ROLE OF OBESITY AND GESTATIONAL DIABETES MELLITUS STATUS ON THE EXPRESSION OF KISSPEPTIN, INFLAMMATORY MARKERS AND OTHER ENDOCRINE SIGNALS, AND THEIR CORRELATION WITH FOETAL OUTCOMES AND PLACENTAL STRUCTURE

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DECLARATION

I, Ezekiel Musa, hereby declare that this thesis is my original work, is not copied from any other person's work (published or unpublished). This work has never previously been submitted in any form in an application for another degree at the University of Cape Town or elsewhere.

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DEDICATION

I dedicate this thesis to God Almighty for His mercy, favour and grace, and to my beloved wife, Glory, and two children, Shalom and Shadrach, for their love, sacrifice and understanding during the period of undertaking this project.

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LIST OF ABBREVIATIONS

ADA	American Diabetes Association
AgRP	Agouti-related peptide
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
APGAR	Appearance, Pulse, Grimace, Activity, and Respiration
B2M	Beta 2 microglobulin
BCA	Bicinchoninic acid
BMI	Body mass index
BSA	Bovine serum albumin
cDNA	Complementary DNA
CSH1	Chorionic somatomammotropin hormone 1
CSH2	Chorionic somatomammotropin hormone 2
CSH	Chorionic somatomammotropin hormone
CVD	Cardiovascular disease
CYP11A1	Cytochrome P450 family 11 subfamily A member 1
CYP19A1	Cytochrome P450 family 19 subfamily A member 1
DIP	Diabetes in pregnancy
EGF	Epidermal growth factor

EIA	Enzyme immunoassay
ELISAs	Enzyme-linked Immunosorbent Assays
EV	Extracellular vesicles
EVT	Extra-villous trophoblast
FC	Foetal capillary
FCR	Fc receptor
FCS	Foetal calf serum
FPG	Fasting plasma glucose
F/P	Foetal birth weight/placenta weight
fPBS	Free phosphate-buffered saline
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GDM	Gestational diabetes mellitus
GHV	Growth hormone variant
GLP-1	Glucagon-like peptide 1
GLUT4	Glucose transporter 4
GnRH	Gonadotropin releasing hormone
GPR-54	G-protein coupled receptor-54
GSIS	Glucose-stimulated insulin secretion
H&E	Haematoxylin and eosin

H_2O_2	Hydrogen peroxide
HAPO	Hyperglycemia and Adverse Pregnancy Outcome
HIP	Hyperglycaemia in pregnancy
HOMA-IR	Homeostatic Model Assessment of Insulin Resistance
hPL	Human placental lactogen
hPL1	Human placental lactogen 1
hPL2	Human placental lactogen 2
HREC	Human research ethics committee
HSD3B1	3-Beta-hydroxysteroid dehydrogenase 1
IADPSG	International Association of Diabetes in Pregnancy Study Groups
ICV	Intracerebroventricular
i.e.	That is
IFN-γ	Interferon-gamma
IGF2	Insulin-like growth factor 2
IL-10	Interleukin 10
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
IRS	Immunoreactive score
IRS-1	Insulin receptor substrate-1

IUGR	Intrauterine growth restriction
IV	Intravenous
KCNQ1	Potassium voltage-gated channel KQT-like 1
kDa	Kilodalton
KISS1	Kisspeptin
KISS1R	Kisspeptin 1 receptor
MAPK	Mitogen-activated protein kinase
MBS	Maternal blood space
MMP	Matrix metalloproteinase
NPY	Neuropeptide Y
OD	Optical density
OGTT	Oral glucose tolerance test
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline tween
PCOS	Polycystic ovary syndrome
PI	Phosphatidylinositol
PLAP	Placental alkaline phosphatase
POMC	Pro-opiomelanocortin
PPAR-α	Peroxisome proliferator-activated receptor alpha

PVDF	Polyvinylidene difluoride
RIPA	Radioimmunoprecipitation
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Real-time polymerase chain reaction
RT-qPCR	Real-time quantitative polymerase chain reaction
SA-HRP	Streptavidin horseradish peroxidase
SDC	Specific diffusion capacity
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	standard error of mean
SBP	Systolic blood pressure
STB	Syncytiotrophoblast
STBEV	syncytiotrophoblast EV
TBST	Tris-buffered saline tween
TDC	Theoretical diffusion capacity
TGF-β	Transforming growth factor beta
TMB	Tetramethylbenzidine
TNFα	Tumour necrosis factor alpha
VEGF	Vascular endothelial growth factor

VEGF-A	Vascular endothelial growth factor A
WHO	World Health Organization

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ABSTRACT

Background: Maternal obesity and gestational diabetes mellitus (GDM) are associated with short and long-term health risks for the mother and child. The placenta produces hormones, including steroids and cytokines, that influence maternal glucose control. Current literature links kisspeptins with glucose-stimulated insulin secretion, and low plasma kisspeptin concentrations have been associated with GDM and markers of insulin resistance. In addition, maternal obesity is characterized by low-grade inflammation and insulin resistance. However, little is known about the effect of maternal obesity and GDM and their interaction on placental kisspeptin and inflammatory marker (TNFα, IL-6) expression, the relationship between placental and circulatory kisspeptin and inflammatory markers with placental villous morphology and maternal and neonatal parameters. There is also a paucity of data on the effect of maternal obesity and GDM on other endocrine signals (leptin, placental lactogen family members), growth factors (IGF2, VEGF), and steroidogenic hormone enzyme gene expression in the placenta.

Aim: This work aimed to examine the effect of maternal obesity and/or GDM on molecular expression (placental, maternal and cord serum) of kisspeptin and inflammatory markers (TNFα, IL-6) and placental morphology, and how these effects relate to maternal and neonatal clinical parameters. Additionally, the work aimed to investigate the effect of maternal obesity and/or GDM on leptin, placental lactogen family members, growth factors, and steroidogenic hormone enzymes gene expression in the placenta.

Methods: This study included 4 groups of South African pregnant women: Non-GDM, Non-obese (n=14); Non-GDM, Obese (n=19); GDM, Non-obese (n=15); GDM-Obese (n=23). At delivery,

the women's placental tissue was fixed and processed for immunohistochemistry and histological assessment as well as snap-frozen for RT-qPCR and Western Blot analysis. Maternal and cord blood were also collected for the measurement of placenta-derived factors by ELISA. Data were compared by two-way ANOVA with Bonferroni multiple comparisons tests.

Results: Maternal obesity and GDM had no effect on placental kisspeptin gene and protein expression, immunostaining or circulatory levels. There was a significant negative correlation between placental *kisspeptin* gene expression and volume of villous syncytiotrophoblasts and theoretical diffusion capacity in non-obese women irrespective of their GDM status. There was a significant inverse correlation between plasma kisspeptin protein and BMI and maternal systolic blood pressure in GDM women regardless of obesity. Cord serum kisspeptin concentration correlated negatively with BMI. Maternal obesity reduced placental *Leptin* and *KISS1R* gene expression in the absence of GDM. Overall, placental TNFα protein abundance by immunostaining was significantly higher in women with obesity irrespective of GDM status. TNFα staining of terminal villi syncytiotrophoblast was increased in women with obesity, while IL-6 staining of terminal villi stroma and foetal vessels was reduced in obese women, significantly so in GDM women. There was a positive correlation between the expression of placental *TNFα* gene and IL-6 protein and maternal diastolic blood pressure in obese non-GDM and GDM women, respectively. In obese non-GDM women, maternal serum TNFα and IL-6 concentrations correlated negatively with placenta weight, foetoplacental ratio and volume of intervillous space, and theoretical and specific diffusion capacity, respectively. Women with obesity showed fewer terminal villi with fewer syncytiotrophoblast, foetal vessels and stroma dependent on GDM diagnosis while GDM influenced intervillous space volume by increasing it in obese groups. Maternal obesity also affected the surface areas for maternal-foetal exchange; the surface area of maternal blood space

and foetal capillary were reduced in women with obesity regardless of GDM status. Again, the physiological diffusion gradients for oxygen transfer at the maternal-foetal interface were significantly reduced by maternal obesity.

Conclusion: This study shows neither maternal obesity nor GDM influences kisspeptin levels, although maternal obesity in the absence of GDM seems to downregulate KISS1R, which may impact the kisspeptin-KISS1R signalling pathway. GDM rather than obesity may have a greater effect on TNF-α mediated maternal circulatory and placental inflammation. This study suggests that placental inflammation and insulin resistance may have a relationship with hypertension. In obese women alone, maternal inflammatory cytokines seem to be associated with altered placental structure and function. Indeed, maternal obesity was shown to compromise placental maturation and decrease the surface area and diffusion capacity required for maternal-foetal nutrient and oxygen exchange. These observations are likely to contribute novel insights into the interplay between metabolic dysfunction, obesity, and inflammation in the pathophysiology of GDM and placental dysfunction.

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CHAPTER TWO- MATERIALS AND METHODS

This chapter provides an overview of the literature in relation to gestational diabetes mellitus (GDM) and maternal obesity with kisspeptin, inflammatory markers and other placental endocrine signals that have potential implications for GDM, as well as an overview of human placenta structure and function. The literature was sourced using the following keywords: GDM, obesity, insulin resistance, glucose-stimulated insulin secretion, placenta, syncytiotrophoblast extracellular vesicles, kisspeptin, kisspeptin 1 receptor (KISS1R), TNFα, IL-6, leptin, VEGF, IGF2, sex steroids and placental lactogen family in PubMed, Google Scholar and simple Google search. The chapter concludes with a justification, research questions, hypotheses, aim and specific objectives of the study.

1.0 Maternal Hyperglycaemia and Gestational Diabetes Mellitus in Pregnancy

GDM is associated with short-term adverse pregnancy outcomes in both the mother and offspring, including increased caesarean section rate, preeclampsia, macrosomia, large for gestational age, perinatal mortality and neural tube defects.[1] GDM is also associated with long-term health consequences, such as the increased risk for type 2 diabetes and cardiovascular disease (CVD) in the mother and future obesity, CVD, type 2 diabetes, hypertension, metabolic syndrome and GDM in the offspring.[1-5]

Prior to 2013, the criteria for GDM diagnosis were based on the maternal risk for diabetes following the pregnancy.[6] In 2013, the World Health Organization (WHO) updated the guidelines, which are based on the association of plasma glucose and adverse maternal and neonatal outcomes during pregnancy, at birth and immediately following it.[7] The evidence underpinning the new guideline came from the Hyperglycemia and Adverse Pregnancy Outcome (HAPO) study, an

international multicentre study in over 25,000 pregnant women.[8] The new WHO guidelines adopted the same diagnostic cut-points for GDM used in HAPO and further recommended that hyperglycaemia first detected at any time during pregnancy be separated into two categories - diabetes mellitus in pregnancy (DIP) or GDM.[9] Hyperglycaemia in pregnancy (HIP) which includes DIP and GDM, is reported as the commonest metabolic disorder encountered during pregnancy.[10]

1.1 Epidemiology and Risk Factors of GDM

The lowered criteria for GDM proposed by the WHO and the International Association of the Diabetes and Pregnancy Study Groups (IADPSG) have resulted in a rise in the reported prevalence of GDM. A systematic review of the prevalence of GDM in Africa published in 2019 found a pooled prevalence of 13.6% using 2013 WHO or ADA or IADPSG diagnostic criteria.[11] In South Africa, two recent studies in the Gauteng Province have, however, reported a widely varying GDM prevalence of 9.1% and 25.8% when using the WHO 2013 and IADPSG glycaemic diagnostic criteria, respectively.[12, 13]

Many risk factors for GDM have been reported. These include overweight and obesity, family and personal history of GDM, westernised diets, excessive gestational weight gain, family history of type 2 diabetes, polycystic ovary syndrome (PCOS), previous unexplained stillbirth, foetal macrosomia, low birth weight and increasing maternal age above 30 years.[14-21] Other risk factors include prediabetes, short stature, multiple gestation, genetic susceptibility, as well as race and ethnicity.[22] These risk factors are intrinsically linked to either impaired pancreatic beta-cell function, insulin resistance, or both. For example, overweight and obesity are intimately associated with excessive calorie intake, which in turn, may overwhelm the capacity of pancreatic beta cells

to secrete insulin, cause beta cell damage, as well as impair insulin signalling pathways in tissues like skeletal muscle and white adipose to respond to the insulin, thereby leading to GDM.[23]

Obesity is a global epidemic with a rising trend in Africa and, in particular, South Africa.[24] There is also a growing prevalence of maternal obesity globally, primarily driven by increasing rates of obesity among women of reproductive age, which increases the risk of adverse pregnancy outcomes.[25, 26, 27] In 2016, a systematic review on the prevalence of maternal obesity in Africa, reported an overall prevalence of 6.5 to 50.7% in the Democratic Republic of Congo and Nigeria respectively, with older and multiparous mothers more likely to be obese. In addition, the prevalence of maternal obesity in the first trimester was reported as 9.0 and 17.9% in Ghana and Nigeria, respectively.[28] Furthermore, a study of 767 pregnant women in South Africa reported an

obesity prevalence of 44%. These data indicate a high prevalence of obesity in pregnancy on the continent.[29]

The major drivers of the obesity epidemic in Sub-Saharan Africa include rapid urbanisation, increased intake of energy-dense foods high in fat and refined sugars, increasing sedentary lifestyle and genetic predisposition.[30] Maternal obesity has significant adverse health impacts on the mother during pregnancy and the developing foetus. Studies have reported a strong association between maternal obesity and GDM development.[31] In the US, maternal obesity is associated with 1.3–3.8 times the risk of developing GDM compared to women with normal body mass index (BMI).[32] Additionally, maternal obesity is associated with early pregnancy loss, preeclampsia, increased caesarean section rate, macrosomia, congenital malformations, intrauterine growth restriction (IUGR) and stillbirth. Furthermore, in the long term, maternal obesity is associated with an increased risk of CVD, obesity, and type 2 diabetes in the offspring, as well as cardiometabolic disease in the mother.[28, 33-35]

There is a consistent link between consuming diets rich in saturated fats, refined sugars, red and processed meats, overnutrition, and a heightened risk of GDM either directly or through their association with obesity.[36, 37] Animal studies have demonstrated that high-fat diets during pregnancy may lead to endothelial damage, oxidative stress and increased placental nutrient transport, altogether strengthening the role of saturated fats in disordered glucose homeostasis, beta cell maladaptation, and consequent development of GDM.[38-40] Similarly, maternal obesity and glucose intolerance during pregnancy have been associated with increased exposure to high-fat diets.[41-43] Meanwhile, over-nutrition impairs beta-cell insulin secretion and/or adaptation due to glucotoxicity, lipotoxicity and oxidative damage of the beta cells, thereby leading to GDM.[44, 45]

Furthermore, Alzamendi et al in 2012, demonstrated that pregnant rats fed with a high fructose diet had significantly higher circulating insulin levels than those fed a normal diet, suggesting that excessive intake of high sugar diets during pregnancy may result in maternal hyperinsulinaemia and increase the risk of developing GDM.[46]

1.3 Maternal Metabolic Adaptations during Normal Pregnancy

1.3.1 Physiological Insulin Resistance

The latter part of a normal pregnancy is characterised by the acquisition of insulin resistance in the mother, promoted by the effects of maternal endocrine glands (pituitary gland and ovary) and placental-derived hormones, including progesterone, oestrogen, human placental lactogen (hPL), leptin, cortisol, and human placental growth hormone (hPGH).[47, 48] Other important mediators of insulin resistance include adipokines such as adiponectin, TNF-α, IL-6 and leptin, expressed by maternal adipose tissue and the placenta. The low-grade insulin resistance promotes endogenous glucose production and lipolysis resulting in a surge in blood glucose and free fatty acid concentrations, thereby contributing to the acquisition of a physiological insulin-resistant state in the mother and consequently increased substrate availability (glucose, lipids) for transport by the placenta to fuel foetal growth.[49]

Throughout pregnancy, hPL increases by about 30-fold, resulting in increased foetal growth and placental size. Studies have reported conflicting roles of hPL in glucose homeostasis, where it either caused glucose stimulating insulin secretion (GSIS) or induced insulin resistance.[47, 50, 51] In the second trimester of pregnancy, hPGH rises by up to 8 fold and has been reported to play a role in the development of insulin resistance.[52] Following glucose and insulin tolerance tests, transgenic mice that overexpressed placental growth hormone showed similar hPGH concentrations to third-

trimester concentrations of human pregnancy. Furthermore, the transgenic mice had increased fasting insulin levels and upregulation of the p85α subunit of phosphatidylinositol (PI) 3-kinase in the skeletal muscle, a molecule involved in the alteration of insulin signalling during normal pregnancy.[53, 54]

Additionally, progesterone induces insulin resistance due to decreased glucose transporter 4 (GLUT4) expression and decreased maximum insulin binding.[47] Plasma leptin during pregnancy is primarily produced by the placental syncytiotrophoblast and plays a role in glucose homeostasis in humans.[55] It may contribute to insulin resistance by inhibiting insulin signalling and insulin-stimulated glucose metabolism. It also correlates positively with fasting insulin levels and Homeostatic Model Assessment of Insulin Resistance (HOMA-IR).[56-58] As pregnancy advances, hyperleptinaemia develops due to leptin resistance, which mirrors insulin resistance.[23]

1.3.2 Beta Cell Adaptations to Normal Pregnancy

Beta-cells primary function is insulin synthesis, storage and secretion in response to rising glycaemia after meals. During pregnancy, there is usually accentuation in plasma insulin levels, primarily due to pancreatic beta cell mass adaptation, characterised by beta cell proliferation, growth and enhanced survival, as well as increased GSIS by the beta cells.[59, 60] Other adaptive metabolic changes in pregnancy include a reduced threshold for GSIS, increased insulin content in the beta cells, increased peripheral glucose utilization, and increased c-AMP metabolism.[59]

Most evidence on beta-cell adaptation in human pregnancy comes from in vitro studies on post-mortem pancreatic tissue. In these in vitro studies, Butler and Van Assche et al demonstrated a 1.4-2.4-fold increase in beta-cell mass and the percentage of beta cell hyperplasia.[61, 62] While, the

mechanisms for beta-cell adaptation to pregnancy are mostly from mouse models, most human islet cells clearly undergo adaptation to pregnancy. It is unclear if the mechanism for beta-cell adaptation in humans mirrors the mechanisms seen in rodents during pregnancy. However, there are suggestions that neogenesis may be the underlying mechanism for beta-cell mass increase in humans, rather than the beta-cell proliferation that is seen in rodents.[63-65] Placenta-derived hormones and maternal tissues that are involved in islets function upregulation modulate these changes.[59] These include hPL and prolactin, which enhance GSIS and stimulate beta cell proliferation and hypertrophy by binding to the prolactin receptor.[59] Parsons et al reported an increase in hPL concentration which correlated with increased pancreatic beta cell proliferation and function.[66]

Other hormones contributing to beta-cell adaptation and GSIS regulation are progesterone and oestrogen, although data are conflicting. Oestrogen has been reported to directly affect pancreatic beta cells, including anti-apoptosis, enhancement of GSIS and islet lipid homeostasis.[67, 68] Conversely, progesterone receptor knock-out female mice showed enhancement in beta-cell proliferation and mass, insulin secretion and improved glucose tolerance.[69]

In summary, normal pregnancy is characterised by pancreatic beta cell adaptation and increased GSIS for regulating glucose homeostasis. The placental-derived hormones are principal mediators of these processes.

