pregnancy progresses, similar to the changes in microbes reported in obesity (63,64). In a separate study, advancing gestation was associated with enhanced

microbial bile acid deconjugation (secondary to an increase in *Bacteroidetes*-encoded bile salt hydrolase), reduced ileal bile acid uptake and therefore lowered FXR induction in enterocytes (65), resulting in increased hepatic bile acid synthesis. Along with reduced ileal FXR activity, studies of pregnant mice showed reduced FGF15, and reduced expression of bile acid transporters late in gestation (65,66). More detailed studies that take account of individual variation in gestational phenotypes are required to delineate the alterations in specific enterotypes with advancing gestation.

Pregnancy hormones have also been shown to influence bile acid homeostasis. Studies in mice demonstrated that, as serum bile acid levels increase during gestation, FXR expression is suppressed. This was associated with the downregulation of bile acid transporters such as BSEP, NTCP and OATP, particularly exemplified in the late stage of pregnancy (67,68). Hormones such as progesterone and oestrogen, whose concentrations increase as gestation progresses, contribute to the changes in bile acid metabolism. Oestrogen and its metabolites inhibit FXR and BSEP, and increase CYP7A1 activity in animal studies (67,69,70). Similarly, BSEP and NTCP are inhibited by sulphated progesterone metabolites, and whilst progesterone sulphates exert partial agonism towards FXR, this prevents bile acid binding and reduces overall activation of FXR (71–73). Therefore, both oestrogen, progesterone and their metabolites contribute to raised bile acids during normal pregnancy.

Metabolic changes also occur during pregnancy to accommodate the demands of the fetus. Serum lipids, particularly triglycerides, and LDL-cholesterol increase as pregnancy progresses (74,75). Insulin resistance is typically seen in pregnancy, which contributes to the stimulation of fatty acid synthesis, and increased lipid release into the serum (76). Enhanced hepatic gluconeogenesis and impaired insulin sensitivity result in higher circulating glucose concentrations during the third trimester. Insulin resistance is normally compensated for by an increase in the size and number of pancreatic islets, thereby enhancing glucose-stimulated insulin secretion (GSIS) (77). High oestrogen levels during pregnancy stimulate hepatic lipogenesis and reduce clearance of circulating triglyceride-rich

lipoproteins (76). Oestradiol acts on the β-cells to enhance GSIS, and is also suggested to be involved in developing maternal insulin resistance and glucose intolerance (78,79). One of the suggested mechanisms of action occurs by oestradiol binding directly to insulin and the insulin receptor to cause insulin resistance (80). Elevated levels of progesterone have also been implicated in contributing to decreased insulin sensitivity, increased insulin resistance and glucose intolerance (79). One mechanism through which this occurs is through inhibition of insulin-induced glucose transporter type 4 (GLUT4) translocation. Progesterone prevents GLUT4 translocation by suppressing the phosphoinositide 3-kinase-mediated pathway, inhibiting Akt phosphorylation and decreasing insulin-induced phosphorylation of Cbl signalling proteins, causing reduced cellular glucose uptake (81).

It is also possible that TGR5 signalling is affected during pregnancy. Activation of TGR5 in enteroendocrine cells causes GLP-1 secretion. During normal pregnancy, fasting serum GLP-1 concentrations increase from the second to third trimester, which is thought to compensate for the increase in glycaemia and insulin resistance (82). With changes in the gut microbiome promoting enhanced hepatic bile acid synthesis, and also increased microbial deconjugation and dehydroxylation of primary bile acids to LCA and DCA, the TGR5 receptor would be further activated, thereby influencing maternal metabolism. GLP-1 secretion is key for pancreatic β-cell adaptations. During normal pregnancy, islet and β-cell area increase to compensate for changes in both mice and humans. In GLP-1 receptor null mice, these islet adaptations are abolished, suggesting that GLP-1 is a key mediator in β-cell mass expansion and related adaptations in pregnancy (83).

Bile acid composition and concentration may also differ in gestational disease states compared to uncomplicated pregnancies, particularly in the metabolic disorders ICP and GDM.

Bile acids in gestational disease

Intrahepatic Cholestasis of Pregnancy

ICP is the most common pregnancy-specific liver disease. Women with ICP most commonly present in the third trimester with pruritus and elevated serum bile acids, which can occur alongside raised liver transaminases. ICP accounts for roughly 1% of pregnancies in Europe and North America, with higher incidence in women of South Asian and South American ancestry, occuring most commonly in Chile and neighbouring countries (4). As well as pruritus, hypercholanaemia and abnormal liver function, maternal features of ICP include impaired glucose tolerance and dyslipidaemia (6). ICP is associated with an increased risk of adverse perinatal outcomes, including preterm birth, meconium stained amniotic fluid and stillbirth (84–86). ICP has a complex aetiology with hormonal and genetic factors. Most women are diagnosed when the concentrations of both oestrogens and progesterone are at their highest in the later stages of pregnancy. Sulphated progesterone metabolites, implicated in the pathogenesis of ICP, are elevated in women with ICP in the third trimester, but are raised before the onset of pruritus (87). Genetic studies have demonstrated pathological variants in genes involved in bile acid synthesis and transport (particularly *ABCB4* and *ABCB11*) in ICP (88,89).

As well as the total serum concentration, the bile acid profile is also altered. In normal pregnancy the CA/CDCA ratio is increased, and this is further amplified in ICP by a larger increase in CA (90,91). This ratio change increases the hydrophilicity of the bile acid pool, due to the extra hydroxyl group on CA. This further reduces FXR activation in ICP as CA is a less potent agonist of FXR (Figure 3), but is likely also beneficial to the liver as CA should exhibit cytoprotection over the more cytotoxic hydrophobic bile acids (92). Activation of TGR5 by bile acids, or other agonists such as progesterone sulfates, may also play a role in the pruritus associated with ICP (87,93,94). As well as maternal effects, bile acids have been directly implicated in fetal arrhythmias, with fetal PR interval elongation and abnormal calcium dynamics reported (95–97). FXR function has also been linked to the pathophysiology of ICP. FXR function is reduced in pregnancy due to the rise in oestrogen and its metabolites, causing a

cholestatic phenotype (67,71). While reduced FXR function is likely to occur in all pregnancies, in

some women gestational changes will exacerbate susceptibility to hypercholanaemia to cause ICP.