1.4 Aetiopathogenesis of GDM

The precise mechanisms underlying the development of GDM are not fully understood, but chronic insulin resistance and pancreatic beta-cell dysfunction are thought to be central pathogenic

processes, with a complex interaction between multiple maternal genes, environmental factors and foeto-placental factors underpinning GDM development.[23, 70-72] There are myriads of potential molecular and cellular mechanisms underlying the pathophysiology of GDM with the involvement of many tissues or organs such as the placenta and the maternal pancreas, adipose tissues, brain, liver, gut, and skeletal muscle (Table 1.1).[72]

1.4.1 Maternal Obesity and Implications for GDM

Maternal obesity is usually characterised by chronic low-grade inflammation, which is associated with insulin resistance- a mechanism that leads to the development of GDM with an established relationship between increased adiposity, inflammation and insulin resistance.[73] Adipokines such as tumour necrosis factor alpha (TNFα) and interleukin 6 (IL-6) influence metabolic function and have been proposed to have pathophysiological significance in GDM. There are, however, conflicting data on TNFα levels in obesity and GDM. Hotamisligil et al observed increased TNFα mRNA and protein levels in adipose tissue of obese individuals, while some studies did not find any significant increase in the circulatory TNFα levels in obese women with or without GDM.[73-76] While Sen et al found decreased TNFα levels in isolated T-cells from obese pregnant women without GDM.[77] Maternal serum IL-6 levels have been shown to be elevated in maternal obesity and GDM regardless of obesity.[78] Intriguingly, Friis et al found that in early to mid-pregnancy, the circulating IL-6 levels increased with rising BMI with the abolishment of this increase in the later stage of gestation.[78, 79]

There are other mechanisms by which maternal obesity leads to GDM. These include leptin resistance and impaired beta-cells insulin secretory function.[80]

1.4.2 Maternal Metabolic Changes in GDM

1.4.2.1 Exaggerated Insulin Resistance

Insulin resistance occurs when there is an inadequate response of tissue or cell insulin receptors to insulin. It is generally due to insulin signalling pathway (PI3K/Akt/mTOR) failure with a resultant decrease in plasma membrane translocation of GLUT4.[81] GLUT4 is a transporter expressed by the syncytiotrophoblast, maternal adipose tissues and skeletal muscle that transports glucose into the cells for energy production.[82] Friedman et al found a significant reduction (65%) in insulin-stimulated glucose transport in human skeletal muscle fibres of obese GDM pregnancies compared to normal glucose tolerant obese pregnancies.[83] Similarly, Garvey and Birnbaum found more severely decreased adipocyte glucose transport in obese GDM compared to non-obese pregnant women.[84]

Proinflammatory cytokines and adiponectin are important modulators of insulin resistance perpetuation. They have been proposed to induce insulin resistance by promoting the accumulation of free fatty acids, sphingomyelin, stress kinases and inflammatory pathways and promotion of serine phosphorylation of insulin receptor substrate-1 (IRS-1).[85-87] Stress kinase activation causes a rise in the phosphorylation of P38 mitogen-activated protein kinase (MAPK) in white adipose tissue and skeletal muscle, leading to insulin resistance.[88]

The placenta and maternal white adipose tissue also produce hormones such as leptin, cortisol, human placental lactogen, oestradiol, progesterone and prolactin that play a role in insulin resistance during pregnancy.[86, 89] These hormones contribute to GDM by causing alteration in glucose and lipid homeostasis leading to rising glycaemia, hyperinsulinaemia, decreased maternal adipose tissues, and elevated postprandial fatty acids.[22]

1.4.2.1.1 Inflammation and its Implications in GDM: Placental TNFα has been reported to be the most important mediator of insulin insensitivity. It has been found to be the only significant predictor of the change in insulin sensitivity from pre-pregnancy to the third trimester of pregnancy relative to leptin, cortisol, hPL, human chorionic gonadotropin, estradiol, progesterone, and prolactin.[90] Carlson and Friedman et al showed a negative correlation between plasma TNFα with insulin sensitivity suggesting a role for inflammation as a mechanism underlying the pathogenesis of GDM.[91, 92]

1.4.2.1.2 Leptin and its Implications in GDM: Leptin is a hormone secreted mainly by adipocytes that acts on the neurons within the arcuate nucleus of the hypothalamus to suppress appetite and increase energy utilisation. Leptin does this by inhibiting appetite-stimulators neuropeptide Y (NPY) and agouti-related peptide (AgRP) and activating the anorexigenic polypeptide pro-opiomelanocortin (POMC).[93] During pregnancy, the human placental syncytiotrophoblast expresses and secretes leptin and is the primary source of plasma leptin.[55] In GDM, there is increased placental expression and secretion of leptin which aggravates inflammation by accelerating the production of CC-chemokine ligands and proinflammatory cytokines.[72, 94] The worsening placental inflammation leads to exaggerated insulin resistance and consequently foetal macrosomia and increased placental weight.[95] The increased placental leptin production in GDM contributes to macrosomia and elevated placental weight due to increased nutrients transport and the suppression of leptin-induced JAK2/STAT3 activation.[96]

1.4.2.1.3 Adiponectin and its Implications in GDM: Adiponectin has multiple effects on insulin sensitivity and insulin secretion. It improves skeletal muscle insulin sensitivity via increased insulin receptor tyrosine phosphorylation and suppression of TNFα.[97] Furthermore, adiponectin

decreases hepatic glucose production by stimulating adenosine monophosphate-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor alpha (PPAR-α), resulting in increased fatty acid oxidation and decreased peripheral fat storage, which enhances insulin sensitivity.[98, 99] Adiponectin also has an effect on insulin secretion. Animal and human studies have shown that adiponectin stimulates insulin secretion by the pancreatic beta cells through upregulation of insulin genes and exocytosis of insulin granules.[100, 101] Wijesekara et al demonstrated increased GSIS and increased insulin gene expression in mice islets following long-term treatment with adiponectin.[101] Adiponectin is inversely correlated with proinsulin-to-insulin ratio, a marker of beta-cell failure, and in addition, it is an independent correlate of the insulin secretion-sensitivity index, another marker of beta-cell function.[102, 103] The placental syncytiotrophoblast predominantly produces low adiponectin levels, with proinflammatory cytokines such as TNFα, IL-6, interferon-gamma (IFN-γ), and leptin acting as important modulators of adiponectin synthesis.[104] In GDM pregnancies, the placental expression and circulatory adiponectin levels are low.[105, 106] For example, Altinova and Ott et al reported lower plasma adiponectin concentrations as well as adipose tissue adiponectin mRNA expression in GDM compared to normoglycaemic pregnant women.[107, 108]

1.4.2.2 Insufficient Beta Cell Adaptations

Beta cell mass expansion (hypertrophy and hyperplasia) and GSIS enhancement are critical compensatory mechanisms that counter insulin resistance and maintain normal glucose tolerance during pregnancy.[109] GDM occurs when there is pancreatic beta cell mass maladaptation and impaired function to rising glycaemia with concomitantly decreased insulin secretion to combat maternal peripheral insulin resistance.[23, 110] Although the precise mechanisms underlying beta-cell

dysfunction are complex, it is thought to be a consequence of chronic hyperinsulinaemia in response to persistent hyperglycaemia and/or a genetic predisposition.[111]

In addition to peripheral insulin resistance, other factors such as glucotoxicity, lipotoxicity, islet inflammation and beta cell apoptosis exacerbate pancreatic beta cell failure culminating in a vicious cycle of hyperglycaemia, insulin resistance, and worsening beta cell dysfunction. Many GDM-associated gene variants, including potassium voltage-gated channel KQT-like 1 (KCNQ1), glucokinase, TCF7L2, GCKR, CDKAL1, SLC30A8, PPARG, IRS1, PRLR and MTNR1B are linked to pancreatic beta-cell function.[71, 112-116] In a Chilean population, Le et al reported that two PRLR SNPs, rs10068521 and rs9292578, were associated frequently with GDM compared to controls with a 2.36 times increased risk of GDM. Also, PRLR knockout newborn mice exhibited a 30% reduction of beta-cell mass.[117, 118] Recently, a large case-control study by Ding et al investigated 2636 GDM women and 6086 non-GDM women from the Nurses Health Study II and the Danish National Birth Cohort and found eight gene variants associated with GDM, including HNF1A, GLIS3, rSLC30A8, REB1, TCF7L2, TCF7L2, GPSM1 and GLIS3.[112]

Table 1.1 Potential Mechanisms Underlying the Development of GDM[72]

Organ/tissue	Mechanisms
Placenta	ınsulin resistance: ↑Proinflammatory cytokines (TNFα, IL-1β, IL-6) ↑Placental leptin ↑Placental transport of nutrients (glucose and fatty acid) ↑Fetal growth
Pancreas	β-cell dysfunction
Brain	↑Appetite ↓Energy expenditure
Adipose tissue	Insulin resistance: ↑Leptin ↓Adiponectin, IL-10 ↑Lipolysis, free fatty acids ↑Proinflammatory cytokines (TNFα, IL-1β, IL-6) ↑Adipocyte hypertrophy and death ↑Lipotoxicity
Muscle	Insulin resistance: Ectopic fat deposition ↓Mitochondrial function ↑ROS
Liver	Insulin resistance: ↑Gluconeogenesis Ectopic fat deposition ↑ROS

IFN-γ: interferon-γ; ROS: reactive oxygen species

1.5 Kisspeptin and Metabolic Changes: Implications for GDM

Currently, there is growing evidence that kisspeptins are important in modulating glucose homeostasis. Kisspeptins are reported to induce GSIS by the pancreas and regulate insulin sensitivity of the liver, skeletal muscle, and white adipose tissues.[119-123] Recently, low plasma kisspeptin concentrations have been associated with GDM and markers of insulin resistance.[122, 124, 125]

Before discussing the metabolic effects of kisspeptin and its role in GDM, kisspeptin evolution, structure, synthesis, and expression and function in placenta formation will be described.

1.5.1 Kisspeptin Evolution, Structure, Synthesis, and KISS1R Signaling

The *KISS1* gene was first isolated by the Welch laboratory in Hershey, Pennsylvania. It is located on chromosome 1q32 5' and undergoes exon translation.[126] Kisspeptin, formerly known as metastin, is the peptide encoded by the *KISS1* gene in humans and is the cognate ligand for the kisspeptin 1 receptor (KISS1R), which is also known as G-protein coupled receptor-54 (GPR-54).[127] Kisspeptin is formed from cleavage of the parent 145 amino acid peptide (kisspeptin-145) by furin or prohormone convertase to the 54 amino acid protein, kisspeptin-54 (metastin).[126, 128] Proteolytic cleavage of kisspeptin-54 results in bioactive kisspeptins (kisspeptin-10, 13, 14) (Figure 1.3)[126] that have a common C-terminal sequence and similar affinity to their receptor KISS1R.[129]

Upon ligand-receptor binding, the downstream intracellular signalling events such as coupling of the $_{Gq}$ subunit, activation of the phospholipase C pathway and stimulation of β-arrestin mediate

kisspeptin's essential biological functions.[126, 130-132] For instance, inhibitors of phospholipase C or p42/44 MAPK result in the loss of potentiation of GSIS while protein kinase C or p38 MAPK inhibitors result in the opposite effect.[133] Kisspeptins and KISS1R mRNA and protein are expressed in the brain, pancreas, placenta, and other tissues, including the adrenal glands, kidneys, adipose tissue, liver, small intestine, and coronary arteries.[128, 134-137]

Kisspeptin has been shown to suppress the metastatic capabilities of various types of tumour cells. In athymic nude mice, transfection of *KISS1* in malignant melanoma cells resulted in inhibition of metastasis.[138, 139] In addition, kisspeptins are essential in regulating pubertal onset and reproduction. Inactivating mutations of the KISS1R (in both humans and mice) result in idiopathic hypogonadotropic hypogonadism.[134, 140-142] Kisspeptins have also been demonstrated to play critical regulatory roles in embryo implantation, trophoblast invasion, maternal immune tolerance and early placental development, all indispensable components of early gestation.[130, 143, 144]

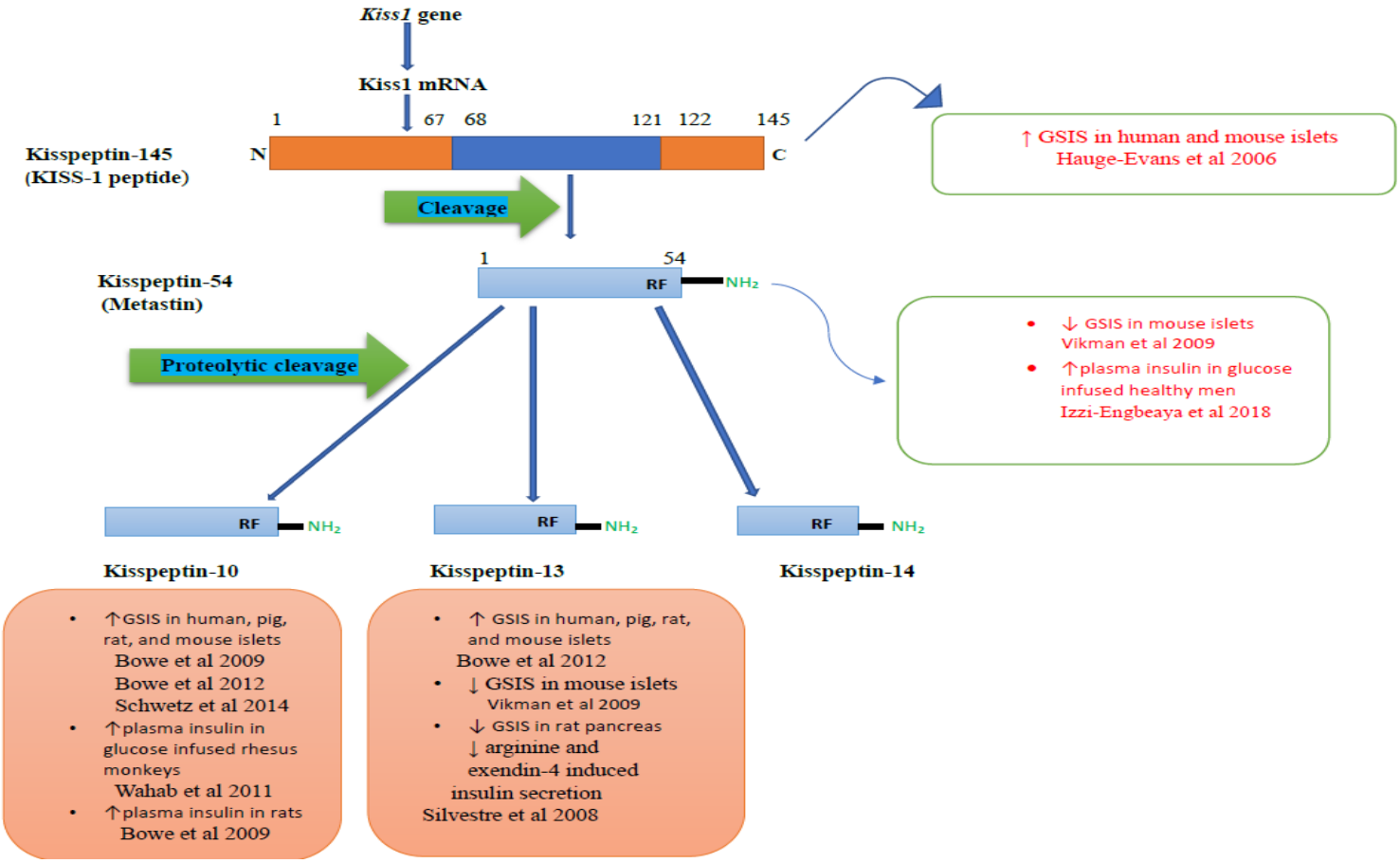


Figure 1.1. Kisspeptin bioactive forms and their putative roles on insulin secretion

1.5.2 Kisspeptin Expression and Function in Placenta Formation (Trophoblast Invasion and Angiogenesis)

Kisspeptin expression has been found in the human placenta and that of other mammalian species (Table 1.2). Kisspeptin mRNA and protein are predominantly expressed by the placental syncytiotrophoblast, and to a lesser degree, the cytotrophoblast. KISS1R mRNA and protein expression have also been reported in syncytiotrophoblasts, but there are conflicting reports regarding their expression in cytotrophoblasts.[130, 145, 146] Placental *KISS1* mRNA expression is highest in the first trimester of human pregnancy when it is thought to be important for regulating placental formation.

As demonstrated by Bilban et al kisspeptin-10 inhibited trophoblast invasion and migration of first trimester villous explants in vitro.[130, 147] More specifically, kisspeptin inhibits trophoblast invasion by downregulating the expression of vascular endothelial growth factor (VEGF) and MMP 1, 2, 3, 7, 9, 10 and 14 and up-regulating the expression of tissue inhibitors of metalloproteinases 1 and 3.[148] Yet normal placentation and successful pregnancies can occur in the absence of a functional kisspeptin-receptor coupling and signalling system in mutant mice as well as in humans.[149] This was demonstrated by Pallais and colleagues who reported that in humans, female patients carrying *KISS1R* loss of function mutation had a successful pregnancy.[150]

It has been proposed that kisspeptins may also complement other known potent regulators of trophoblast invasion. These include oxygen tension, cytokines (leukaemia inhibitory factor, epidermal growth factor (EGF), transforming growth factor-beta (TGF-β), TNFα, IL-1, 6, 10, and 15), and hormones (oestradiol, gonadotropin-releasing hormone (GnRH), neurokinin B and insulin-like growth factor 2 (IGF2))[151, 152], but the evidence for this is limited. Santos et al

examined the expression of kisspeptin/kiss1r, angiogenic and immunological mediators in the maternal-foetal interface of domestic cats during pregnancy and found a positive correlation between placental kisspeptin and TNFα expression. In contrast, they reported a negative correlation between placental kiss1r and IL-6 and IL-10.[153] Meanwhile, Gorbunova et al demonstrated that kisspeptin significantly increased IL-10 production in CD4+T lymphocytes cultures.[144] These findings show that kisspeptin/kiss1r correlate with immunological mediators and suggest likely functional links of kisspeptin and immune function.