Ursodeoxycholic acid (UDCA) is a hydrophilic secondary bile acid, normally used to treat a variety of

cholestatic liver disorders. UDCA lowers serum levels of bile acids, acting on BSEP, MDR3 and

multidrug resistance-associated protein 4, which improves biliary secretion of bile acids (98,99).

Other effects include protection of the liver from bile acid-induced apoptosis, anti-inflammatory

actions and stabilisation of the 'biliary bicarbonate umbrella' (100). UDCA treatment also alters the

bile acid pool, constituting approximately 60% of total bile acid measurements in treated women

and replacing more harmful bile acids (101). UDCA is a commonly used treatment for ICP, with

studies demonstrating reductions in maternal features of ICP, such as itch, hypercholanaemia,

elevated transaminases and adverse outcomes (102,103). However, a recent trial demonstrated that

UDCA did not reduce the frequency of a composite endpoint that perinatal death, spontaneous and

iatrogenic preterm birth and admission to the neonatal unit for more than 4 hours (104). Ongoing

research is evaluating whether UDCA may be of benefit to a subgroup of women with ICP, or only

those at risk of specific adverse pregnancy outcomes.

Gestational Diabetes Mellitus

GDM is characterised by the pathological development of insulin resistance and hyperglycaemia

during pregnancy, which resolves following delivery. Due to the lack of consensus and diagnostic

standard for GDM worldwide, there is a large variation in the prevalence of GDM which makes it

challenging to compare across countries and regions. Although pre-existing diseases such as obesity

contribute to the likelihood of developing GDM, multiple risk factors are implicated in its

pathogenesis, including age, ethnicity, family history of diabetes, smoking and genetic susceptibility

(105). With many women choosing to have children at a later stage of their life and maternal obesity

rates increasing worldwide every year (105), the prevalence has been rising, regardless of the

diagnostic criteria. The pathophysiology of GDM is multifactorial. While the metabolic adaptations of

normal pregnancy described above occur in all pregnancies, GDM occurs when the islets cannot

meet the heightened insulin demand, and the β-cells become defective, resulting in hyperglycaemia

(105). GDM typically occurs in the third trimester when insulin resistance is at its highest and

peripheral insulin sensitivity at its lowest (105).

GDM is associated with both short- and long-term complications. Shorter-term consequences

include accelerated fetal growth, macrosomia, neonatal hypoglycaemia, and jaundice (106–108).

Longer-term complications include increased risk of developing T2DM in both the mother and

offspring (106,109–111), and an increased risk of developing metabolic syndrome, cardiovascular,

kidney and liver diseases for the mother (105,106). A recent study examining 11-12 year old

offspring of women with GDM determined that these children were also at increased risk of

hyperglycaemia, diabetes and obesity (109,112).

Initial treatment for GDM involves a lifestyle modification immediately after diagnosis, often

involving diet and exercise (113). If hyperglycaemia is not resolved within 1-2 weeks,

pharmacological treatment is initiated. Metformin and/or insulin are often given as first line

treatments, with sulfonylureas sometimes given as an alternative, depending on different country

guidelines (113). The Metformin in Gestational Diabetes (MiG) trial demonstrated that mothers

randomised to metformin, compared to insulin, had reduced maternal weight gain and gestational

hypertension (114). However, the rate of large for gestational age (LGA) offspring was not affected

and the children had more subcutaneous fat at 2 years of age after maternal metformin treatment

(115). Furthermore, metformin use has been associated with greater childhood size, adiposity and

inferior cardiometabolic health (116). These studies have raised concerns that metformin, currently

used by many women with GDM, does not adequately prevent adverse perinatal outcomes, and may

have negative long-term effects on the metabolic health of the children (117). However, a recent

study has provided more reassuring data: the 3-5 year old children of obese women randomised to

take metformin in pregnancy had lower gluteal and tricep circumferences, lower systolic blood

pressure and improved left ventricular diastolic function compared to the children of obese women

randomised to placebo (118). Thus, more research is required to establish whether maternal

metformin treatment improves long term cardiometabolic outcomes for exposed fetuses. Indeed,

even insulin treatment (the "gold-standard" pharmacological approach) was not shown to be of

definitive benefit for GDM offspring in the most recent Cochrane review, and was thought to

possibly increase the risk of raised blood pressure compared to oral treatments (119). The

sulfonylurea, glibenclamide, has not been shown to be superior to insulin treatment in randomised

trials (120), or as an add-on therapy to metformin (121). Sulfonylurea use is also linked with higher

rates of LGA babies and neonatal hypoglycaemia compared with offspring of GDM women treated

with insulin or metformin (122). Thus, while the recommended treatments should be prescribed for

women with GDM, the potential long-term effects for the child should be taken into careful

consideration, and there is a need for more effective intervention strategies to be developed, likely

with consideration of individual risk factors as GDM is a heterogeneous disorder. However treatment

for GDM does not seem to improve the long-term effects seen in children (123,124), although

current postpartum studies evaluating children born from GDM women are of relatively short

duration.

Impact of bile acids in ICP and GDM

ICP and GDM have some similarities; both are gestational metabolic disorders associated with

maternal dyslipidaemia. Although bile acids and their receptors have a greater impact on ICP, it is

plausible that bile acid signalling also influences the risk of GDM, and modulation of bile acid

pathways may be of benefit in both conditions.

There is an increasing research focus on the relationship between bile acids and the risk of T2DM, in

particular the relevance of the CA:CDCA ratio and CYP8B1 function (125,126). However, studies of

bile acids in GDM are comparatively limited. As described earlier in this article, elevated serum bile

acids and changes in the bile acid pool also occur in normal pregnancy and are heightened in ICP.