Although there are few data on placental kisspeptin regulation, some mechanisms have been proposed. Hiden et al reported that placental kisspeptin expression is regulated by a negative feedback mechanism involving ligand-receptor coupling and down-regulation of KISS1R expression in syncytiotrophoblasts.[154] In addition, Takino et al found that active MMP 2, MMP 9, MT1-MMP, MT3-MMP and MT5-MMP cleaved kisspeptins at Gly118-Leu119 residues, resulting in the abolishment of the ligand's functional activity.[155] Lastly, Oride et al demonstrated that oestradiol, GnRH, and neurokinin B increased rat placental kisspeptin mRNA expression, similar to the endocrine regulation of kisspeptin expression by the KNDy (Kisspeptin, Neurokinin-B and Dynorphin) neurons in the brain.[156, 157]

Plasma kisspeptin levels markedly rise in the second and third trimester of pregnancy, reaching approximately 7000 times higher concentrations in the latter, compared to the first trimester. This marked surge in circulatory kisspeptin concentrations in the third trimester is likely to result from greater placental production with increasing placental mass as pregnancy advances.[158, 159] Given their placental source of expression, maternal plasma kisspeptin concentrations unsurprisingly fall to pre-pregnancy concentrations within five days post-partum.[158]

Lower circulatory kisspeptin concentrations have been linked with disorders of defective deep placentation such as miscarriage, intrauterine growth restriction and preeclampsia.[160-164] In preeclampsia, cross-sectional studies have reported low circulatory kisspeptin concentrations, despite elevations in placental kisspeptin protein expression.[163, 165, 166] Similarly, in GDM, lower plasma kisspeptin concentrations and increased placental kisspeptin expression have been described.[120, 162, 167] There is, however, a paucity of data on the effect of maternal obesity on the placental expression of kisspeptin and maternal plasma kisspeptin levels. One underpowered study reported a decrease in circulatory kisspeptin levels in obese pregnant women with a BMI of ≥ 40 kg/m^2 who subsequently developed preeclampsia in their index pregnancies.[168]

Kisspeptin has been demonstrated to play a role in placenta invasion, migration, and development, with abnormal placental and circulatory levels showing significant implications for adverse pregnancy outcomes.

Table 1.2 Kisspeptin Expression in Human and other Mammalian Placenta During Pregnancy

Species	Cells or tissues	Findings	References
Human	villous syncytiotrophoblasts, villous cytotrophoblasts, Decidua	Decreased expression in recurrent spontaneous abortion compared with voluntary abortion	Wu et al, 2014[146]
Human	villous syncytiotrophoblasts, villous cytotrophoblasts	Decreased expression in recurrent spontaneous abortion compared with elective abortion	Park et al, 2012[145]
Human	villous syncytiotrophoblasts, placental bed and decidua parietalis of healthy human pregnancies	Higher expression in the placenta compared to the placental bed and decidua parietalis	Matjila et al, 2013[169]
Human	villous cytotrophoblasts	Increased expression in early placenta	Janneau et al, 2002[147]
Human	Placenta (early and term pregnancy, preeclampsia)	Reduced expression in preeclampsia compared to normal term pregnancy	Cartwright et al, 2012[170]
Human	Placenta (term pregnancy, preeclampsia); placental bed and decidua parietalis	Increased expression in placentae of pregnancies complicated by preeclampsia	Matjila et al, 2016[163]
Human	Placenta (term pregnancy)	Two surges of circadian rhythm (04:00 h and 12:00 h, respectively)	De Pedro et al, 2015[171]
Human	villous syncytiotrophoblasts	localisation and immunoreactivity to the syncytiotrophoblasts	Horikoshi et al, 2003[158]
Human	villous syncytiotrophoblasts, villous cytotrophoblasts; Extravillous trophoblast cells	Higher expression in first trimester trophoblasts than at term gestation.	Bilban et al, 2004[130]
Human	Placenta	Higher expression preterm placenta than the term placenta	Torricelli et al, 2008[172]
Mouse	Decidualizing stromal cells	Increased dynamically from Day 5 onward	Zhang et al, 2014[173]
Rat	Trophoblast giant cells	Expression gradually decreased as the placenta matures	Terao et al, 2004[174]
Rat	Trophoblast giant cells	Increased expression in the labyrinth and junctional zones placenta on day 22 > Day 16	Mark et al, 2013[175]
Dog	Superficial uterine glands, Syncytiotrophoblast	Not changed in expression between pre-implantation and post-implantation	Schafer-Somi et al, 2017[176]
Cat	Luminal epithelial cells and uterine glands, stroma cells	Not investigated	Tanyapanyachon et al, 2018[177]

1.5.3 Kisspeptin and Maternal Beta Cell Adaptations

The initial demonstration of kisspeptin and KISS1R expression in the pancreatic β and α cells led to the exploration of their potential role in insulin secretion, with findings strongly implicating kisspeptin in the regulation of pancreatic hormonal secretion.[136] As seen in Table 1.3 and Figure 1.3, data from numerous in vivo and in vitro studies [120, 136, 178, 179] suggest that kisspeptin plays a role in GSIS, that different kisspeptins may play varying physiological roles in glucose homeostasis, and that the impact of kisspeptins on glycaemia and metabolism may be influenced by sexual dimorphism.[180] Lastly, that kisspeptins are likely implicated in the pathophysiology of GDM (Table 1.4).

1.5.4 Glucose Dependency and Effect of Kisspeptins on Insulin Secretion

In vitro studies have shown that kisspeptins enhance GSIS in human and animal pancreatic islets (Table 1.3).[119, 133, 136, 178] More specifically, in the pig, rat, and mouse, kisspeptin-10 and -13 potentiated insulin secretion by direct activation of islet cells in 20 mmol/L glucose (Table 3).[133] Similarly, kisspeptin-54 induced GSIS in human islets and pancreatic cell lines cultured at high (17 mM) but not low glucose concentrations (3 mM).[121] In vivo studies have shown that intravenous (IV) kisspeptin-10 administration in rats and glucose-infused adult male monkeys (both fed and fasting states) resulted in a significant increase in plasma insulin concentration (Table 3).[119, 181] However, in glucose-deprived rhesus monkeys, IV kisspeptin-10 administration did not affect plasma insulin levels in either fed or fasting states, emphasizing a glucose-dependent mechanism. Additionally, a glucose-dependent (17 mmol/L) increase in plasma insulin secretion has been reported following kisspeptin-54 infusion in humans.[121] These findings suggest that kisspeptins stimulate insulin secretion in a glucose-dependent fashion, simulating an incretin-like

effect, further supporting previous data that hyperglycaemia may be the stimulus for kisspeptin-induced GSIS (Figure 1.3).[119]

1.5.5 Differential Effects of Kisspeptins on Pancreatic β-cell Responses

Contrary to some studies' reported GSIS effects of kisspeptin-10 and -13, Vikman et al found that kisspeptin-13 and kisspeptin-54 (at doses ranging from 10 nM to 1μM) inhibited insulin secretion in the presence of lower concentrations of glucose (2.8 mmol/L) in islets isolated from mouse pancreas.[179] In addition, the inhibitory effect of kisspeptin-13 on mouse islets was almost abolished at higher glucose concentrations.[179] At 11.1 mmol/L of glucose concentration, inhibition of insulin secretion was only observed with high doses of kisspeptin-13 and kisspeptin-54, but no inhibition of insulin secretion was noted at 16.7 mmol/L glucose irrespective of kisspeptin-13 concentrations.[179] Additionally, kisspeptin-13 selectively inhibited glucose, arginine, and exendin-4 induced insulin secretion, but not somatostatin and glucagon secretion in the rat pancreas (Table 1.3).[182] Furthermore, Song et al in 2014, demonstrated the inhibitory effects of synthetic kisspeptin 54 (at nanomolar concentrations) on insulin secretion in mice (Table 1.3), or at least improved insulin secretion in Kiss1R-/- mice.[183] Song et al further suggested the involvement of hepatic kisspeptin in beta cell adaptation, where in diabetic mice, hepatic kisspeptin 1 inhibited GSIS, with improvement in the in vivo glucose tolerance and GSIS augmentation in hepatic kisspeptin knock-out mice fed on a high fat diet.[183] These data suggest that different kisspeptins may have varying physiological effects in the glucose-dependent regulation of pancreatic beta cell function at particular doses and specific glucose concentrations.

1.5.6 Kisspeptin and Maternal Insulin Resistance

In non-pregnant women, Kolodziejski et al have reported an inverse relationship between plasma kisspeptin concentrations and markers of insulin resistance, such as BMI, fasting insulin, HOMA-IR, ghrelin and leptin (Table 1.4).[122] Furthermore, non-pregnant women who are insulin-resistant due to obesity and/or PCOS have significantly lower plasma kisspeptin concentrations than their non-obese equivalents (Table 1.4).[123, 122] Although there is evidence of a correlation between lower plasma kisspeptin levels and insulin resistance, the observational nature of the studies precludes the establishment of a causal link between decreased circulatory kisspeptins and reduced insulin sensitivity.

In late pregnancy, the marked rise in plasma kisspeptin concentrations coincides with peak insulin resistance in the mother. Whether the surge in kisspeptin in late gestation plays a role in insulin resistance, enhancing insulin secretion by the pancreatic beta cells and increasing energy expenditure requires further exploration.[158]

Table 1.3 Summary of Studies Conducted on the Role of Kisspeptin in Insulin Secretion and Regulation of Appetite

Model	Species	Effect of Kisspeptin	Reference
Pancreatic islet cells in vitro	Mouse	↑ GSIS ↑ GSIS ↓ GSIS ↑ GSIS ↑ GSIS	Schwetz et al. 2014 [178] Bowe et al. 2012 [133] Vikman et al, 2009 [179] Bowe et al, 2009 [119] Hauge-Evans et al, 2006 [136]
	Rat	↑ GSIS	Bowe et al. 2012 [133]
	Pig	↑ GSIS	Bowe et al. 2012 [133]
	Human	↑ GSIS	Bowe et al. 2012 [133]
	Human	↑ GSIS	Hauge-Evans et al, 2006 [136]
	MIN-6 Cells	↓ GSIS	Hauge-Evans et al, 2006 [136]
Pancreas in vitro	Rat	↓ GSIS ↓ Arginine and exendin-4 induced insulin secretion	Silvestre et al, 2008 [182]
In vivo	Rhesus monkeys	↑ GSIS ↔ basal insulin levels in both fed and fasting	Wahab et al, 2011 [181]
	Wistar rats	↑ insulin secretion (IV) ↔ insulin secretion (ICV)	Bowe et al, 2009 [119]
	Mice	↑ GSIS ↓ GSIS	Bowe et al, 2019 [120] Song et al, 2014[183]
	Human	↑ GSIS	Izzi-Engbeaya et al, 2018 [121]
	Mice	↓ food intake, meal frequency, time spent on meals, total mealtime ↑ inter-meal interval and satiety	Stengel et al, 2011 [184]

GSIS – glucose-stimulated insulin secretion, IV- intravenous, ICV-intracerebroventricular

Table 1.4. Summary of Cross-sectional Studies that show Correlations Between Plasma Kisspeptin concentrations and Markers of Insulin Secretion, Insulin Resistance and Appetite Regulation

Population	Sample size (n)	Findings	Reference
Adult nondiabetic individuals	261	- highest kisspeptin tertile had significantly lower insulinogenic and disposition indices than other tertiles - inverse correlation between kisspeptin and OGTT-derived indices of glucose-stimulated insulin secretion - positive correlation between kisspeptin concentration and age, BP, 2- h glucose	Andreozzi et al, 2017 [185]
Women in first, second, and third trimesters: Type 1 diabetes Healthy control Women in second and third trimester: GDM Gestational hypertension	 16 25 20 18	- ↓↓ kisspeptin levels in GDM women in the second and third trimesters than controls - ↓↓ kisspeptin levels in women with type 1 diabetes than controls in all the trimesters.	Cetkovic et al, 2012 [162]
Pregnant women	91	- ↓↓ kisspeptin levels in women with GDM compared to non- GDM - positive correlation between kisspeptin levels and basal β-cell secretory function. - positive correlation between kisspeptin concentrations and oral glucose-stimulated insulin levels - No significant correlation between kisspeptin and fasting insulin levels.	Bowe et al, 2019 [120]
Non- obese non-pregnant women: BMI <25 kg/m^2 Obese non-pregnant women: BMI >35 kg/m^2	 15 15	- ↓ kisspeptin levels in the obese than non-obese women. - negative correlation between kisspeptin and BMI, HOMA-IR, fasting insulin, ghrelin, and leptin. - positive correlation between kisspeptin and adiponectin levels.	Kolodziejski et al, 2018 [122]
Obese and overweight non-pregnant women with PCOS Normal-weight women with PCOS Obese and overweight women without PCOS (controls)	28 28 13	- ↑↑ kisspeptin levels in normal-weight women with PCOS and obese controls - negative correlation between plasma kisspeptin with BMI, HOMA-IR and fasting insulin	Panidis et al, 2006 [123]

↑↑- markedly higher, ↓↓- significantly lower

1.5.7 Kisspeptin and other Maternal Metabolic Effects (Appetite Regulation)

Several hormones and neuropeptides such as leptin, adiponectin, ghrelin and glucagon-like peptide 1 (GLP-1) are established key players in appetite regulation and energy balance, while less is known about kisspeptin.[180, 186-189] Kolodziejski et al reported that plasma kisspeptin concentrations are negatively correlated with ghrelin and leptin but positively correlated with hormones involved in glucose homeostasis (adiponectin and glucagon-like peptide 1).[122] These associations suggest that altered circulating kisspeptin concentrations may alter appetite, as well as nutrient utilization, and potentially lead to obesity and disordered glucose homeostasis. This hypothesis is supported by studies showing that kisspeptin administration reduces body weight, suppresses appetite and increases bowel movement (Table 1.3).[180, 184] However, sexual dimorphism in the effect/action of kisspeptin was demonstrated by Tolson et al who found that female *KISS1R* knock-out mice showed less energy utilization, glucose intolerance, reduced physical activity and increased body weight relative to their male counterparts, independent of differences in sex steroids.[180] These data suggest a sex-dependent difference and that dysregulated kisspeptin pathways in females could be an important underlying mechanism governing appetite regulation, hyperglycaemia and obesity.

1.5.8 Kisspeptin and GDM and Maternal Obesity

Work in experimental animals has demonstrated the essential role of kisspeptin-KISS1R signalling in glucose homeostasis during pregnancy. In pregnant mice, Bowe et al showed that administration of KISS1R antagonist (kisspeptin-234) resulted in a reduced GSIS and impaired glucose tolerance.[120] Further, compared to controls, β cell-specific *KISS1R* knockout mice showed more impaired glucose tolerance and lower insulin secretory responses 30 minutes post glucose challenge in pregnant compared to non-pregnant states, suggesting a more physiological effect in pregnancy.[120]

Two cross-sectional studies have reported lower circulating kisspeptin concentrations in GDM compared to non-GDM women in advanced pregnancies.[120, 162] In the first, Cetkovic et al reported significantly lower plasma kisspeptin concentrations in the second and third trimesters of women with GDM (n = 20) compared to non-GDM (n = 25) (Table 1.4).[162] In the second trimester, Bowe et al reported lower plasma kisspeptin levels in GDM than non-GDM pregnancies in a cohort of 91 pregnant women between 26 to 34 weeks gestation (Table 1.4), and further, a weak positive correlation between plasma kisspeptin and basal β cell secretory function as assessed by HOMA2-%β in pregnant women.[120]

The in vivo and in vitro studies suggest that kisspeptin may be a mediator of insulin secretion during pregnancy. Indeed Bowe et al suggested that kisspeptin-stimulated beta cell mass adaptation is a potential compensatory mechanism for combating the pregnancy-associated increasing peripheral insulin resistance with advancing gestation.[120] Thus, low kisspeptin concentrations in GDM may compromise this potential compensation. These data suggest kisspeptin could contribute to the pathogenesis of GDM through insulin resistance or insulin secretion, or a combination of both. However, studies of circulating kisspeptin concentrations in obese women with and without GDM have yet to be conducted. Further, whether pregnant women with kisspeptin signalling disruption may develop GDM secondary to unsuppressed appetite, excess weight gain, reduced energy utilisation, beta cell dysfunction and disordered glucose balance requires study.[120, 180]

1.6 Other Mechanisms Driving Maternal Metabolic Changes with Implications for GDM Development

1.6.1 Angiogenic Factors (VEGF), IGF2, Sex Steroids, Placental Lactogen Family, (Produced by Placenta)

Angiogenesis is the formation of new blood vessels from the existing vasculature, which involves endothelial cell migration, proliferation and differentiation.[190] It is an important pathway for placental development as the placenta is a highly vascular organ. The placental vasculature is indispensable for the maintenance of continuous and competent foeto-maternal nutrient, oxygen and waste exchange in early placentation and implantation, as well as throughout gestation.[191, 192] Pro-angiogenic factors such as vascular endothelial growth factor A (VEGF-A) are critical in early pregnancy, such that VEGF-A receptor knockouts in rodents result in embryo demise during embryogenesis, secondary to dysregulated angiogenesis.[193, 194]

Some studies have examined the role of kisspeptin in angiogenesis. Francis et al demonstrated that kisspeptin-10 down-regulates VEGF-A in primary cultures of human trophoblasts, indicating an anti-angiogenic effect.[148] In both animal and human studies, respectively, Cho and Ramaesh et al showed that kisspeptin suppressed VEGF production and microvessels sprouting in mice avascular cornea.[195, 196] Cho and Ramesh et al utilising chicken chorioallantoic membrane and VEGF–induced mouse corneal micropocket assays demonstrated the antiangiogenic effect of kisspeptin-10 in vivo. Furthermore, using complementary ex vivo and in vitro assays, Ramaesh et al demonstrated that kisspeptin 10 inhibited new vessel formation in placental arteries.[196] Decreased placental and circulatory levels of VEGF-A and placental growth factor (PIGF), a VEGF homolog, are increasingly being associated with disorders of placentation such as preeclampsia and IUGR.[197, 198]