Furthermore women with ICP have an increased risk of developing GDM (5,127,128). Total and

individual bile acid species have been found to be higher in women with GDM in the third trimester

(129). Elevated total bile acids were also found in women in their first trimester who went on to develop GDM (130,131). However, other studies found a reduction in bile acids in GDM women recruited from the first and second trimester (132,133). These differences could be due to ethnicity, the heterogeneous aetiology of GDM or variations in the method used to assay bile acids. The composition of the bile acid pools also differs between these studies, and some report alterations in the concentrations of minor bile acids that may not be of relevance to clinical metabolic phenotypes. This discrepancy between studies of bile acids in GDM warrants further investigation. It is important for future studies to use consistent measurement techniques in large cohorts of women with GDM, and to match the BMI in women with uncomplicated pregnancies, alongside ethnic group, gestational week of pregnancy and feeding/fasting blood sampling, as these factors all influence the concentration of specific bile acid species in the serum.

It is possible that changes in FXR and TGR5 activity could affect GDM susceptibility. Since both receptors play a role in regulation of glucose homeostasis, changes to normal receptor function are also likely to affect glucose metabolism. Consistent with this, mice deficient of FXR or TGR5 develop gestational impaired glucose tolerance, and FXR$^{-/-}$ mice have insulin resistance in pregnancy (134). It is plausible both FXR and TGR5 could contribute to the pathophysiology of GDM and could be the link between the increased risk of developing GDM in ICP women.

Bile acid receptors and therapeutics for gestational diseases

Due to the increasing evidence of the involvement of FXR and TGR5 in energy metabolism, manipulation of these receptors could be key in improving metabolic disease such as diabetes. As a result of the cytotoxic nature of hydrophobic bile acids, concerns have been raised as to the safety of using bile acids as pharmaceuticals. To avoid this issue, much research has evaluated semi-synthetic analogues or synthetic TGR5 or FXR agonists as potential therapeutics for metabolic diseases.

Studies on targeting FXR regulation of metabolism appear to be contradictory. The FXR agonist obeticholic acid (OCA) has delivered promising results in clinical trials for liver-based metabolic

diseases and T2DM in non-pregnant adults, including improving insulin sensitivity (135). However, in mouse models of GDM, OCA did not produce the full effects seen in other studies; whilst treatment reduced plasma cholesterol, glucose tolerance was not improved (136). Research into FXR-specific therapeutics for gestational disorders such as ICP or GDM is also currently lacking. However, FXR agonism provides some benefits in mouse models of hypercholanaemia, with improved fetal bile acid profiles (137,138).

Likewise, TGR5 agonism has been researched as a therapeutic for ameliorating symptoms of diabetes, with TGR5 agonists in diabetic mice improving glucose homeostasis (41). However, to date, no research has looked specifically at the impact of TGR5 receptor activation on biochemical features of GDM. Several novel TGR5 agonists exist and many show encouraging effects when used in animal models of diabetes (139–144). One recent example used a novel, orally administered TGR5 agonist, RDX8940. This induced incretin secretion and improved insulin sensitivity with minimal side effects in western diet-fed mice (144). Another study which used another novel TGR5 agonist, WB403, on a model of T2DM mice improved glucose tolerance and decreased fasting blood glucose. WB403 administration caused changes at the islet level, increasing pancreatic β-cells in mice (142). Clinical trials using TGR5 selective agonists have also been carried out with promising results (145). Side effects are a major concern for novel TGR5 agonists due to TGR5 having broad multi-organ expression. However, using organ-restricted agonists would avoid these side effects and would consequently likely have acceptable safety profiles. Currently TGR5 agonists with low intestinal absorption rates have been designed that may have therapeutic value. Studies have shown their ability to cross cell membranes and that they show specificity for TGR5 receptors without contributing to systemic absorption (146,147). Lasalle *et al.* 2017 designed a gut-restricted TGR5 agonist named compound 24. When used in diet-induced obese and insulin resistant mice, sustained GLP-1 release and decreased fasted plasma insulin levels were observed with low systemic levels of compound 24 detected. Many of these agonists could have the potential in alleviating GDM symptoms, though further studies are required to explore the relationship between TGR5 and GDM.

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Bile acids for the treatment of diabetes are currently being investigated. A recent study of UDCA treatment in people with T2DM and chronic liver disease resulted in weight loss and reduction of HbA1c over 12 weeks (148). Furthermore, a meta-analysis showed significant reductions in fasting plasma glucose, HbA1c and plasma insulin concentrations in UDCA treated people with non-alcoholic fatty liver disease (a disorder linked with T2DM and previous GDM) (149,150). The improvements seen in UDCA treatment for T2DM could translate as a plausible treatment for those who have GDM and future studies could look to see if these improvements in T2DM could be replicated in GDM patients.

Conclusions

Bile acids are signalling molecules that influence energy metabolism. Emerging research is revealing that bile acids and their receptors contribute to modulation of bile acid, lipid and glucose metabolism and that they influence the pathophysiology of diseases, including the gestational disorders ICP and GDM. Research into FXR has expanded the knowledge of not only how the body maintains tight control of bile acid production and export but has revealed additional roles for this nuclear receptor in lipid and glucose metabolism. Conflicting studies exist detailing how FXR activation alters glucose homeostasis and further research is necessary to clarify its role in disorders of glucose homeostasis. On the other hand, TGR5 stimulation in the gut, and associated release of GLP-1, is likely to be valuable for modulating gestational diseases in which women display glucose intolerance. Due to TGR5 expression in numerous organs, organ-specific TGR5 agonists could be an attractive option for amelioration of symptoms GDM. While the relationship between ICP and bile acids is well established, the potential relationship between bile acids and susceptibility to GDM is currently less well understood. More detailed investigation of the impact of therapeutic targeting of bile acid receptors is likely to provide data to establish whether this will improve metabolic derangements in ICP and GDM, and therefore future maternal and child health.

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Figure legends

Figure 1: Enterohepatic Circulation of Bile Acids

Schematic detailing the formation and export of bile acids into the intestinal tract. Bile acids are modified from primary to secondary forms by and deconjugation and 7α-dehydroxylase produced by gut bacteria. The majority of bile acids are reabsorbed through enterocytes (approximately 95%), with the remainder (~5%) excreted in the faeces. Reabsorbed bile acids in the L-cells activate TGR5 on the basolateral side which potentiates GLP-1 release. The reabsorbed bile acids are transported back to the liver via the portal vein, completing the enterohepatic cycle. FXR, Farenesoid X receptor; TGR5, Takeda G-protein coupled receptor 5; GLP-1, Glucagon-like peptide-1; ASBT, Apical sodium-dependent bile acid transporter; OST α/β, Organic solute transporter α/β; NTCP, Taurocholate co-transporting polypeptide; OATP, Organic anion transporting polypeptides; FGFR4, Fibroblast growth factor receptor 4; CYP7A1, Cholesterol 7α-hydroxylase; MDR3, Multidrug resistance protein; BSEP, Bile salt export pump; ABCG5/8, ATP-binding cassette transporters G5/G8 heterodimer; FGF19/15, Fibroblast growth factor 19/15.

Figure 2: Bile acid structures

Pathways displaying the formation and structures of primary and secondary bile acids in their unconjugated form, derived from cholesterol.

Figure 3: Order of potency of bile acids for FXR and TGR5

Potency of bile acids to FXR and TGR5. CDCA and LCA most potent to FXR and TGR5 respectively, with CA being least potent for both receptors. 3D structures created using PerkinElmer ChemDraw. EC50 refers to the concentration that gives half-maximal response.
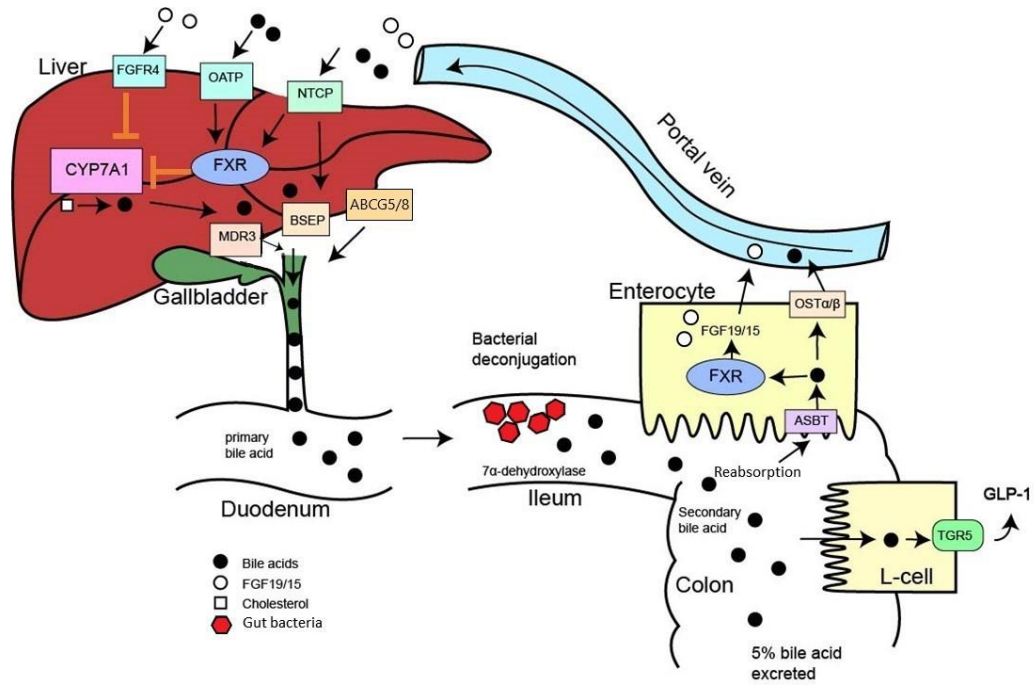
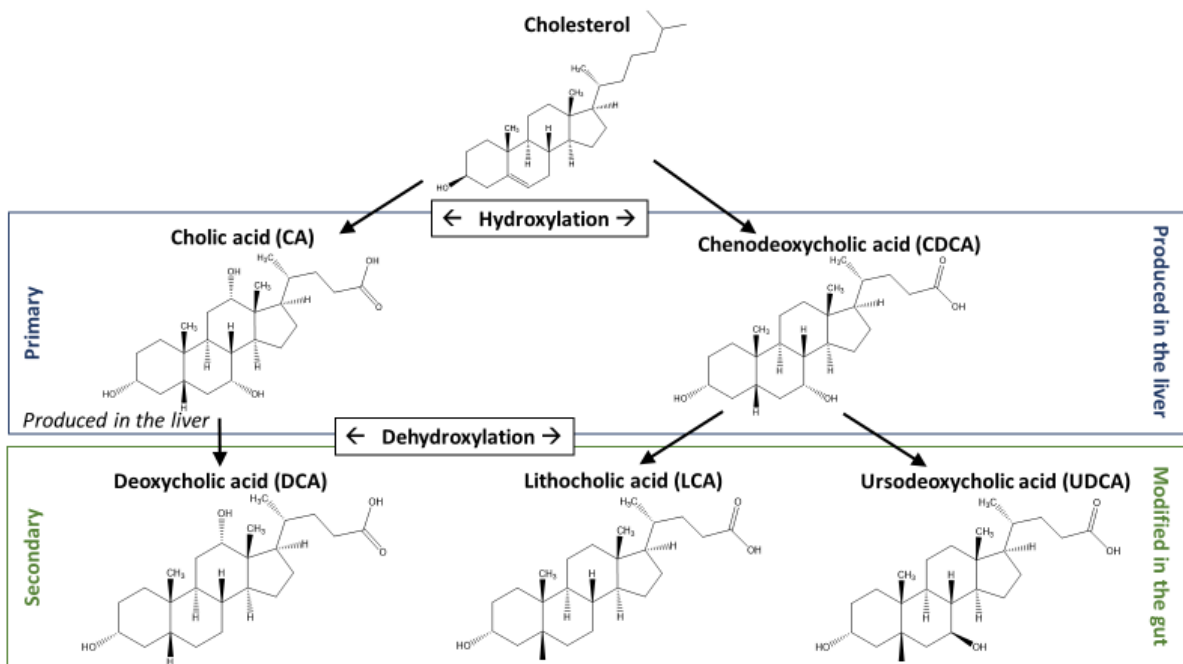Figure 1: Enterohepatic Circulation of Bile Acids.
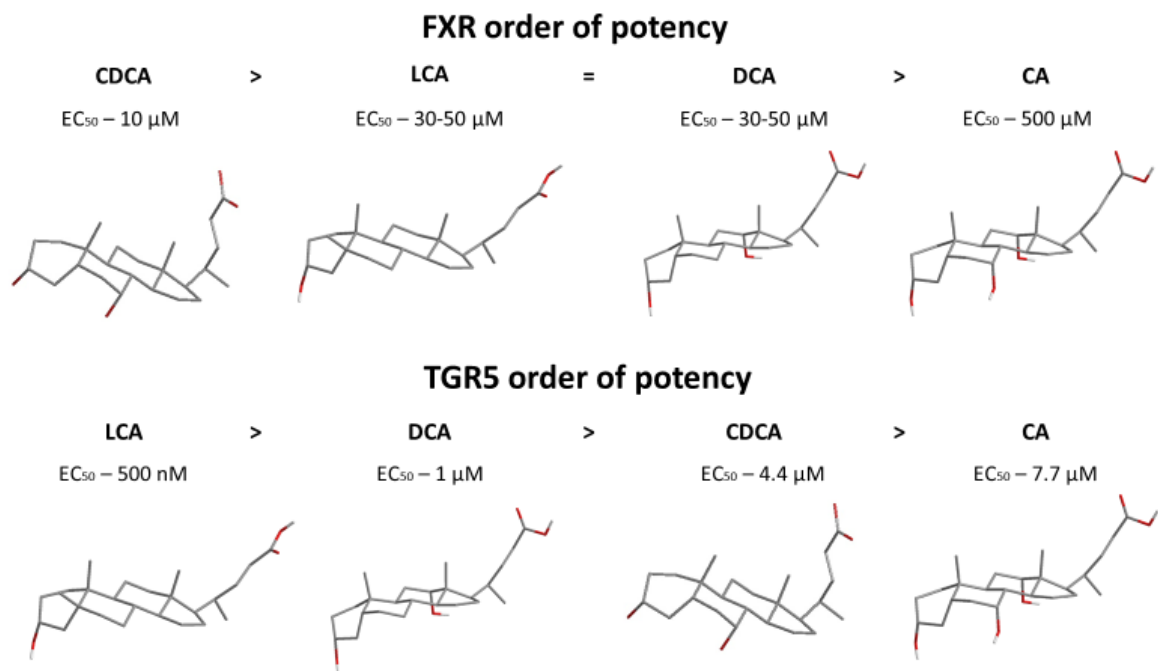