1.6.1.1 VEGF and GDM: There are conflicting data on VEGF expression in GDM. Meng et al showed significantly decreased expression of VEGF-A mRNA and protein, as well as degenerative alterations of placental terminal villi vasculature in GDM women compared to normal pregnancies.[199] However, Akarsu et al a showed higher syncytiotrophoblast and stromal VEGF immunoreactivity in GDM than a control group.[200] On the other hand, Lappas et al showed that pre-existing maternal obesity and GDM did not affect VEGF mRNA expression in placental and omental adipose tissue when they studied four cohorts of women, namely non-GDM non-obese, non-GDM obese, GDM non-obese, and GDM-obese).[201]

1.6.1.2 IGF2 and GDM: During gestation, IGF2 is produced by the placenta and plays a major role in promoting foetal growth and placental development.[202, 203] Animal studies in pregnancy have shown that placental lactogens, prolactin, human growth hormone variant (hGHV) and IGF2 modulate maternal insulin sensitivity and glycaemia.[204, 205] In addition, in humans, disturbed expression of these placental proteins have been associated with GDM and abnormal foetal growth.[117, 206, 207] Su et al reported that the expression of IGF2 is significantly higher in women with GDM compared to normoglycaemic women in both the cord blood and placenta. In addition, there was a significantly higher placental and cord IGF2 expression in macrosomic infants born to normoglycaemic women than in the normal birth weight group.[207] Maternal insulin resistance induced by placental and maternal hormones and growth factors has been shown to promote increased placental and foetal weight. Additionally, decreased placental function (using birth weight to placental weight ratio) and increased IGF2 expression are essential contributors to foetal macrosomia in GDM.[208, 209]

1.6.1.3 hPL and GDM: hPL, also known as human chorionic somatomammotropin, is synthesised by syncytiotrophoblasts. Ryan and Grumbach et al demonstrated that hPL plays an important role in GSIS and systemic insulin resistance during pregnancy to enhance maternal glycaemia for foetal growth and development.[47, 210] Maternal obesity disrupts the expression of both human placental lactogen 1 (hPL1) and human placental lactogen 2 (hPL2). Jin et al found that the expression of *hPL* and *GHV* mRNA was significantly decreased (75%) in the term placentae of women with obesity (pre-pregnancy BMI > 35 kg/m^2) in comparison to their non-obese counterparts (BMI: 20–25 kg/m^2).[211] There are, however, limited data on hPL and GDM. For example, Mills et al showed no significant difference in placental hPL mRNA between GDM and normal pregnancies.[212] Similarly, cross-sectional studies have reported no difference in the plasma levels of hPL in GDM and normal pregnancy.[213-216] While hPL has been reported to have a role in GSIS and insulin resistance; the available data did not record differences in placental and circulatory hPL levels between GDM and non-GDM.

1.6.1.4 GHV and GDM: hGHV is expressed in the syncytiotrophoblast and invasive extravillous trophoblasts of the human placenta and secreted directly into the maternal circulation. It plays a significant role in maternal metabolic adaptation to pregnancy and foetal growth, particularly by enhancing beta cell insulin secretion.[217, 218] Maternal GHV levels are impacted by foetal sex and obesity, with studies reporting an inverse relationship between circulating GHV concentrations and BMI at various stages of pregnancy.[219] On the other hand, no studies have found an association between maternal plasma GHV, placental GHV mRNA and GDM.[220-222] Available data suggest that GHV may be an important endocrine signal regulating maternal GSIS and foetal growth during pregnancy, with obesity significantly influencing maternal circulating levels.

1.6.1.5 Prolactin and GDM: The role of prolactin, produced by the anterior pituitary, is well-established in lactation. In addition, prolactin is involved in metabolic functions such as appetite regulation, adipose tissue function, and glucose homeostasis.[223, 224] In pregnancy, prolactin regulates beta cell mass expansion, and it also induces a certain degree of insulin resistance essential to shunt glucose to the developing foetus by stimulating passive transportation of glucose across the placenta.[225] Prolactin has been proposed to induce insulin resistance via a number of mechanisms such as lipotoxicity, down-regulation of insulin receptors and a defect in insulin-receptor signalling.[226] In a prospective study, GDM women had higher prolactin levels in the first trimester compared to controls.[227] In a non-pregnant state, prolactin levels have been associated with a lower risk of type 2 diabetes, but during pregnancy, higher prolactin levels contribute to deteriorating glycaemia due to insulin resistance and increased body weight.[228, 229]

1.6.1.6 Sex Steroid Hormones and GDM: In humans, the placenta is the main source of sex steroid hormones (oestrogens and progesterone) during pregnancy. It has been suggested that sex steroids, in concert with other hormones, contribute to insulin resistance in advanced pregnancy with resultant development of GDM.[47] In addition, some animal studies have reported the effect of progesterone and oestrogen on beta cell proliferation and insulin secretory function; however, whether this role is stimulatory or inhibitory remains debatable.[69, 230-232]

There are conflicting data on the association between sex steroids and GDM. Couch and Montelongo et al reported an association between progesterone and oestradiol and the risk of GDM, while Li et al showed no association between progesterone and GDM risk.[227, 233, 234] Significantly lower progesterone levels were reported at 10–14 weeks gestation in GDM associated pregnancies, with an inverse relationship with glucose and insulin levels[227] but,

increased progesterone and oestrogen concentrations have been found in the second trimester in GDM pregnancies.[235]

In summary, most placental-derived hormones and growth factors with important roles in maternal beta cell mass adaptation and GSIS regulate metabolic events to enhance the delivery of nutrients (especially glucose) to the developing foetus and facilitate placenta development. While disruption in their levels may lead to GDM, the available data on these placental factors' roles in GDM development are conflicting. Further, knowledge of interactions between placental kisspeptin and other placental-derived hormones is limited.

1.6.2 Adipokines; Leptin, Adiponectin and Proinflammatory Cytokines; TNFα, IL-6, IL-1β and GDM

As mentioned earlier, maternal obesity is characterised by low-grade inflammation due to increased TNFα, IL-6, IL-12, high sensitivity C-reactive protein (hsCRP), increased leptin and decreased adiponectin. This leads to the perpetuation of inflammation in pregnancy, contributing to GDM.[236-238]

During pregnancy, women with hyperglycaemia tend to show increased placental lysate TNFα, IL-6, interleukin-8 (IL-8) and leptin expression but decreased adiponectin.[239] Higher IL-6, IL-8, and TNFα levels were found in placental homogenates of women with mild hyperglycaemia, GDM and type 2 diabetes, respectively.[240] Kuzmicki et al demonstrated an increase in placental *TNFα* gene expression in women with GDM but no placental *adiponectin* gene expression.[241] Similarly, Moreli et al reported increased placental expression of TNFα and TNFα/IL-10 ratio in women with GDM and type 2 diabetes. In addition, alterations of placental and serum TNFα/IL-10 ratio and

TNFα were correlated with hyperglycemia severity.[242] Yet Kleiblova et al reported no significant difference in the placental expression of *IL-6*, *TNFα* and *leptin* genes in GDM and normal pregnancy.[243] While, in prediction models for GDM, IL-6 levels at 11-14 weeks of gestation were higher in GDM compared to normal pregnancies even after correcting for maternal obesity, supporting its role in GDM development irrespective of body mass index (BMI).[237]

Tsiotra et al reported a significantly higher leptin mRNA expression in the subcutaneous adipose tissue than visceral adipose tissue and placental expression and a positive correlation between circulatory leptin and maternal BMI. In addition, three-fold higher visceral adipose tissue *leptin* mRNA expression and higher plasma leptin concentrations have been demonstrated in obese GDM and normoglycaemic women compared to their non-obese GDM and normoglycaemic counterparts.[244] These findings suggest that maternal subcutaneous adipose tissue-derived leptin may be a significant contributor to GDM development.

Available data indicate a linkage between maternal obesity, placenta and pancreatic beta cells in GDM pathogenesis. Although the data linking adipokines and proinflammatory cytokines with GDM are conflicting principally due to differences in the study protocol, gestational age at sampling and assay methods, increased placental and circulatory proinflammatory cytokines and decreased adiponectin predominantly have been associated with GDM.

The previous sections described the central role of the placenta in the aetiopathogenesis of GDM through its expression and secretion of hormones that are essential for pancreatic beta cells adaptation and function and insulin resistance. The following section will describe the placenta

structure and function and the role of placental extracellular vesicles and their biological substances in GSIS and GDM development.

1.7 Human Placenta Structure and Function

The placenta is the interface connecting the foetus to the maternal uterus and performs functions crucial for maintaining foetal wellbeing and normal gestation.[245, 246]

The human placenta weighs about 500g at term, has a 12-44 square meter surface area, and expresses many genes and proteins. The placenta develops from the trophectoderm of the blastocyst and is a highly specialised organ. The trophoblast stem cell of the placenta is the cytotrophoblast, which further differentiates into the invasive extra-villous trophoblast (EVT) or proliferates and then fuses to the syncytiotrophoblast.[246, 247] During placental development, the primary villi are formed by the penetration of the cytotrophoblasts into the cords of the syncytiotrophoblast. In contrast, the appearance of the mesenchymal core within the expanding villus leads to the formation of the secondary villi, with the mesenchymal core of the secondary villus being directly surrounded by a layer of the cytotrophoblast cells and peripherally by the syncytiotrophoblasts. Late in the third week of gestation, the terminal villi are formed when the blood vessels penetrate the mesenchymal core of the secondary villi and in the presence of newly formed branches.[248] The syncytiotrophoblast layer is the only layer that has direct contact with the maternal blood and is essential for foetal gaseous exchange and nutrients, as well as hormone synthesis (Figure 1.1).[249, 250] The intervillous space is between foetal chorionic villi where maternal spiral arteries enter directly and fill it with maternal blood. It is the primary site where nutrients, oxygen and waste exchange take place.[251] The EVT, which invades the maternal decidua, becoming interstitial and subsequently endovascular trophoblast, is crucial for transforming

maternal spiral arteries and promoting blood flow to the placenta (Figure1.2).[249] Failure to transform deep myometrial maternal spiral arteries results in disorders of deep placentation such as preeclampsia, IUGR and preterm delivery.[94, 252, 253] In a rat model of lifelong maternal obesity, Hayes et al reported an alteration of placental spiral artery remodelling.[254]

The placenta receives blood supply from maternal and foetal circulatory systems without a direct connection between the two systems. The human uteroplacental circulation begins with the maternal blood perfusing the intervillous space through decidual spiral arteries for nutrient and oxygen exchange with foetal blood as maternal blood baths the terminal villi in the intervillous space.[255] In the maternal placental circulation, deoxygenated blood moves through the placenta's intervillous space and is drained through uterine veins to the maternal circulatory system.[255] In contrast, foetal-placental circulation involves the umbilical vein that transports nutrient-rich and oxygenated blood from the placenta to the foetal circulation. The umbilical arteries carry deoxygenated nutrient-depleted blood from the foetus to the placenta.[256]

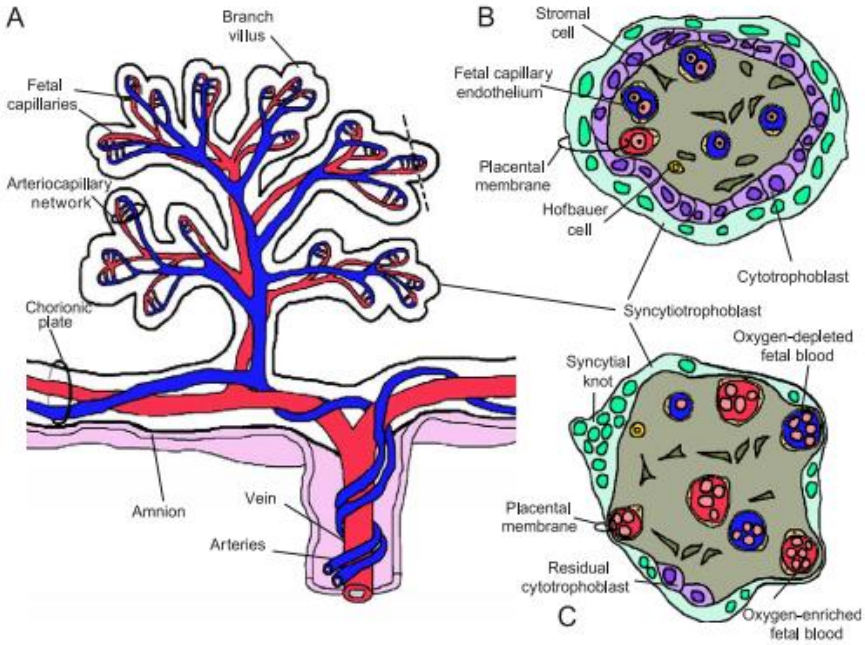


Figure 1.2. Foetal placental circulation (A), coronal section of chorionic villous at 10 weeks (B) and chorionic villous section at term (C). Adapted from Gude et al.[245]

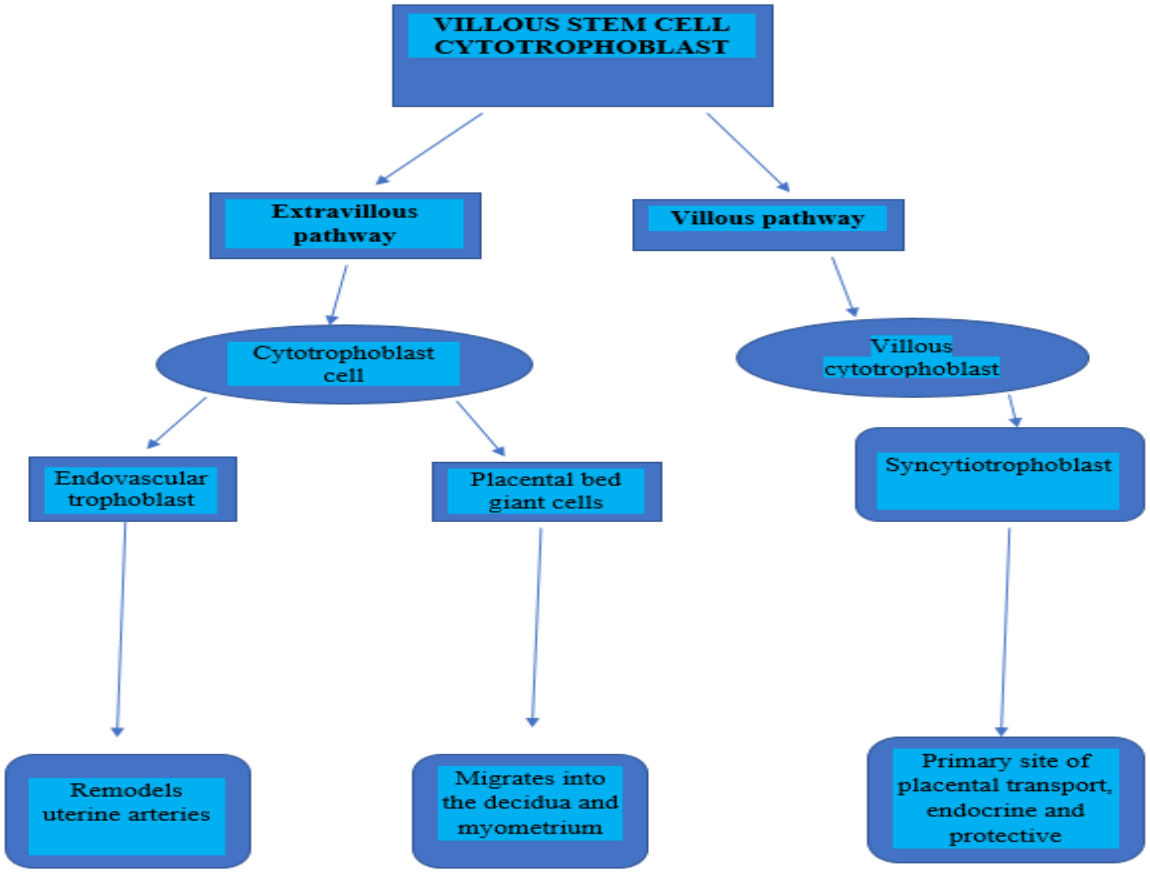


Figure 1.3. Trophoblast differentiation and function

1.7.1 Placental Extracellular Vesicles

The placental syncytiotrophoblast produces and releases extracellular vesicles (EV) known as syncytiotrophoblast extracellular vesicles (STBEV) into the maternal circulation.[257] STBEV contain a mixed cargo of proteins, lipids, glycans, mRNA and miRNA when released to exert their biological function.[258-261] There are three distinctive EV subtypes based on their sizes: exosomes (50-200nm), microvesicles (100nm-1µm) and apoptotic blebs (1-5µm).[262] EV are involved in cell to cell interaction through diverse mechanisms, such as direct binding to cell membrane receptors, fusion with its target and endocytosis with the optimal release of cargo to the cellular cytosol (Figure 1.4).[262]

STBEV have been found to rise in the maternal circulation as pregnancy advances, which mirrors the increasing peripheral insulin resistance, suggesting they may be involved.[263, 264] It has been proposed that STBEV play a role in the pathophysiology of maternal and foetal morbidity such as preeclampsia, GDM and IUGR.[265-268] Salomon et al reported several-fold increases (approximately 2.2-fold, 1.5-fold, and 1.8-fold) in placental derived exosome levels in GDM compared to normal pregnancies across the gestational ages.[268] They also observed significantly increased proinflammatory cytokine release from endothelial cells following incubation by exosomes isolated from GDM pregnancies.[268] Kandzija et al showed STBEV released from normal placenta were dipeptidyl peptidase IV (DPPIV) positive and dose-dependently inhibited by a glucagon-like peptide-1 (GLP-1) analogue, vildagliptin.[269] They further demonstrated greater DPPIV activity in STBEV from perfused placentae and an eightfold increase in the circulatory DPPIV-bound STBEV in women with GDM.[269] In non-pregnant mice infused with small EV acquired from GDM women, glucose intolerance was noted. While enhanced GSIS was observed in mice infused with healthy pregnancy small EV compared to nonpregnant mice with small EV.[270] These findings

suggest STBEV may contain important biological substances that play a role in GSIS and GDM development. But currently, there are no data on kisspeptin as a possible STBEV-bound molecule that may potentially regulate pancreatic beta cell insulin secretion.