Figure 2: Bile acid Structures.

Figure 3: Order of potency of bile acids for FXR and TGR5

Appendix 2

TRPM3α2 plasmid insert sequence (pcDNA3.1) (https://www.ncbi.nlm.nih.gov/nuccore/AJ544534)

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181   caccggctca gaaatcctgg attgaaagag cattttataa aagagaatgt gttcacatca
241   taccgagcac caaagacccc cataggtgtt gctgtggacg cctcataggc cagcatgttg
301   gactcactcc cagtatctct gtgcttcaga atgagaaaaa tgagagtcgc ctctcccgaa
361   atgacatcca gtctgagaag tggtctatca gcaaacacac tcagctcagc ccaacagacg
421   cttttgggac cattgagttc caaggaggtg gccattccaa caaagctatg tatgtccgag
481   tatctttga tacgaaacct gatctcttgc tacacctgat gaccaaggaa tggcaactgg
541   agcttcccaa acttctcatc tccgtgcacg gagggctgca gaactttgaa ctccagccca
601   aactcaagca agtcttcggg aagggggctca tcaaagcagc catgacaact ggagcctgga
661   tcttcactgg aggggtcaac acaggtgtca ttcgtcatgt tggagatgcc ttgaaagacc
721   atgcatctaa gtctcgaggg aagatatgta ccataggtat cgcccctgg ggaattgtgg
781   aaaaccagga agacctgatt ggaagagatg tagtccgacc ataccagacc atgtccaatc
841   ctatgagcaa gctcaccgtt ctcaatagca tgcactccca cttcatcctg gctgacaatg
901   ggaccacggg gaaatacgga gcagaggtga aactccgcag acaactggaa aagcacattt
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Supplementary Table 1: Individual and total bile acids in Cohort 1.

Bile acid	Cohort 1: HAPO study						Significance
	FPG ≤ 4.3 mmol/l			FPG ≥ 5.1 mmol/l			
	Median	25%	75%	Median	25%	75%	
TBA	2.150	1.265	3.540	2.490	1.335	4.130	NS
UDCA	0.000	0.000	0.019	0.000	0.000	0.030	NS
CDCA	0.048	0.000	0.177	0.096	0.022	0.225	NS
DCA	0.516	0.216	1.733	0.634	0.218	1.761	NS
CA	0.145	0.088	0.322	0.156	0.091	0.347	NS
LCA	0.053	0.044	0.063	0.055	0.043	0.064	NS
GUDCA	0.019	0.000	0.038	0.019	0.000	0.040	NS
GCDCA	0.199	0.098	0.341	0.221	0.133	0.453	NS
GDCA	0.121	0.047	0.220	0.149	0.061	0.270	NS
GCA	0.155	0.109	0.258	0.199	0.130	0.355	*
GLCA	0.024	0.000	0.035	0.025	0.000	0.032	NS
TUDCA	0.00	0.000	0.000	0.000	0.000	0.000	NS
TCDCA	0.049	0.023	0.107	0.061	0.031	0.111	NS
TDCA	0.040	0.000	0.080	0.046	0.001	0.102	NS
TCA	0.026	0.000	0.066	0.036	0.005	0.096	NS
TLCA	0.000	0.000	0.023	0.000	0.000	0.004	NS

Fasting serum samples from cohort 1 (HAPO study) were assayed for bile acids in women from the lowest (n=87) and highest (n=85) quartiles of FPG. Significant differences indicated by: * $P < 0.05$, as determined by Mann-Whitney U test. Data expressed as median and interquartile range. NS; no significance, TBA; Total bile acids.