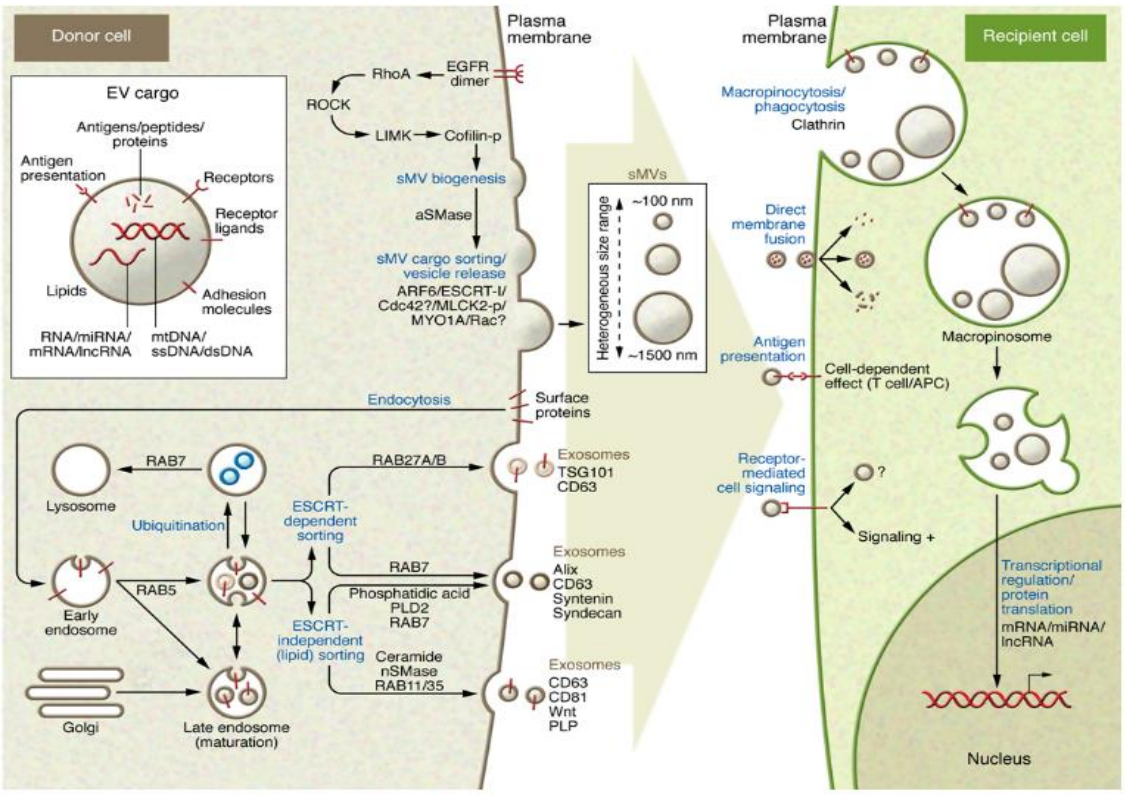


Figure 1.4. Mechanisms involved in cellular interactions mediated by extracellular vesicles. Adapted from Xu et al (2016).[271]

1.8 Justification of the Study

GDM is common and is associated with short and long-term adverse pregnancy outcomes for both the mother and offspring.[2]

Obesity, characterized by insulin resistance, is a major driver of the diabetes epidemic, and by implication, GDM.[272, 273] In the later stages of normal pregnancies, insulin resistance is present. This is purported to be induced by the release of placental hormones such as progesterone, oestrogen, growth hormone, hPL, and cortisol.[47] However, inflammatory cytokines, such as TNFα, IL-6 and IL-1β, thought to play a role in the pathogenesis of insulin resistance in obesity, are also produced by the placenta and may contribute to the elevated risk of GDM in women with obesity.[85, 274]

Limited data suggest that recently discovered kisspeptin, which is expressed by the placenta and whose concentrations rise dramatically in the maternal circulation in the second and third trimesters, may play a role in the pathogenesis of GDM.[275] However, little is known about the effect of maternal obesity and GDM and their interaction on (i). kisspeptin and inflammatory markers (TNFα, IL-6); (ii). the relationship between placental and circulatory kisspeptin and inflammatory markers with placental villous morphology and maternal and neonatal parameters. There are also no data on STBEV kisspeptin's role in mechanisms underlying GDM development.

1.9 Research Questions

In women with obesity and /or GDM, are there pathophysiological perturbations in kisspeptin and other placental gene or protein expression, placental morphology, and extracellular vesicle content?

1.10 Hypotheses

We hypothesise that there are significant alterations in placental kisspeptin, proinflammatory cytokines and other endocrine signals in pregnant women with obesity and GDM, independently and in combination, compared to those without obesity and/or GDM, that may have physiological implications for the mother and neonate.

1.11 Overall Aim

To examine the effect of maternal obesity and GDM on the physiological changes within the placenta (genes and protein expression and morphology) and maternal and foetal blood. This with a view to advancing our understanding of the mechanisms underpinning GDM.

1.12 Specific Objectives

To determine in non-obese and obese South African women with and without GDM:

1. The placental expression of kisspeptin, other endocrine signals (leptin, placental lactogen family members), proinflammatory cytokines (TNF-α, IL-1 and IL-6), growth factors (IGF2, VEGF), and steroidogenic hormone enzymes

2. The maternal and cord concentrations of kisspeptin and proinflammatory cytokines

3. Correlations between placental kisspeptin and proinflammatory cytokine genes and their respective maternal and cord concentrations

4. The placental villous morphology, including villi maturation, vascularity and the surface area for exchange

5. Examine placental extracellular vesicle kisspeptin protein expression in women with and without GDM

6. Correlations between placental expression of kisspeptin, proinflammatory markers and placental villous morphology and maternal and neonatal parameters

7. Correlations between circulatory concentrations of kisspeptin, proinflammatory markers and placental villous morphology and maternal and neonatal parameters

This chapter details the methods used to address the objectives described in the preceding chapter. The study design was cross-sectional, and two study populations were included. The first provided samples and data for studies 1-4, and the second provided samples for study 5.

2.1 Studies 1 to 4:

2.1.1 Patient Population:

The study population for the first four studies comprised of women attending antenatal care and delivery at the University of Cape Town's Groote Schuur and Mowbray Maternity Hospitals between October 2018 and June 2019. Mowbray Maternity hospital is a secondary level hospital providing amongst other services, screening of high-risk women for GDM and initial non-pharmacological management of GDM. Groote Schuur Hospital (GSH) is a tertiary-level hospital where the Department of Obstetrics and Gynaecology has a diabetes clinic and in-patient service dedicated to providing comprehensive treatment to high-risk pregnant women with diabetes, other medical diagnoses, or complicated HIP.

2.1.2 Inclusion Criteria: Pregnant women above 18 years of age who had undergone a standard OGTT at 24-28 weeks gestation following a risk-based screening strategy, were identified as having either GDM or normoglycaemia and were either obese or non-obese were eligible to participate in these studies. Obesity was defined according to WHO criteria as maternal BMI ≥ 30 kg/m^2 and non-obese as maternal BMI < 30 kg/m^2 at booking.[276] GDM diagnosis was based on the IADPSG diagnostic criteria and National Institute for Health and Care Excellence (NICE) diagnostic criteria adopted by the study hospitals where the participants were recruited.[277]

2.1.3 Exclusion Criteria: Pregnant women aged less than 18 years or diagnosed with pre-gestational diabetes, multiple gestations, pregnancy secondary to assisted reproduction with gonadotropins or in-vitro fertilization, HIV with unsuppressed viral load, preeclampsia and who did not give consent were excluded from the study.

2.1.4 Ethical Consideration

This study received ethical approval from the Human Research Ethics Committee (HREC) of the Faculty of Health Sciences, University of Cape Town, South Africa, with the Reference Number: HREC: 463/2018 (studies 1-4, 6 and 7) (Appendix 1). In addition, the ethical principles of the Helsinki Declaration and South African Guidelines for Good Clinical Practice and the Medical Research Council Ethical Guidelines for Research for the study of human subjects were observed. Study participants gave written informed consent after being provided with information regarding the study with an opportunity to ask questions. Participants received no financial inducement or payment to participate in the study; those who declined to participate in the study were not in any way denied standard care. Confidentiality was maintained throughout and beyond the study period (Appendix 2).

2.1.5 Study Participants

Ninety-six women were recruited consecutively: 41 with GDM and 55 without GDM. Of these, 25 women were excluded due to lack of oral glucose tolerance test (OGTT) results, incomplete clinical information, incomplete samples or withdrawal of consent at delivery. The final sample size was 71. There were 38 women with GDM and 33 without GDM (Figure 2.1) who were sub-categorised into the following groups:

(1) Non-obese and (2) obese women without GDM (3) Non-obese and (4) obese women with GDM.

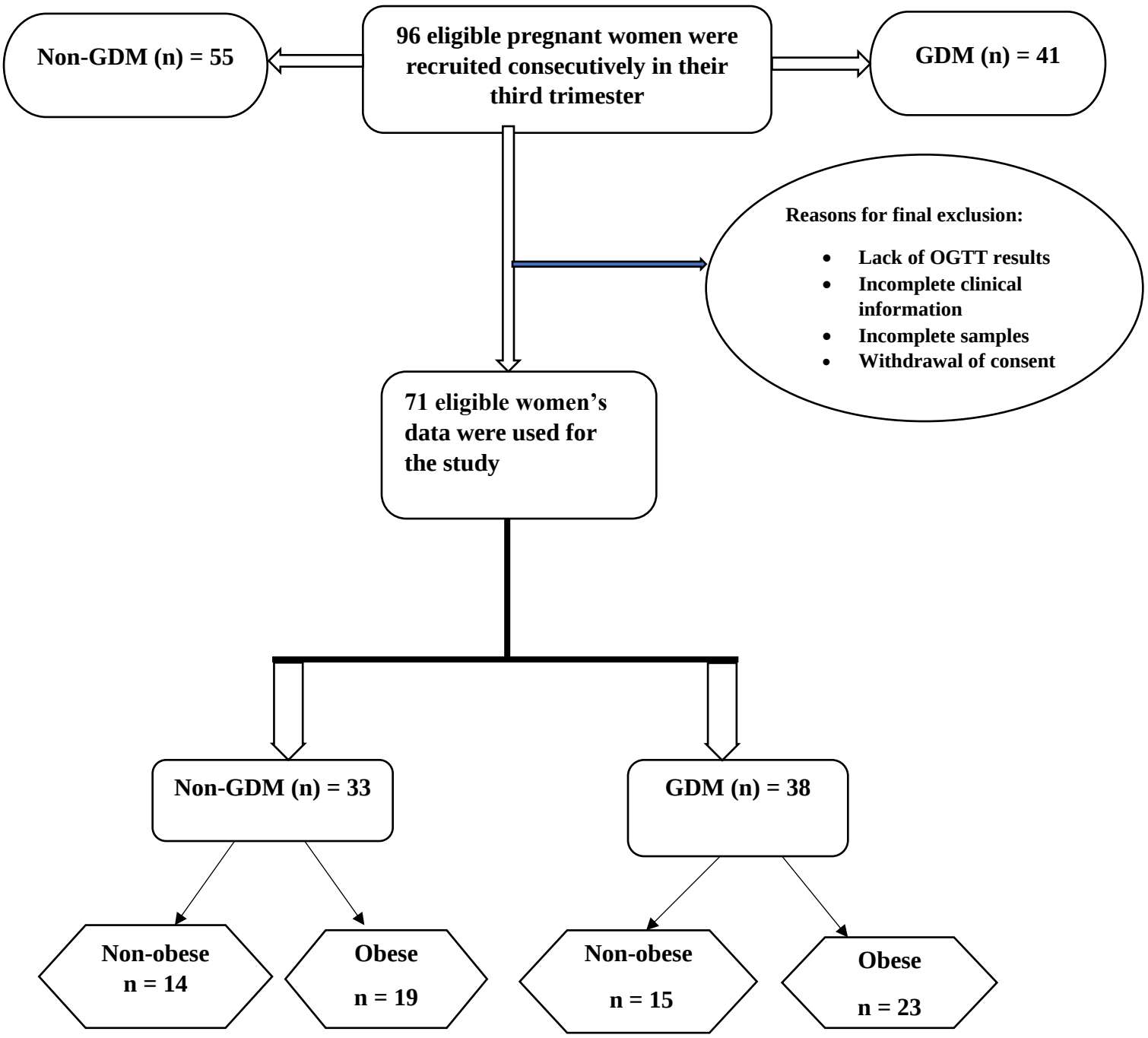


Figure 2.1. Flow chart of the study participants

2.1.6 Study Location:

This study was conducted through a collaboration between the Departments of Medicine (Division of Endocrinology) and Obstetrics and Gynaecology, as well as the University of Cambridge's Centre for Trophoblast Research, Department of Physiology, Development & Neuroscience, United Kingdom. This study was funded by the University of Cape Town Faculty of Health Sciences Research Stimulus Grant and Lister Institute of Preventative Medicine and Medical Research Council (University of Cambridge).

2.1.7 Sample Size Determination

Two sample size calculations were performed. As the determination of placental kisspeptin expression in women with/without GDM and with/without obesity was one of the key objectives of this project, it formed an important consideration for the sample size calculation. At the onset of this study, there were no published data on placental kisspeptin mRNA expression in women with GDM and obesity; hence, our sample size was informed by the data from a study in women with preeclampsia:

In this, Qiao et al examined the expression of *KISS-1* mRNA in the placentae of Chinese women with early-onset preeclampsia and reported a 2.2-fold upregulation in expression compared to placentae from normotensive controls.[166] The calculated minimum sample size using the formula for placental mRNA expression studies[278] is as follows:

$$n = \frac{16 \times \sigma^2}{\delta^2}$$

Where n = minimum sample size (desired)

σ = standard deviation of such log fold-changes from different samples {represented as $Log_2(3)$}

δ = desired detectable effect size/fold change (expressed as the Log_2 fold-change

$\sigma = \log_2 (3) = 1.6$

$\delta = \log_2 (2.2) = 1.1$

$n = 16 \times 1.6^2/1.1^2 = 40.96/1.21$

$n = 34$

As expression of placental inflammatory markers formed part of the study objectives, a study by Radaelli et al which reported a 3-fold increase in placental TNFα mRNA expression among women with GDM compared to those without GDM, was used to calculate the second sample size.[279] Using this study, the minimum sample size was calculated using the formula above:

$$n = \frac{16 \times \sigma^2}{\delta^2}$$

$\sigma = \log_2 (5) = 2.3$

$\delta = \log_2 (3) = 1.6$

$n = 16 \times 2.3^2/1.6^2 = 84.64/2.56$

$n = 33$

The minimum sample sizes calculated above were 34 and 33 as indirect estimations. In order to take potential attrition into consideration, 41 women with GDM and 55 without GDM were recruited for this study.

2.2 Clinical Assessment Methods

Following enrolment, socio-demographic, pregnancy and medical information were obtained by questionnaire and review of medical records (Appendix 3). These data included age, ethnicity, pre-pregnancy weight if known and if not, weight and height at booking for calculation of BMI, previous medical history, relevant family history, gestational age at which diagnosis of GDM was made, results of the OGTT, current gestational age by dates or ultrasound, medical conditions, glycaemic control, therapy used during pregnancy and weight just prior to delivery. Additionally, neonatal APGAR scores, anthropometric measures, the presence of congenital anomalies, and placental weights were obtained.

2.3 Biological Samples

2.3.1 Blood Samples

At delivery, samples were drawn from the antecubital vein of the mother as well as the umbilical cord for serum kisspeptin, TNFα, and IL-6 estimations. The samples were immediately centrifuged (12000 rpm) at room temperature for 5 minutes, and sera were collected and stored at -80℃ until assayed.

2.3.2 Placental Tissues Collection

Immediately after delivery, four random samples of the placenta (central portion) free of membranes or clots were taken and placed into separate plain tubes for gene and protein studies, immunohistochemistry and stereological analyses. The samples for gene analyses were immediately treated with RNA later (RNAlater®, Cat # R0901, Sigma-Aldrich® Co. LLC, USA), snap-frozen in liquid nitrogen and stored at -80℃. For morphological and immunohistochemical

studies, samples were incubated in 10% neutral buffered formalin or 4% paraformaldehyde, embedded into paraffin wax blocks and sectioned on a microtome (5µm).

2.4 Laboratory Methods

2.4.1 Placental Tissue Gene Expression Studies

In order to study the gene expression of endocrine signals (kisspeptin, KISS1R, leptin, placental lactogen family members (GHV, hPL1, hPL2, hPL)), growth factors (IGF2, VEGF), proinflammatory cytokines (TNFα, IL-6, IL-1β) and steroidogenic hormone enzymes (HSD3B1, CYP11A1, CYP19A1), ribonucleic acid (RNA) was extracted from the placental tissue and cDNA synthesized prior to quantitative RT-PCR studies.

2.4.1.1 RNA Extraction

Total RNA from placental tissue was extracted using the GeneJet RNA purification kit (ThermoFisher Scientific, USA) according to the manufacturer's protocol with slight modification. About 30 mg of thawed placental tissue was sliced into fine pieces with a sterile scalpel on dry ice and transferred into a nuclease-free 2 ml tube containing the kit's lysis buffer supplemented with β-mercaptoethanol and two steel beads. The tissue was disrupted further on a bead mill homogeniser (Omni Bead Ruptor 24, Omni International, USA) for a maximum of 40s. Proteinase K enzyme solution (supplied and diluted based on manufacturer's guideline, 600 µl) was added to the resulting homogenate and incubated at room temperature for 10 min. The homogenates were then centrifuged at 12 000 x g for 10 min at 4℃. Next, the supernatant was collected in fresh 1.5 ml RNase-free microcentrifuge tubes, 450 µl of absolute ethanol was added to the solution and mixed by upward and downward pipetting. This column was centrifuged at 12 000 x g for 1 min and the eluent discarded. This step was repeated for any residual supernatant from the lysis and

Proteinase-K digestion step. Next, the column was washed with 700 µl of Wash Buffer 1 (supplied in the kit and supplemented with ethanol as directed in the manual) by centrifugation at 12 000 x g for 1 min. Another wash step was performed with 600 µl Wash Buffer 2 (also supplied in the kit and supplemented with ethanol) using the same centrifugation parameters as the previous step, followed by a final wash with 250 µl of Wash Buffer 2 with centrifugation at the same speed but for 2 min. Finally, the column was dried by a further centrifugation step at maximum speed for 1 min. The dried column was then removed from the collection tube of the column and placed in a sterile, RNase-free 1.5 ml microcentrifuge tube. The extracted RNA was eluted from this column with 60 µl of nuclease-free water, quantified using a nucleic acid quantifier (Biodrop, Biotek Instruments, USA), and stored at – 80 ℃ until downstream application.

2.4.1.2 Complementary DNA Synthesis

The synthesis of single-stranded complementary DNA (cDNA) from the extracted RNA was achieved with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA) following the manufacturer's protocol. About 2 µg of RNA per reaction was used to synthesise cDNA (in a nuclease-free PCR tube) utilising supplied reverse transcriptase (RT), RT buffer, dNTPs, RT Random primers and Nuclease-free water. The nuclease-free tube was placed in a thermal cycler (BioRad CT100, USA) with the following cycling conditions for cDNA synthesis: RT activation at 25 ℃ for 10 min, cDNA synthesis at 37 ℃ for 120 min, and RT inactivation at 85 ℃ for 5 min. The synthesis of cDNA was confirmed by reverse-transcription polymerase chain reaction (RT-PCR) with *GAPDH* primers. The synthesised cDNA was stored at -20 ℃ until further use.

2.4.1.3 Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

Gene expression studies were conducted using SYBR Green chemistry and mastermix on the ABI 7500 Fast Real-Time PCR instrument from Applied Biosystems. For a single sample, 15 µl reaction (3 dilutions - 1:10, 1:20 and 1:100 were each run in triplicate with negative controls), the following were added: 5 µl H_2O, 6.5 µl PCR mastermix, 0.25 µl of both forward and reverse primers and 3 µl of cDNA sample. After mixing, each sample, as well as a sample devoid of reverse transcriptase (negative control) and reference cDNA (positive control), was added to 96-well MicroAmp FAST Optical PCR plates (Applied Biosystems, Warrington, UK) and sealed with nuclease-free MicroAmp optical adhesion film. The standard thermal cycling protocol was conducted as follows: 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 95 seconds and 60 °C for 1 min. The following primer pairs were used as internal gene expression controls: *Glyceraldehyde 3-phosphate dehydrogenase* (*GADPH)*, *Actin* and *Beta 2 Microglobulin* (*β2M)* (Table 2.1). The ΔΔCt method was employed for relative gene expression analysis.

Table 2.1 Primers Sequences used for Real-Time qPCR Reactions

Gene Name	Gene entry	Forward 5′- 3′	Reverse 5′- 3′	T°	bp	References
Endocrine signals and Growth factors						
IGF2	NM_000612.6	CCCCTCCGACCGTGCT	TCATATTGGAAGAACTTGCCCA	58-61	64	Chen 2018
VEGF	NM_001025366.3	CGA GGG CCT GGA GTG TGT	CGC ATA ATC TGC ATG GTG ATG	61-57	57	Li 2015
KISS1	NM_002256.4	ACT CAC TGG TTT CTT GGC AGC	ACC TTT TCT AAT GGC TCC CCA	59-60	70	Cockwell 2012
KISS1R (GPR54)	NM_032551.5	GGTGCTGGGCGACTTCAT	CACACTCATGGCGGTCAGAGT	55-60	91	Matjila 2016
Leptin	NM_000230.3	TTC ACA CAC GCA GTC AGT CTC	CTG CCA GTG TCT GGT CCA TC	60-62	107	Nogues 2019
Placental lactogen family						
GHV	NM_002059.5	GTTTGAAGAAGCCTATATCCTG	TCACCCTGTTGGAAGGTGTT	55-60	107	Vakili 2013, Jin 2018
CSH1 (hPL1)	NM_001317.6	GGCTTCTAGGTGCCCGAGTA	GCACTGGAGTGGCACCTTCA	61-62	77	Ganguly 2015, Jin 2018
CSH2 (hPL2)	NM_022645.2 NM_022644.3	CAGCAAGTTTGACACAAACTCA	AGAAGCCACAGCTACCCTCT	58-60	146	Ganguly 2015
Both	NM_001317.6 NM_022644.3	CATGACTCCCAGACCTCCTTC	TGCGGAGCAGCTCTAGATTG	59-60	97	Kiwashima 2014, Li 2019
Pro-inflammatory						
IL-6	NM_000600.5	AAATTCGGTACATCCTCGACGG	GGAAGGTTCAGGTTGTTTTCTGC	60-61	112	Seno 2017
IL-1β		ACAGATGAAGTGCTCCTTCCA	GTCGGAGATTCGTAGCTGGAT	59-59	73	Li 2004
TNFα	NM_000594.4	CATCTATCTGGGAGGGGTCTTC	AGGAGGGGGTAATAAAGGGATT	58-59	188	Mezouar 2019

Gene name	Gene entry	Forward 5′- 3′	Reverse 5′- 3′	T°	bp	References
Sex steroids genes						
CYP11A1	NM_000781.3	AGGAGGGGTGGACACGAC	TTGCGTGCCATCTCATACA	61-57	60	Noyola 2017
HSD3B1	NM_000862.3	AGAGGCCTGTGTCCAAGCTA	TTTTGCTGTGTGGGTATGGA	61-58	152	Shin 2017
CYP19A1	NM_000103.4	GAATTCATGCGAGTCTGGATCT	TCATTATGTGGAACATACTTGAGGA	58-59	76	Noyola 2017
House-keeping genes						
Beta actin (*actin*)	NM_001101.5	GGACTTCGAGCAAGAGATGG	AGCACTGTGTTGGCGTACAG	59-61	234	Shin 2017
GADPH	NM_002046.7	CGAGATCCCTCCAAAATCAA	GTTCACACCCATGACGAACA	55-58	171	Hogg 2014
Beta-2 microglobulin (β2M)	NM_004048.3	TGCTGTCTCCATGTTTGATGTATCT	TCTCTGCTCCCCACCTCTAAGT	60-61	86	Nogues 2019

2.4.2 Placental Protein Expression Studies

2.4.2.1 Protein Extraction

The frozen placental tissue was homogenised by sonication (Soniprep 150, UK) for 20 seconds in Radioimmunoprecipitation (RIPA) lysis buffer containing 50mM Tris-HCl (pH 8), 1% Triton x-100, 150 mM NaCl, 0.1% sodium dodecyl sulphate (SDS), 0.5% sodium deoxycholate with a protease and phosphatase cocktail inhibitor (Halt protease and phosphatase inhibitor, Thermo Scientific, USA). The placental lysates were then centrifuged at 15000 revolutions per min relative centrifugal force for 20 minutes at 4 °C (Labnet International, NJ07095, USA). The supernatant was collected, and the protein concentration of the supernatant was measured employing the bicinchoninic acid (BCA) method using the Pierce protein assay kit (Thermo Scientific, Rockford, USA).

2.4.2.2 Protein Quantification

A BCA protein assay was performed using the Pierce BCA protein assay kit (Thermo Scientific, Rockford, USA) to quantify the protein concentration of the placental lysate samples. Serial dilutions of bovine serum albumin (BSA) were done using RIPA buffer, as demonstrated in Table 2.2.

The working reagent was prepared with a mixture of 50 parts of reagent A and one part of reagent B giving a clear green solution.

A flat bottom 96-well microplate was used for the procedure. Standards (25 µl) and 10 µl of samples were pipetted in duplicate on a 96-well microplate. Thereafter, 50 µl of RIPA buffer was added to the samples, and 200 µl of working reagent was aliquoted to both standards and

samples. The microplate was briefly shaken, covered with parafilm and incubated at 37°C for 30 minutes.

The samples were placed in a microplate reader (RT-2100C, Microplate Reader, Germany), and absorbance was read at an optical density (OD) of 562 nm. A standard curve of OD versus protein concentration was plotted, and protein concentrations were interpolated.

Table 2.2. Bovine Serum Albumin Serial Dilutions

Vial	Volume of diluent (µl)	Volume of BSA (µl)
A	0	300 of stock
B	125	375 of stock
C	325	325 of stock
D	175	175 of vial B dilution
E	325	325 of vial C dilution
F	325	325 of vial E dilution
G	325	325 of vial F dilution
H	400	100 of vial G dilution
I	400	0

2.4.2.3 Western Blotting

Western blotting was carried out to determine kisspeptin, TNFα and IL-6 protein expression in placental lysates. The experiments were performed in two batches, and four sets of gels (two gels for GDM non-obese and obese and two gels for non-GDM non-obese and obese) were run simultaneously, resulting in four separate membranes.

Placental lysates were diluted in 4X Laemmli buffer (BioRad, USA) and water to a final protein concentration of 20 µg/well (TNFα, IL-6, KISS1R) and 30 µg/well (kisspeptin). Prepared samples were denatured at 95 ^{0}C for 10 minutes using a heating block, followed by centrifugation at 6000 revolutions per minute (rpm). Protein samples were separated with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions using 10-well 4–15% Mini-PROTEAN Tris-Glycine precast- protein gels from BioRad. A pre-stained protein ladder (Cat #26616, ThermoFisher) was used for marking the molecular weights of proteins in kilodalton (kDa), and the gels were run at 110-150V for 60 minutes. After the completion of SDS-PAGE electrophoresis, proteins in the gel were electroblotted onto polyvinylidene difluoride (PVDF) membrane (Hybond-P, Amersham Biosciences) using a semi-dry technique (Semi-dry Blotter, Invitrogen) and run at 25 V for 45 min. Membrane protein transfer was confirmed by placing and agitating the PVDF membrane in 0.2% ponceau red solution and demonstrating protein bands. The membrane was washed with tris-buffered saline tween (TBST) (0.1% tween) and subsequently blocked with 5% milk TBST on a shaker for 60 minutes at room temperature.

To study kisspeptin protein expression in placental lysates, membranes were incubated overnight at 4 ^{0}C with rabbit polyclonal anti-kisspeptin antibody (ab19028, Abcam, UK) at a dilution of 1:500 in 5% milk TBST. For the investigation of IL-6, TNFα, and KISS1R protein

expression, membranes were incubated with 1:800 rabbit polyclonal anti-IL-6 antibody (ab6672, Abcam, UK), 1:1000 mouse polyclonal anti-TNF-alpha antibody (Ab1793, Abcam, UK, a gift from Dr Hlumani Ndlovu) and 1:1000 rabbit polyclonal anti-GPR54 (GT100374, GeneTex Inc, USA) respectively in 5% milk TBST overnight at 4^0C (Appendix 4). For each of the two sets of membranes, one membrane was incubated in the primary antibody. The second membrane was incubated in the primary antibody-omitted blocking buffer (5% milk TBST) during the first experiment or already visualised antibody-incubated membrane stripped off to serve as negative controls. Subsequently, both membranes were incubated in a rocker overnight at 4 ^{0}C.

Goat anti-rabbit antibody (SC-2004, Santa Cruz Biotechnology, USA) in 5% milk TBST for 60 minutes was used as secondary antibody for detection of kisspeptin, KISS1R, IL-6 and GAPDH. A different goat anti-mouse antibody (Cat. #1706516, BioRad Laboratory, USA) was utilised as a secondary antibody for the detection of TNF-α (Appendix 4). Protein bands were visualised by chemiluminescence using Clarity™ Western ECL substrate (Cat. #1705061, BioRad, USA) and exposed on a C400 Azure Biosystems Imaging System. Thereafter, membranes were washed with TBST before transfer into stripping buffer and shaking on a rocker for 15 minutes at room temperature. Lastly, the membranes were washed thrice with TBST for 5 minutes, followed by blocking in 5% milk TBST and subsequent detection of the protein of interest or protein loading control.

To confirm that the same protein concentration was loaded in each lane, incubation with rabbit polyclonal antibody against GAPDH (SC-25778, Santa Cruz Biotechnology, USA) at 1:1000 in 5% milk TBST overnight at 4 ^{0}C was utilised. GAPDH is a housekeeping protein that is

constitutively expressed in many tissues, including the placenta and hence was used as a protein loading control.

The captured images of all western blots were downloaded, and the signal intensity of protein bands was quantified using GelQuantNet software. Finally, the concentrations of each protein of interest were normalised using corresponding GAPDH expression. The western blots buffer formulation details are shown in Appendix 5.

2.4.3 Histology and Immunohistochemistry

Sections of wax-embedded placental tissue blocks (5µm) were first stained with haematoxylin and eosin (H&E) using a standard protocol and subsequently subjected to immunohistochemistry to examine kisspeptin KISS1R, TNFα, and IL-6 protein expression utilising their respective antibodies (see below). All slides were batch-stained to minimise "inter-test" as well as inter-observer staining variability.

2.4.3.1 Kisspeptin, KISS1R, TNFα and IL-6 Immunostainings

The sections were dewaxed thrice - in xylene followed by rehydration in 3 washes of 100% alcohol, 2 washes of 96% alcohol, 1 wash of 70% alcohol and lastly, immersed in running water and washed for 1 minute. The slides were then incubated for 15 minutes in 3% hydrogen peroxide (H_2O_2) in distilled water (for blocking endogenous peroxide activity), followed by a quick rinse in water. Next, antigen retrieval was performed using a pressure cooker with slides in 0.01M sodium citrate buffer (pH 6) for 3 minutes. Afterwards, the slides were washed in water, rinsed in TBST, and the tissue circled with a PAP pen to create a hydrophobic barrier.

To alleviate the non-specific binding of the primary antibody, blocking was performed by adding 3% BSA for 30 minutes, 5% BSA for 60 minutes and 5% BSA for 15 minutes for kisspeptin and TNFα, KISS1R, and IL-6 immunostainings, respectively.

The slides were incubated with 100 µl of their respective primary antibodies along with two negative controls (primary antibody omitted) (1- non-GDM, 1- GDM). For kisspeptin immunostaining, the slides were incubated with primary kisspeptin (KISS1) mouse monoclonal antibody (LS-B15903, LifeSpan BioSciences Inc, USA) (1:100 dilution in TBS) in a humidifying chamber at 4 ^{0}C overnight. For KISS1R immunostaining, the slides were incubated with KISS1R rabbit polyclonal primary antibody (Ab1212, a gift from Prof Robert Millar) at 1:200 dilution in 5% BSA in a humidifying chamber at room temperature for 60 minutes. For TNFα immunostaining, the slides were incubated with TNFα mouse monoclonal primary antibody (Ab1793, Abcam, UK) at 1:100 dilution in 5% BSA, along with two negative controls (primary antibody omitted) (1- non-GDM, 1- GDM) in a humidifying chamber at 4 ^{0}C overnight. For IL-6 immunostaining, the slides were incubated with IL-6 rabbit polyclonal primary antibody (ab6672, Abcam, UK) at 1:100 dilution in 5% BSA in a humidifying chamber at 4 ^{0}C overnight.

The following morning the slides were washed in TBST, 2 drops of anti-mouse EnVision+System-HRP (K4001, Dako, USA) were added, and the slides were incubated for 30 minutes at room temperature for kisspeptin and TNFα immunostainings. The same number of drops of anti-rabbit EnVision+System-HRP (K4003, Dako, USA) were added and incubated for 30 minutes at room temperature for KISS1R and IL-6 immunostainings.

For all four proteins, the slides were then re-washed in TBST, liquid DAB+ Substrate Chromogen System (K3468, Dako, USA) was added, and the slides were incubated for 10 minutes at room temperature. The slides were then rinsed with tap water and placed in Mayer's haematoxylin for 5 minutes for nuclei counterstaining. After that, the slides were rinsed off in tap water, followed by a quick submersion in Scott's water and rinsing in running tap water for 5 minutes. Slides were dehydrated in decreasing alcohol concentrations from 70% to 100%, then xylene and coverslips were applied with entellan (Sigma-Aldrich). An Olympus VS120 Digital Slide Scanner (Olympus Corporation, Tokyo, Japan) at X 40 magnification (pixel resolution of the resulting images was 0.16µm/pixel) was used for scanning whole slides with stained tissue sections after visualisation with a light microscope.

2.4.3.2 Histological Analysis by Stereology

Wax-embedded placental tissue sections ((5µm thickness) were de-waxed and stained in haematoxylin and eosin (H&E) according to standard protocols. Slides with stained sections were scanned to create electronic images and analysed to determine the structure of the placenta. Test point systems were superimposed and used on each placental image at random positions and used to estimate the component volume and surface densities. 5X magnification images were used to calculate volume densities within the placenta. Stem villi, intermediate villi, terminal villi, intervillous space and intervillous fibrin-type fibrinoid were evaluated on each image (Grid 7 x 5 = 35 points). Similarly, a quadratic array of 13 x 9 = 117 was used to estimate volume densities within terminal villi images (syncytiotrophoblast (STB), stroma, foetal capillaries) obtained at 20X magnification. Surface densities were estimated using 16 cycloids per image to count chance intersections between test lines and the surface traces of peripheral (intermediate and terminal) villi and their foetal capillaries. Component densities were converted into absolute volumes and surfaces using the volume of the placenta as the

reference space. Placental volumes were derived from weights taking tissue density to be 1.05 g/cm^3. Fixed estimates were then converted to fresh volumes and surfaces using appropriate correction factors. Analyses were performed blinded to the group. Dr Esteban Salazar Petres, (a postdoctoral fellow in Dr Sferruzzi-Perri's laboratory, University of Cambridge, UK) conducted the stereological analyses as part of the collaboration between the Universities of Cambridge and Cape Town.

2.4.3.3 Immunohistochemistry Semi-quantitative Analysis

To determine differences in immunoreactivity of kisspeptin, KISS1R, TNFα and IL-6 protein between the four study groups, two methods were performed. Using a Macro designed with ImageJ, the percentage of DAB-stained area related to the H&E-stained area of terminal villi area was quantified in 10 images at 20X magnification and data were expressed as DAB area/terminal villi area. In addition, an immunoreactive score (IRS), a semiquantitative method,[280] was used to evaluate DAB staining area and intensity of the STB, stroma and foetal capillaries of terminal villi within the section. To perform this analysis, score values were assigned to evaluate the percentage of positive staining area, with the score range from 0 (no staining), 1 (<10% of positive area), 2 (10-50% of positive area), 3 (51-80% of positive area) to 4 (>80% of positive area). In addition, the intensity of staining was scored as 0 = no DAB positivity, 1 = mild DAB positivity, 2 = moderate DAB positivity, 3 = intense DAB positivity. The final IRS score was calculated by multiplying both scores, with a final IRS score from 0-1 = negative, 2-3 = mild, 4-8 = moderate and 9-12 = strongly positive. Analyses were performed blinded to the group.