Supplementary Table 2: Individual and total bile acids in Cohort 2.

Bile acid	Cohort 2: GDM study						Significance
	Non-GDM			GDM			
	Median	25%	75%	Median	25%	75%	
TBA	1.363	0.764	1.860	1.500	0.639	2.509	NS
UDCA	0.010	0.005	0.020	0.020	0.010	0.030	NS
CDCA	0.030	0.013	0.090	0.030	0.016	0.051	NS
DCA	0.120	0.056	0.310	0.135	0.069	0.370	NS
CA	0.040	0.030	0.110	0.050	0.030	0.085	NS
LCA	0.010	0.005	0.020	0.010	0.005	0.020	NS
GUDCA	0.016	0.004	0.030	0.020	0.008	0.035	NS
GCDCA	0.267	0.140	0.450	0.220	0.120	0.470	NS
GDCA	0.110	0.056	0.252	0.140	0.057	0.295	NS
GCA	0.151	0.088	0.301	0.180	0.096	0.345	NS
GLCA	0.000	0.000	0.010	0.010	0.000	0.010	NS
TUDCA	0.000	0.000	0.000	0.000	0.000	0.000	NS
TCDCA	0.110	0.056	0. 200	0.100	0.053	0.215	NS
TDCA	0.050	0.020	0.084	0.080	0.022	0.150	NS
TCA	0.070	0.030	0.140	0.100	0.042	0.155	NS
TLCA	0.000	0.000	0.000	0.000	0.000	0.000	NS

Fasting serum samples from cohort 2 were assayed for bile acids in women with non-GDM (n=64) and GDM (n=25). No significant differences were found using Mann-Whitney U tests. Data expressed as median and interquartile range. NS; no significance, TBA; Total bile acids.

**Supplementary Table 3: Individual and total bile acids in Cohort 3 with BMI ≤25 kg/m^2.**

Bile acid	Cohort 3: 11-13 weeks, BMI ≤25 kg/m^2						Significance
	Non-GDM			GDM			
	Median	25%	75%	Median	25%	75%	
TBA	3.143	1.609	4.960	3.132	2.365	4.799	NS
UDCA	0.008	0.003	0.025	0.009	0.003	0.032	NS
CDCA	0.056	0.027	0.101	0.060	0.022	0.122	NS
DCA	0.128	0.062	0.287	0.208	0.120	0.353	NS
CA	0.061	0.036	0.166	0.057	0.035	0.125	NS
LCA	0.014	0.008	0.020	0.015	0.011	0.022	NS
GUDCA	0.060	0.023	0.103	0.057	0.023	0.133	NS
GCDCA	1.131	0.484	1.552	1.046	0.743	1.661	NS
GDCA	0.294	0.086	0.842	0.461	0.283	0.882	NS
GCA	0.320	0.134	0.684	0.342	0.186	0.523	NS
GLCA	0.017	0.007	0.050	0.024	0.002	0.044	NS
TUDCA	0.002	0.000	0.008	0.004	0.000	0.008	NS
TCDCA	0.201	0.086	0.386	0.234	0.163	0.343	NS
TDCA	0.090	0.025	0.184	0.129	0.069	0.255	NS
TCA	0.081	0.032	0.201	0.104	0.054	0.174	NS
TLCA	0.004	0.002	0.013	0.007	0.002	0.013	*

Fasting serum samples from cohort 3 (BMI ≤25 kg/m^2) were assayed for bile acids in women who either developed GDM (n=50) or had uncomplicated pregnancies (n=50). Significant differences indicated by: * $P < 0.05$, as determined by Mann-Whitney U test. Data expressed as median and interquartile range. NS; no significance, TBA; Total bile acids.

**Supplementary Table 4: Individual and total bile acids in Cohort 3 with BMI ≥35 kg/m².**

Bile acid	Cohort 3: 11-13 weeks, BMI ≥35 kg/m²						Significance
	Non-GDM			GDM			
	Median	25%	75%	Median	25%	75%	
TBA	2.717	1.868	4.299	3.144	1.916	4.599	NS
UDCA	0.0148	0.005	0.035	0.019	0.008	0.033	NS
CDCA	0.068	0.034	0.214	0.071	0.040	0.225	NS
DCA	0.193	0.072	0.338	0.134	0.064	0.283	NS
CA	0.066	0.041	0.304	0.090	0.044	0.175	NS
LCA	0.011	0.008	0.015	0.011	0.008	0.018	NS
GUDCA	0.062	0.023	0.115	0.067	0.032	0.122	NS
GCDCA	0.826	0.519	1.380	1.192	0.618	1.929	NS
GDCA	0.277	0.125	0.546	0.312	0.144	0.533	NS
GCA	0.284	0.142	0.509	0.435	0.159	0.636	NS
GLCA	0.011	0.007	0.019	0.012	0.007	0.022	NS
TUDCA	0.004	0.002	0.008	0.002	0.000	0.009	NS
TCDCA	0.177	0.111	0.352	0.189	0.087	0.477	NS
TDCA	0.076	0.034	0.133	0.072	0.028	0.127	NS
TCA	0.082	0.057	0.154	0.103	0.038	0.240	NS
TLCA	0.002	0.000	0.004	0.002	0.000	0.006	NS