2.4.4 Enzyme-linked Immunosorbent Assays (ELISAs)

ELISA was used to quantify circulatory maternal and cord kisspeptin, TNFα and IL-6 levels. Due to significant differences in experimental protocol, the serum kisspeptin quantification method will be presented separately, while TNFα and IL-6 methods will be shown together with slight modifications in some steps.

2.4.4.1 Maternal and Cord Serum Kisspeptin Quantification

Maternal and cord serum kisspeptin concentrations were measured using a human KiSS-1 (112-121) amide / Kisspeptin-10 / Metastin (45-54) enzyme immunoassay (EIA) kit (EK-048-56, Phoenix Pharmaceuticals, Inc, USA) according to the manufacturer's instructions. In brief, 50 µl of standard, unknown serum samples and positive controls were added in duplicates into wells of a microplate pre-coated with a secondary antibody, and nonspecific binding sites were blocked. This was followed by adding 25 µl of primary antibody and 25 µl of biotinylated peptide per well and incubating at room temperature for 2 hours. The microplate was washed 4 times with 350 µl 1x assay buffer per well before 100 µl of the secondary antibody and streptavidin horseradish peroxidase (SA-HRP) solution were added per well and incubated at room temperature for 1 hour. Following this, the microplate was washed 4 times with 350 µl of 1x assay buffer per well. A hundred microlitre of Tetramethylbenzidine (TMB) substrate solution per well was then added for 1 hour at room temperature. The reaction was terminated with 100 µl of 2N HCl per well, and the absorbance was read at OD 450 nm using a SPECTRAmax PLUS 384 microplate reader. The assay standard curve comprising OD absorbance versus standards concentration was plotted, and kisspeptin concentrations in the serum samples were interpolated from the standard curve. The assay standard curve is shown in Appendix 6. The detection range of the human kisspeptin EIA assay is 0-100 ng/ml (linear

range 0.08-1.22 ng/ml) with intra-assay variation and inter-assay coefficient variations of <10% and <15%, respectively.

2.4.4.2 Maternal and Cord Serum TNFα and IL-6 Quantification

Maternal and cord serum TNFα and IL-6 levels were measured using a human TNFα ELISA kit (ab181421, Abcam, UK) and human IL-6 ELISA kit high sensitivity (ab46042, Abcam, UK) respectively. In brief, 50 µl of all samples and standards were added into each well of the pre-coated microplate in duplicates for TNFα immunoassay. In contrast, for IL-6 immunoassay, 100 µl of standard (including blank controls), serum samples and 1X control solutions were added in duplicates to appropriate wells of the pre-coated microplate. As the number of subsequent steps were different for TNFα and IL-6 immunoassays, they will be presented separately.

For TNFα, next was the addition of 50 µl of antibody cocktail to each well with the plate sealed and incubated for 1 hour at room temperature on a plate shaker. The microplate wells were washed 3 times with 350 µl 1x wash buffer pipetted, while the excess liquid was removed by blotting the microplate against clean paper towels. Next, 100 µl of TMB Development Solution was added to each well and incubated at room temperature for 10 minutes in the dark. The reaction was terminated by adding 100 µl of Stop Solution to each well, and the microplate was shaken for 1 minute to mix.

For IL-6 immunoassay, 50 µl of 1X biotinylated anti-IL-6 was then added to all wells. The microplate was covered and incubated for 3 hours at room temperature. After this, each well was washed three times with 300 µl 1X wash buffer and excess liquid aspirated before applying 100 µl of 1X Streptavidin-HRP solution to each well. The microplate was incubated for 30

minutes at room temperature and then washed three times by adding 300 µl 1X wash buffer into each well and aspirating in between wells. Chromogen TMB substrate solution, 100 µl per well, was then added, and the microplate was incubated in the dark for 12-15 minutes at room temperature. The reaction was stopped with 100 µl of Stop Reagent pipetted into each well.

After stopping reactions for TNFα and 1L-6, the OD was read at 450 nm for TNFα and 450 nm and 620 nm (reference wavelength) for IL-6 using the SPECTRAmax PLUS 384 microplate reader. The assay standard curve of the absorbance OD against the concentration of the standards was plotted, and the TNFα and IL-6 concentrations of the serum samples were interpolated from their respective standard curves. The standard curves are shown in Appendix 7 and 8.

The detection range of human TNFα ELISA assay is 15.63 pg/ml-1000 pg/ml with a sensitivity of 4.32 pg/ml with intra-assay variation and inter-assay coefficient variations of 2.5% and 3.1%, respectively. The detection range of human IL-6 ELISA high sensitivity assay is 1.6 pg/ml-50 pg/ml (sensitivity of 0.8 pg/ml) with intra-assay variation and inter-assay variations of 4.4% and <14%, respectively.

2.5 Study 5:

This was a preliminary study to determine kisspeptin protein expression in syncytiotrophoblast extracellular vesicles (STBEV) from GDM and non-GDM pregnancies. It was undertaken at the Vatish Laboratory, Nuffield Department of Women's and Reproductive Health, University of Oxford, United Kingdom, in collaboration with the University of Cape Town. The work was Funded by Newton RCUK PhD Partnership Fellowship.

The study utilised six archival syncytiotrophoblast extracellular vesicles (STBEV) samples (GDM- 3, non-GDM- 3) and eight placental lysates samples (GDM- 4, non-GDM- 4), all from the Vatish Laboratory. The use of the archival samples was covered by the University of Oxford ethics committee approval number: 07/H0607/74.

The STBEV samples were obtained from a placental dual lobe perfusion system as was described by Dragovic et al in 2015.[281] The placentae were perfused for 3 hours, and perfusates from the maternal side were collected and centrifuged twice at 1500 ×g for 10 min at 4°C to remove red blood cells and cellular debris. Subsequently, the separation of STBEV based on their sizes was achieved following successive centrifugations, then the spinning of collected supernatant was performed at 10,000 ×g for 35 min at 4°C to the pellet syncytiotrophoblast medium/large extracellular vesicles (200 nm - 1 μm). Ultracentrifugation of resuspended medium/large STBEV in sterile PBS with the passage of the remaining supernatant through a 0.2 μm Stericup filter and underwent spinning at 150,000 ×g for 125 min at 4°C resulted in pellet smaller size syncytiotrophoblast extracellular vesicles (50–200 μm). Thereafter, the STBEV protein concentrations were quantified by BCA protein assay.

2.5.1 Study 5 Laboratory Methods

2.5.1.1 Western Blotting

As the laboratory methods for western blot and immunostaining in this study have some differences from the previously described protocols, they are presented separately below.

Placental lysates and STBEV were diluted in 4X Laemmli buffer (Cat #1610747, BioRad, UK) and water to a final protein concentration of 30 µg/well and 20 µg/well, respectively. The prepared samples were denatured at 95 ^{0}C for 10 minutes and then centrifuged for 1 minute. Protein samples were separated by SDS-PAGE under reducing conditions using 10-well NuPAGE® 4–12% Bis-Tris Gels from ThermoFisher. A pre-stained protein ladder (10 to 180 kDa, ThermoFisher) was used for marking the molecular weights. Gels were run at 110-150V for about 60 minutes, and then proteins in the gel were electroblotted onto a PVDF membrane (Immun-Blot® PVDF Membrane, BioRad, UK) at 25 V for 45 min using a semi-dry setup (Semi-dry Blotter, Invitrogen). Membrane protein transfer was checked using ponceau red staining. Membranes with successfully transferred proteins were washed with TBST before blocking (5% milk TBST), on a shaker, for 60 minutes at room temperature.

Membranes were first incubated with mouse anti-KISS-1 mouse monoclonal antibody (sc-101246, Santa Cruz, USA) at a dilution of 1:200 in 5% milk TBS overnight at 4 ^{0}C and then incubated in conjugated goat anti-mouse secondary antibody (Cat #A32723, ThermoFisher) at 1:4000 dilution in 5% milk TBST for 60 minutes. Protein bands were visualised by chemiluminescence using EZ-ECL substrate (ECL Western Blotting Detection Reagent Cytiva, RPN2209) and exposed on G: BOX (Syngene, UK) in the darkroom.

After protein band visualisation, membranes were washed with TBST, transferred into stripping buffer (ThermoFisher, UK) and shaken on a rocker for about 15 minutes at room temperature. The membranes were then washed 3 times with TBST for 5 minutes, followed by blocking in 5% milk TBST. To confirm that the same protein concentration was loaded in each lane, a 1:1000 Anti-PLAP antibody (in-house antibody) at Prof Vatish's lab was then applied and visualised as described above.

2.5.1.2 Immunoprecipitation

Immunoprecipitation is a technique that is utilised for the purification of a specific protein of interest. It involves the incubation of antibody for the protein of interest with a cell extract allowing for antibody binding to the protein in a solution.[282] Subsequently, the antibody-antigen complex is pulled out of the sample using protein A/G-coupled Dynabeads.[282] Immunoprecipitation technique was used to investigate kisspeptin protein co-localisation with placental alkaline phosphatase (PLAP) in placenta derived STBEV.

The Dynabeads M-280 sheep anti-Mouse IgG was conjugated to anti-KISS-1 mouse monoclonal antibody (sc-101246, Santa Cruz, USA) and anti-PLAP antibody (in house antibody), while Dynabeads coated with anti-IgG2α and anti-IgG1 antibody served as control. Dynabeads were vortexed for about 30 seconds, and 50 µl containing 25 µg of sample protein was transferred to a tube followed by the addition of 1 ml of Washing Buffer (Ca^{2+} and Mg^{2+} free Phosphate-Buffered Saline (fPBS) with resuspension of beads pellet in free PBS with the tube attached to a magnet. Following Dynabeads washing, 6 µg of anti-KISS-1, anti-PLAP, anti-IgG2α, and anti-IgD1 antibodies were added to respective tubes containing Dynabeads and incubated at 4 ^{0}C overnight. The following day, the magnet was used to aggregate Dynabeads, and the supernatant was discarded. Thereafter, 15.51 µl of STBEV sample was

added to 10 µl of anti-human Fc receptor (FCR) blocking reagent and 74.49 µl fPBS to make a total volume of 100 µl and incubated for 10 minutes. Next, 20 µl of the prepared pool was added to the four respective antibodies coated Dynabeads, followed by the addition of fPBS and incubation overnight at 4 ^{0}C. On day 3, the magnetic separator was used to separate bound and unbound STBEV by placing the tubes on a magnet for 5 minutes, followed by 2 washes with 1 ml of wash and one wash with PBS. The Dynabeads pellets containing antigen-positive STBEV and respective supernatants were collected and used for western blotting with gel layout, as outlined in Figure 2.2.

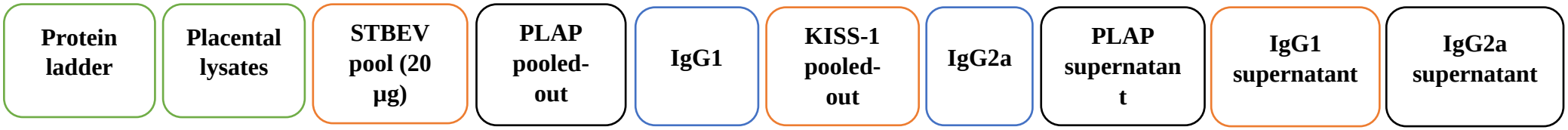


Figure 2.2. Western Blot Gel Layout following Immunoprecipitation

2.5.1.3 Validation of Kisspeptin Antibody for the Localisation of Syncytiotrophoblast in Normal Placenta

To validate the kisspeptin antibody used during western blotting of STBEV and immunoprecipitation, kisspeptin immunostaining of normal placental tissue slides to localise syncytiotrophoblasts was performed. The slides were dewaxed in Histoclear® and rehydrated in graded alcohol, followed by 5 minutes of wash in PBS. Antigen retrieval was performed using a microwave with slides in 0.01M sodium citrate buffer at pH 6 for 10 minutes (1 minute on high power 100 and 9 minutes on low power 10) and thereafter allowed to cool for 20 minutes. The slides were incubated for 5 minutes in 3% hydrogen peroxide (H_2O_2) in PBS at room temperature to block endogenous peroxide activity to limit background staining and then rinsed with distilled water. Slides were then incubated in a blocking solution (1% foetal calf serum (FCS) PBST) at room temperature for 1 hour in a humidifying chamber after the section on slides was circled with a PAP pen. Anti-KISS-1 mouse monoclonal antibody (sc-101246, Santa Cruz, USA) in 1% FCS PBST at 1:25 dilution along with one negative control (blocking solution) were added to the tissue sections and incubated at 4 ^{0}C overnight in a humidifying chamber.

The slides were washed 3 times each for 3 minutes in PBS the following morning, followed by incubation with anti-mouse secondary antibody for 1 hour at room temperature, and then washed 3 times each for 3 minutes. Next, Vectastain ABC (70µl) was added to the sections and incubated in a humidifying chamber for 30 min at room temperature. Again, the slides were washed 3 times (5 minutes each) with PBST and, after that, stained with DAB. A subsequent wash with PBST and water was done before counterstaining with haematoxylin. The sections were then dehydrated with degrading concentrations of alcohol and Histoclear. Finally, the coverslips were applied with Depex mounting medium (Sigma Aldrich, UK) and visualised using a Leica DM 2500 microscope.

2.6 Statistical Analysis

Data were analyzed using the GraphPad Prism software version 8 for Windows, San Diego, California, USA. Data were reported as mean and standard error of mean (SEM). The Shapiro-Wilk test assessed the distribution of continuous variables. A two-way analysis of variance (ANOVA) was used to compare clinical data, gene expression data of quantitative RT-PCR, western blotting, immunostaining, stereological analysis results and ELISAs for four groups (non-GDM non-obese, non-GDM obese, GDM non-obese, GDM obese), with a Bonferroni's multiple comparisons test to identify significant differences between the individual groups. Pearson's correlation coefficients were used to assess associations between variables. P-values of < 0.05 were considered statistically significant.

3.0 Results

The results of the studies undertaken in this thesis are presented by obesity and GDM status, i.e. in four groups- non-GDM non-obese, non-GDM obese, GDM non-obese and GDM obese. In addition, the non-GDM non-obese group serves as a control group.

3.1 Socio-Demographic, Clinical and Metabolic Characteristics of the Study Participants

3.1.1 Maternal Socio-demographic Characteristics

The mean ages of participants within the four study groups did not differ either by GDM or obesity status (Table 3.1). However, more participants in the non-GDM non-obese group were fully employed compared to the other three groups, in whom about two-thirds were students. The majority of women across the four groups achieved high school education although, a greater proportion of GDM non-obese women had completed tertiary level education compared to the other three groups.

3.1.2 Maternal Clinical Characteristics

The mean weight and BMI at booking, as well as weight at delivery, were significantly higher in the two obese than the two non-obese groups, but these measures did not differ by GDM status (Table 3.2). There was a significant effect of BMI but not GDM on maternal percentage weight gain (P_{BMI} = 0.0002), absolute weight gain (P_{BMI} = 0.0179) and maternal weight gain velocity (P_{BMI} = 0.0112), with the weight gains being lower in the two obese groups. BMI had a significant effect on systolic blood pressure (SBP) of the study participants (P_{BMI} = 0.0014), and this effect was significant by pairwise comparison when comparing the two GDM groups, with the obese GDM group having a significantly higher SBP than GDM non-obese (p = 0.0331).

3.1.3 Maternal History of Risk Factors for GDM

The most important risk factors for GDM among the study groups were family history of diabetes, previous stillbirths, and foetal macrosomia, while previous GDM was an important risk factor in the GDM groups. There was no difference in the frequency of stillbirths among the four groups. Meanwhile, foetal macrosomia and family history of GDM and diabetes largely showed an increasing frequency in the order of the groups- non-GDM non-obese, non-GDM obese, GDM non-obese, GDM obese. Past history of preeclampsia was the least significant risk factor for GDM among the four groups. Only 7% of the non-GDM non-obese group and none of the other three groups reported a past history of preeclampsia (Table 3.2).

3.1.4 Maternal Metabolic Characteristics

As expected, the GDM groups had significantly higher mean FPG and 2-hour glucose concentration post-OGTT than their non-GDM counterparts (P_{GDM} = <0.0001), but there was no significant effect of BMI nor the interaction between GDM and BMI on these variables (FPG and 2-hour glucose; Table 3.2). A higher proportion of non-obese than obese GDM women was treated with metformin alone (72.7% vs 59.1%); contrariwise, a greater proportion of GDM obese women required insulin in addition to metformin than their non-obese GDM counterparts (40.9% vs 27.3%, respectively). None of the GDM groups received insulin alone for lowering blood glucose during pregnancy (Table 3.2).

Table 3.1. Socio-demographic Characteristics of the Study Participants

	Non-GDM Non-obese (n=14) Mean±SEM	Non-GDM Obese (n=19) Mean±SEM	GDM Non-obese (n=15) Mean±SEM	GDM Obese (n=23) Mean±SEM	P value interaction	P value GDM	P value BMI
Age (years)	30.64 ± 2.03	29.90 ± 1.27	30.57 ± 1.55	32.04 ± 1.12	0.4523	0.4818	0.8060
Occupation:							
Full employment (%)	42.86	36.84	33.33	30.43			
Informal employment (%)	0.00	0.00	6.67	4.35			
Unemployed (%)	14.29	0.00	0.00	4.35			
Student (%)	42.86	63.16	60.00	60.87			
Level of Education:							
Tertiary (%)	24.43	21.05	33.33	21.74			
High school (%)	71.43	73.68	60.00	78.26			
Primary (%)	0.00	5.26	0.00	0.00			
None (%)	7.14	0.00	6.67	0.00			

Differences in maternal age between groups were determined using two-way ANOVA and Bonferroni's multiple comparisons test.