Fasting serum samples from cohort 3 (BMI ≥35 kg/m²) were assayed for bile acids in women who either developed GDM (n=50) or had uncomplicated pregnancies (n=50). No significant differences were found using Mann-Whitney U tests. Data expressed as median and interquartile range. NS; no significance, TBA; Total bile acids.
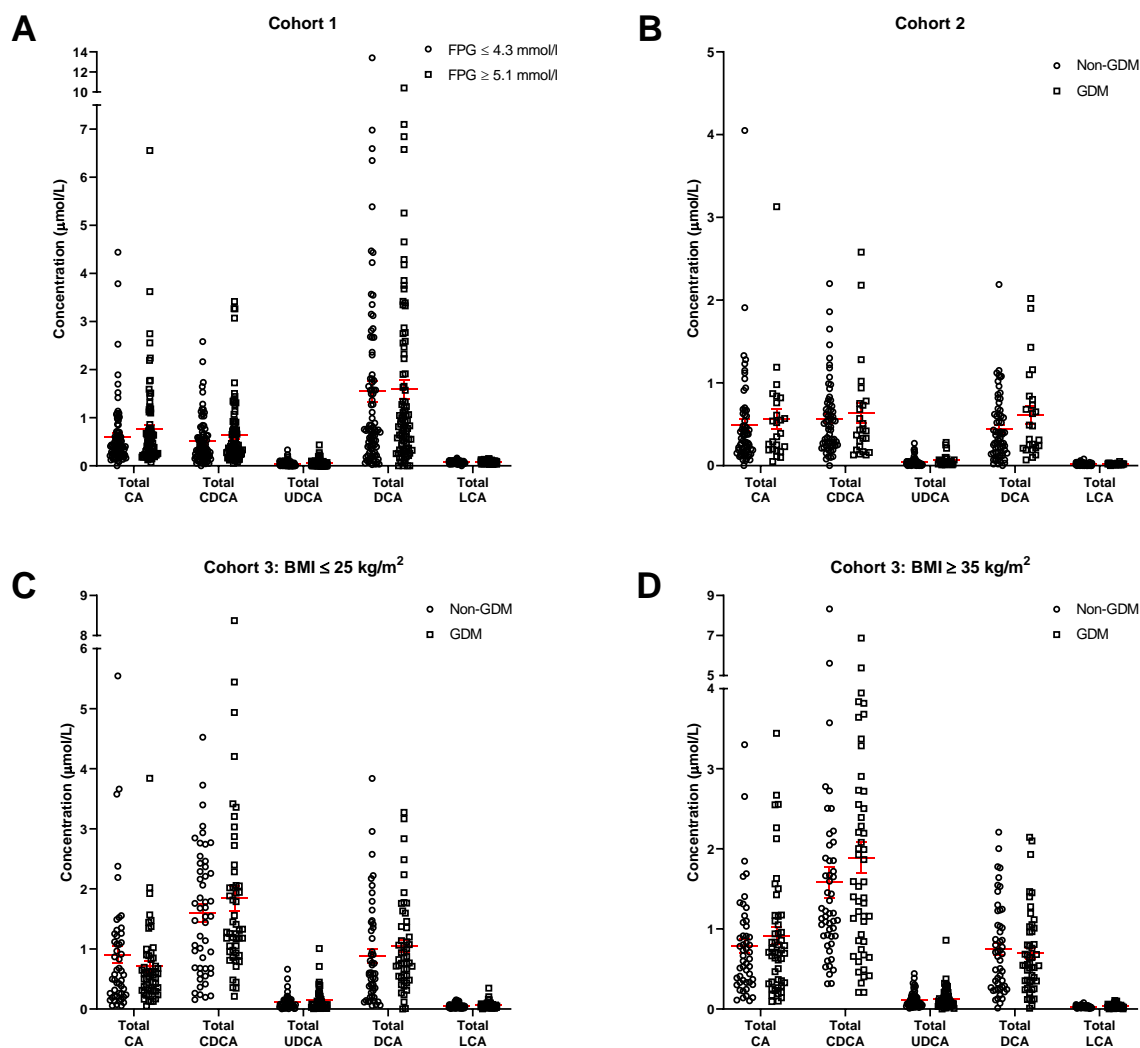
Supplementary Figure 1: Total conjugated and unconjugated forms of each bile acid in the first 3 cohorts.

The unconjugated and conjugated forms of each bile acid were totalled up and analysed for differences in the first 3 cohorts. **(A)** cohort 1 containing samples from the lower (n=87) and upper quartile of FPG (n=85). **(B)** were cohort 2 having women with GDM (n=25) and non-GDM (n=64). Cohort 3, samples from early pregnancy, 11-13 weeks, and were separated according to whether they developed GDM or not in late pregnancy. They were further divided by BMI: **(C)** were women with BMI ≤25 kg/m^2 and **(D)** were women with BMI ≥35 kg/m^2, n=50 per group. Data expressed as mean ± SEM. Data expressed as mean ± SEM.
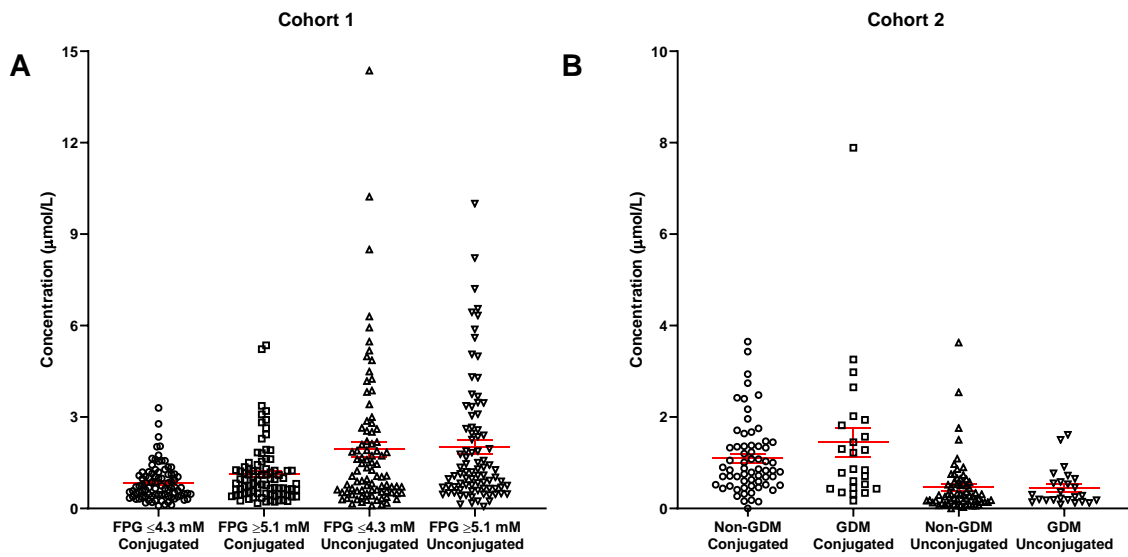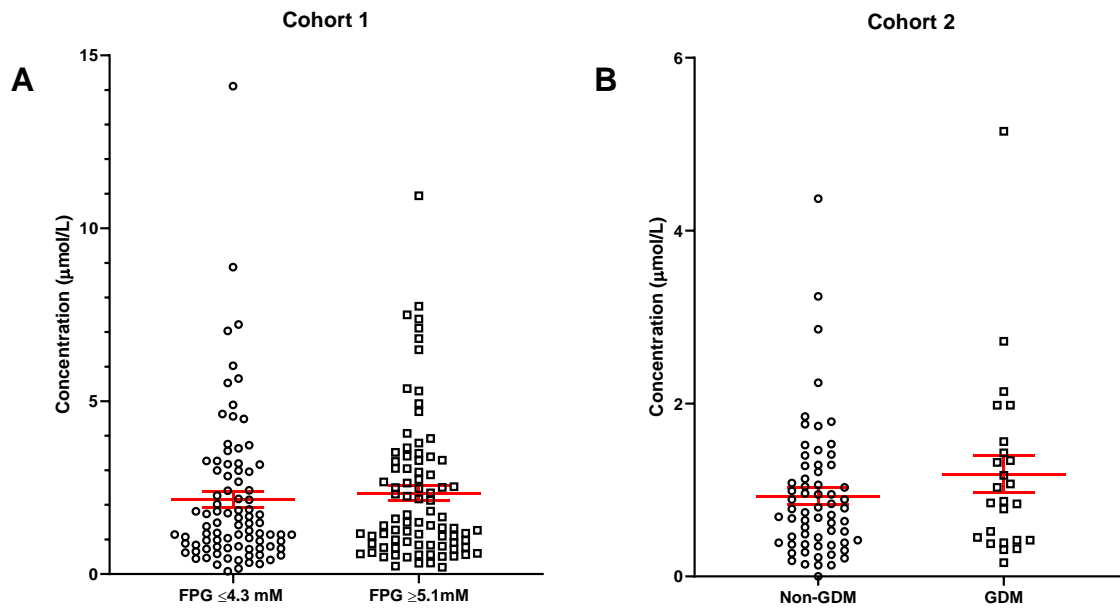
Supplementary Figure 2: Differences in concentrations of conjugated and unconjugated bile acids in cohorts 1 and 2.

Total conjugated and unconjugated bile acids were analysed for changes in all cohorts. **(A)** shows the results from cohort 1 containing samples from the lowest quartile of FPG (n=87) and the upper quartile (n=85). **(B)** were samples from cohort 2 having women with GDM (n=25) and non-GDM (n=64). Data expressed as mean ± SEM.
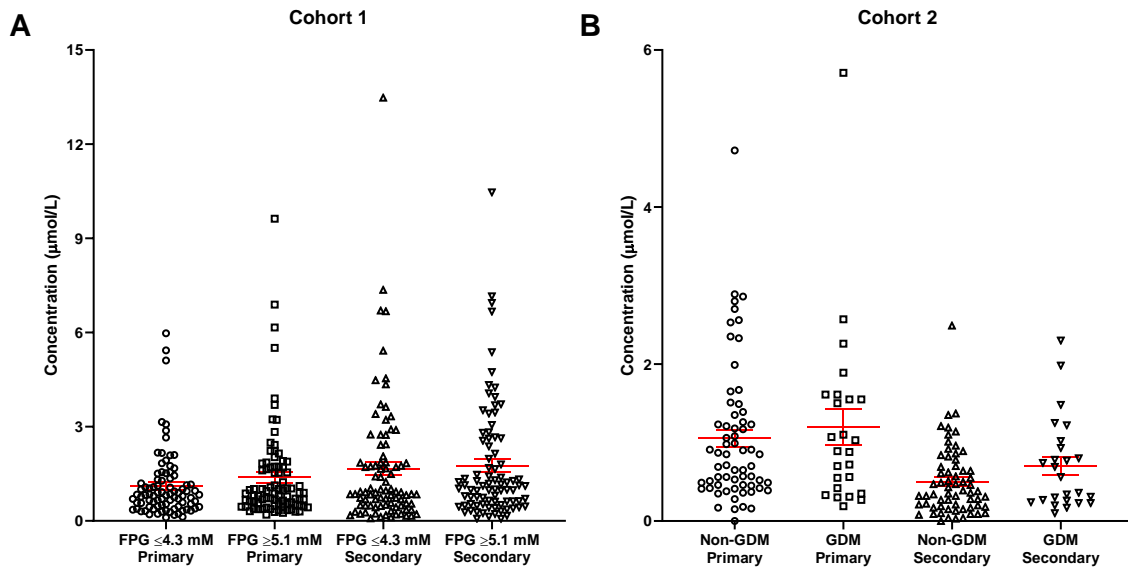
Supplementary Figure 3: Differences in concentrations of primary and secondary bile acids in cohorts 1 and 2.

Total primary and secondary bile acids were analysed for changes within each of the cohorts. **(A)** displays the results from cohort 1 containing samples from the lowest quartile (n=87) and the upper quartile (n=85). **(B)** were samples from cohort 2 having women with GDM (n=25) and non-GDM (n=64). Data expressed as mean ± SEM.

Supplementary Figure 4: Differences in 12α-hydroxylated bile acids in cohorts 1 and 2.

Total 12α-hydroxylated bile acids were calculated and analysed for differences in all cohorts. **(A)** were results from cohort 1 containing samples from the lowest quartiles of FPG (n=87) and the upper quartile (n=85). **(B)** were from cohort 2 having women with GDM (n=25) and non-GDM (n=64). Data expressed as mean ± SEM.