$P < 0.05$ is considered statistically significant

Table 3.2. Clinical and Metabolic Data of the Study Participants

	Non-GDM Non-obese (n=14) Mean±SEM	**Non-GDM Obese (n=19) Mean±SEM**	**GDM Non-obese (n=15) Mean±SEM**	**GDM Obese (n=23) Mean±SEM**	**P value interaction**	**P value GDM**	**P value BMI**
Maternal height (m)	1.61 ± 0.02	1.59 ± 0.02	1.60 ± 0.02	1.63± 0.01	0.2047	0.5042	0.7116
Maternal weight at booking (kg)	67.00± 2.90	99.42 ± 3.73*	68.53 ± 2.89	101.26 ± 2.72@	0.9617	0.6043	**<0.0001**
Gestational age at GDM diagnosis (weeks)	29.64 ± 1.81	29.44 ± 1.18	27.15 ± 1.86	26.13 ± 1.36	0.7916	0.0665	0.6956
Maternal weight at delivery (kg)	80.83 ± 3.07	104.97 ± 4.08*	77.01 ± 3.23	106.37 ± 2.61@	0.4494	0.7242	**< 0.0001**
BMI at booking (kg/m2)	25.72 ± 0.79	38.13 ± 1.66*	26.52 ± 0.57	37.72 ± 1.05@	0.4956	0.7255	**< 0.0001**
Weight gain (Kg)	12.07 ± 1.59	5.59 ± 2.08	8.49 ± 2.08	4.92 ± 1.97	0.4802	0.3122	**0.0179**
Weight gain (%)	17.75 ± 2.52	5.91 ± 1.92*	13.05 ± 3.26	5.51 ± 2.14	0.3854	0.3047	**0.0002**
Weight gain (Kg/weeks)	1.48 ± 0.49	0.62 ± 0.23	1.56 ± 0.64	0.28 ± 0.29	0.6076	0.7476	**0.0112**
Systolic Blood Pressure (mmHg)	114.80 ± 2.98	122.22 ± 3.01	113.43 ± 2.60	124.26 ± 2.21@	0.5379	0.9015	**0.0014**
Diastolic blood pressure (mmHg)	71.79 ± 3.05	76.56 ± 2.23	72.64 ± 2.80	77.17 ± 2.22	0.9632	0.7754	0.0756
Risk factors of GDM							
Preeclampsia (%)	7.14	0.00	0.00	0.00			
Fetal macrosomia (%)	7.14	15.79	20.00	26.09			
Stillbirths/miscarriage (%)	28.57	26.32	33.33	21.74			
Premature delivery (%)	7.14	0.00	6.67	0.00			
Family history of diabetes mellitus (%)	14.29	36.84	33.33	69.57			
Family history of GDM (%)	0.00	5.26	26.67	17.39			

Metabolic characteristics:							
FPG (mmol/L)	4.29 ± 0.08	4.5 ± 0.07	6.99 ± 0.92	6.87 ± 0.26	0.7000	**< 0.0001**	0.9298
OGTT: 2-hour glucose (mmol/L)	5.36 ± 0.22	5.75 ± 0.27	9.79 ± 0.93	10.47 ± 0.57	0.7809	**< 0.0001**	0.3191
HbA1c (%)	-	-	6.94 ± 0.92	6.27 ± 0.25			
Mean FBG at last trimester (mmol/L)	-	-	5.45 ± 0.82	5.40 ± 0.18			
GDM treatment:							
Metformin (%)			72.73	59.09			
Metformin + Insulin (%)			27.27	40.91			
Insulin (%)			0.00	0.00			

Differences between groups were determined using two-way ANOVA and Bonferroni's multiple comparisons test.

*: difference comparing Non-GDM Non-obese vs Non-GDM Obese

$^{@}$: difference when comparing GDM Non-obese vs GDM Obese

Abbreviations. BMI: Body mass index, FPG: fasting plasma glucose, OGTT: Oral glucose tolerance test

$P < 0.05$ is considered statistically significant

3.2 Infant Clinical Outcomes

As seen in Table 3.3, GDM and BMI had no significant effect on the mean birth weight, APGAR score at 1 and 5 minute, head circumference, placental weight, or placenta/infant birth weight ratio. However, there was a significant effect of GDM (P_{GDM} = 0.0011), but not BMI or the interaction between GDM and BMI (P_{BMI} = 0.2513, $P_{GDM+BMI}$ = 0.7099, respectively) on the infant length at delivery. The ponderal index of the infants was significantly influenced by both GDM and BMI (P_{GDM} = <0.0001, P_{BMI} = 0.0259) with no effect of interaction between the two ($P_{GDM+BMI}$ = 0.8850) (Table 3.3). In addition, GDM but not BMI was associated with a reduction in the gestational age of infants (P_{GDM} = 0.0005, P_{BMI} = 0.9935), with no significant differences identified by pairwise comparisons for the study groups (Table 3.3).

There was a difference in the mode of delivery between the two GDM and non-GDM groups, where the majority of infants in both GDM groups were delivered by caesarean section compared to their non-GDM counterparts. The proportion of female infants was highest in the non-GDM non-obese women (71.4%) and decreased across the other 3 groups: non-GDM obese (63.2%), GDM non-obese (53.3%) and GDM obese women (47.8%) (Table 3.3).

Table 3.3. Mode of Delivery and Infants' Clinical Outcomes of the Study Subjects

	Non-GDM Non-obese (n=14) Mean±SEM	Non-GDM Obese (n=19) Mean±SEM	GDM Non-obese (n=15) Mean±SEM	GDM Obese (n=23) Mean±SEM	P value interaction	P value GDM	P value BMI
Weight (kg)	3.24 ± 0.16	3.35 ± 0.09	3.34 ± 0.14	3.49 ± 0.09	0.8690	0.3023	0.2506
APGAR score at 1 minute	8.6 ± 0.24	8.6 ± 0.28	8.2 ± 0.35	8.2 ± 0.26	0.9593	0.1954	0.9676
APGAR score at 5 minutes	9.7 ± 0.13	9.7 ± 0.11	9.7 ± 0.13	9.5 ± 0.20	0.8690	0.3023	0.2506
Length (cm)	52.5 ± 1.1	51.4 ± 0.7	49.6 ± 0.6	49.1 ± 0.6	0.7099	**0.0011**	0.2513
Head circumference (cm)	35.4 ± 0.5	35.4 ± 0.3	35.1 ± 0.4	34.3 ± 0.2	0.8838	0.4181	0.4711
Placental weight (kg)	630.4 ± 43.0	611.5 ± 26.3	611.8 ± 26.8	673.5 ± 30.3	0.2547	0.4415	0.5764
Placenta/infant birth weight ratio	0.19 ± 0.04	0.18 ± 0.03	0.18 ± 0.04	0.19 ± 0.03	0.1746	0.8378	0.6443
Ponderal index (kg/cm^3)	2.22 ± 0.11	2.48 ± 0.10	2.76 ± 0.10	2.99 ± 0.10	0.8850	**<0.0001**	**0.0259**
Gestational age (weeks)	39.2 ± 0.6	39.1 ± 0.3	38.0 ± 0.2	38.1 ± 0.1	0.6727	**0.0005**	0.9935
Mode of delivery							
Cesarean section (%)	42.86	36.84	73.33	47.83			
Vaginal delivery (%)	42.86	57.89	26.67	52.17			
Instrumental delivery (%)	7.14	0.00	0.00	0.00			
Sex							
Female (%)	71.43	63.16	53.33	47.83			
Male (%)	28.57	36.84	46.67	52.17			

Study participants were divided in four groups: Non-GDM Non-obese (n = 14), Non-GDM Obese (n = 19), GDM Non-obese (n =15), GDM Obese

(n = 23). Data are represented AS means ± SEM. Differences between groups were determined using two-way ANOVA and Bonferroni's multiple comparisons test. Abbreviations. APGAR: Appearance, Pulse, Grimace, Activity, and Respiration.

$P < 0.05$ is considered statistically significant.

3.3 The Placental Expression of Kisspeptin, other Endocrine Signal, Proinflammatory Cytokine, Growth Factor, and Steroidogenic Hormone Enzyme Across the Four Study Groups; GDM (non-obese, obese) and Non-GDM (non-obese, obese)

3.3.1 The Placental Expression of Kisspeptin, other Endocrine Signal, Proinflammatory Cytokine, Growth Factor, and Steroidogenic Hormone Enzyme Genes Across the Four Study Groups; GDM (non-obese, obese) and Non-GDM (non-obese, obese) Using Quantitative RT-PCR

3.3.1.1 Placental Kisspeptin and other Endocrine Signal Genes: Although there was no effect of GDM on placental *kisspeptin* gene expression overall, there was a near statistical tendency for obesity ($P_{BMI} = 0.0623$) to increase *kisspeptin* gene expression in both obese GDM and non GDM groups compared to their non-obese counterparts-(non-GDM obese (1.98 ±0.56) and GDM obese (2.75 ±0.66) compared to their non-GDM non-obese (1.47 ±0.43) and GDM non-obese (1.05 ± 0.52) groups respectively) (Figure 3.1). On the other hand, obesity reduced both *KISS1R* and *leptin* mRNA expression by the placenta in the obese groups ($P_{BMI} = 0.0166$, $P_{BMI} = 0.0073$, respectively) with no significant independent effect of GDM. In the non-GDM group, the pairwise comparisons revealed that mean *leptin* mRNA expression was significantly lower in the obese than in the non-obese group (0.58 ±0.17 vs 4.89 ±1.50, p = 0.0333) (Figure 3.1). While there was a similar trend in the GDM group, the difference between the obese and non-obese women was not statistically significant.

There was also no significant effect of GDM and BMI on the expression of placental lactogen family genes (*GHV, CSH1, CSH2, CSH*; Figure 3.1); however, their mean expression tended to be higher in both obese groups compared to their respective non-obese counterparts, with

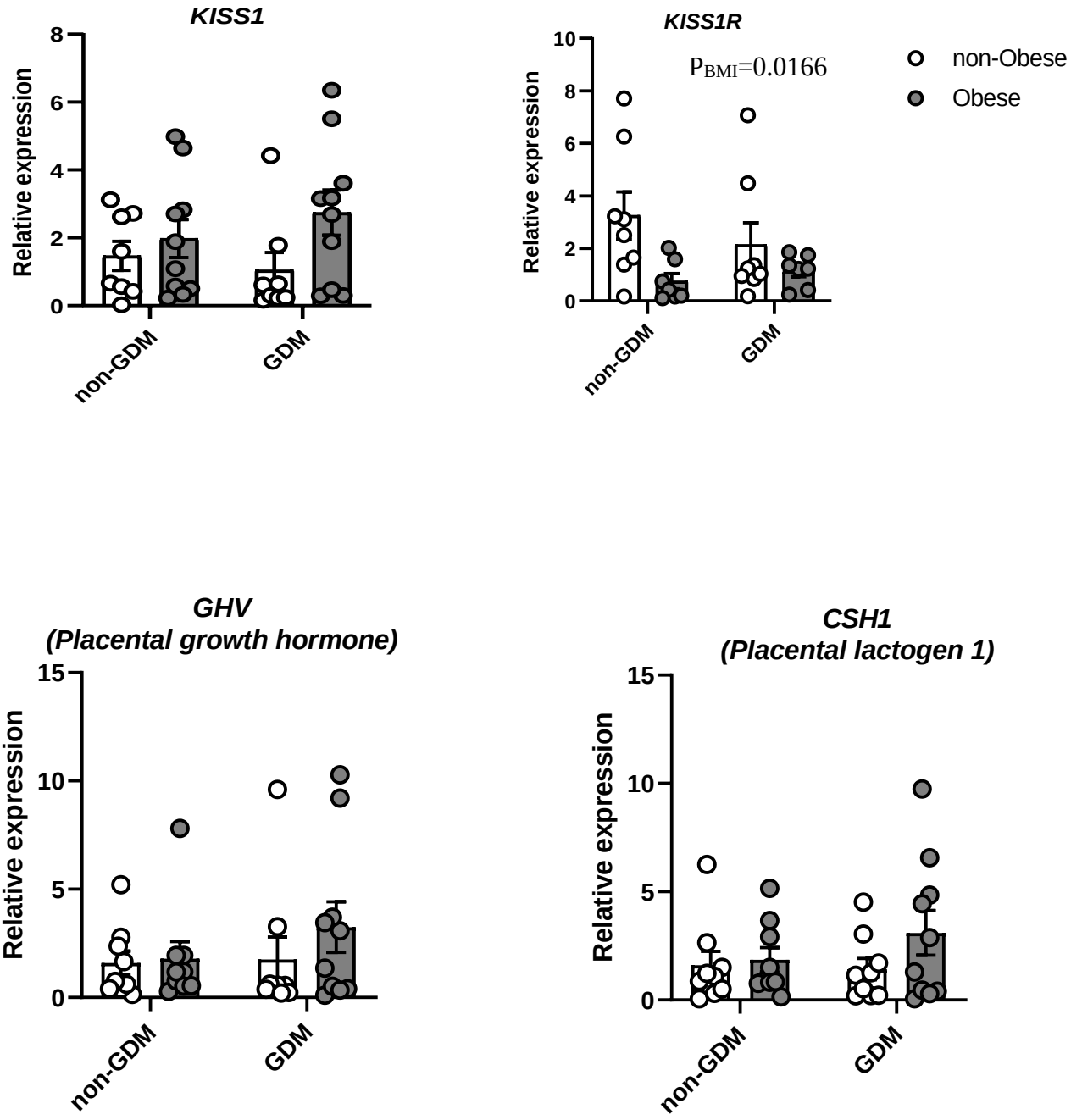
the exception of *CSH* where expression was higher in obese GDM than non-obese GDM but not for non-GDM groups.

3.3.1.2 Placental Proinflammatory Cytokine Genes: The expression of the proinflammatory cytokine genes, *TNFα, IL-6, and IL-1β* was not influenced by either GDM, BMI or their interaction. Also, there was no significant difference in the relative expression of any of the proinflammatory cytokine genes among the four groups. However, in GDM women, those who were obese had non-significant higher relative expressions of the proinflammatory cytokine genes when compared to the non-obese group (Figure 3.2).

3.3.1.3 Placental Growth Factor Genes: Neither GDM nor BMI influenced the placental expression of the growth factor genes, *IGF2* and *VEGF*. Pairwise comparisons also showed no difference in *IGF2* and *VEGF* gene expression between the four groups (Figure 3.3).

3.3.1.4 Placental Steroidogenic Enzyme Genes: GDM and BMI did not influence the placental expression of the steroidogenic enzyme genes (*HSD3B1, CYP11A1, CYP19A1),* nor did pairwise comparison show a significant difference in the expression of placental steroidogenic genes between the four groups. However, the placenta from the obese non-GDM and GDM women had a higher relative expression of the *CYP19A1* gene compared to their non-obese counterparts (Figure 3.4).

Kisspeptin and other endocrine signals



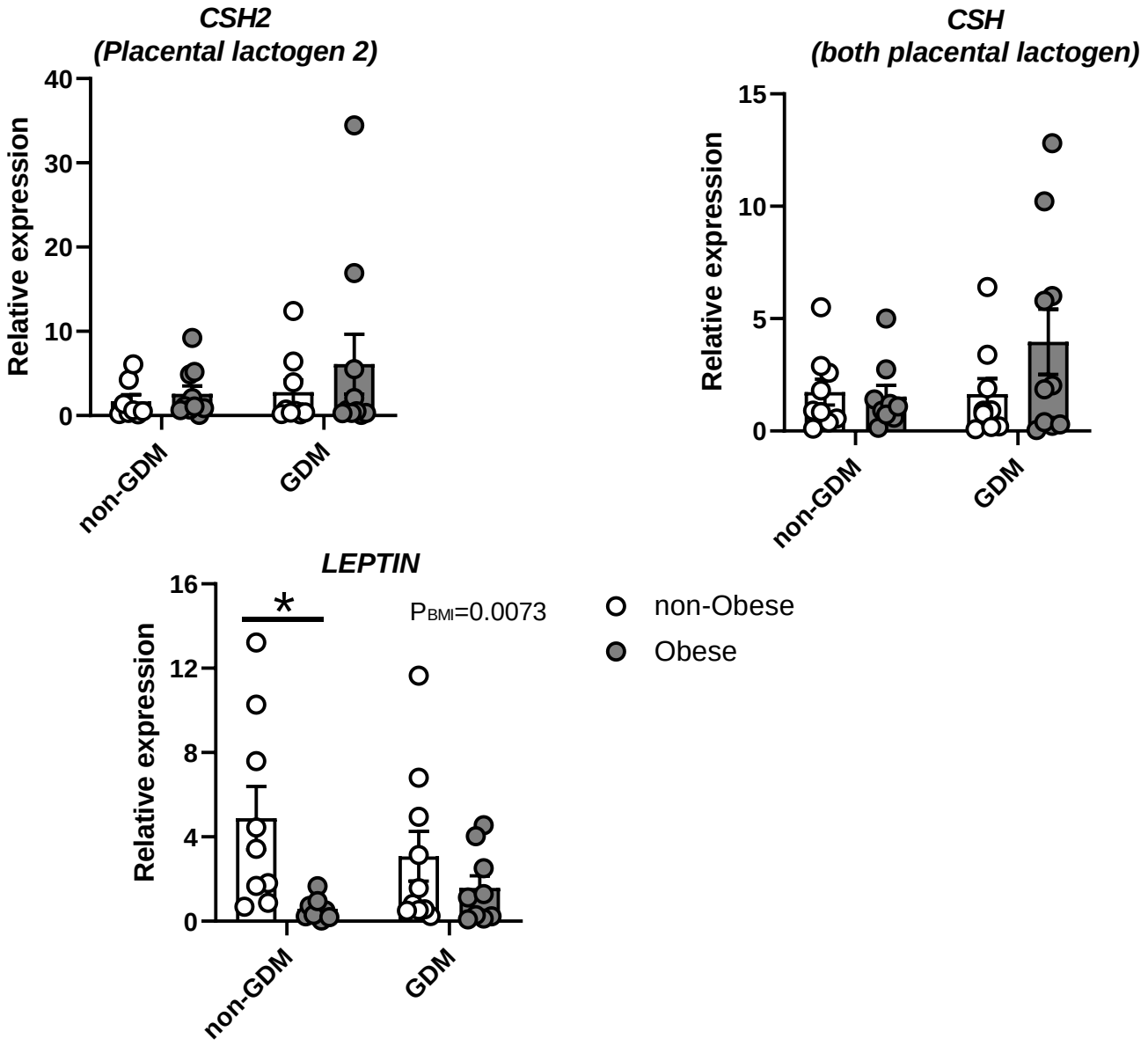


Figure 3.1. Relative gene expression of endocrine signals genes (*KISS1, KISS1R, GHV, CSH1*, *CSH2, CSH, Leptin*) in placental tissue of non-GDM non-obese (n = 9), non-GDM obese (n = 10), GDM non-obese (n = 10) and GDM obese (n = 10) women. Data are shown as individual values and mean ± SEM of relative expression normalised to the mean of *GADPH, B2MA* and *BACTIN* Ct values. Differences were determined by two-way ANOVA and Bonferroni multiple comparison tests.

P_{BMI} = statistically significant effect of BMI on the expression of *KISS1R* and *leptin* genes.

*: significant difference between non-GDM non-obese and non-GDM obese group.

P <0.05 is statistically significant.

Proinflammatory cytokines

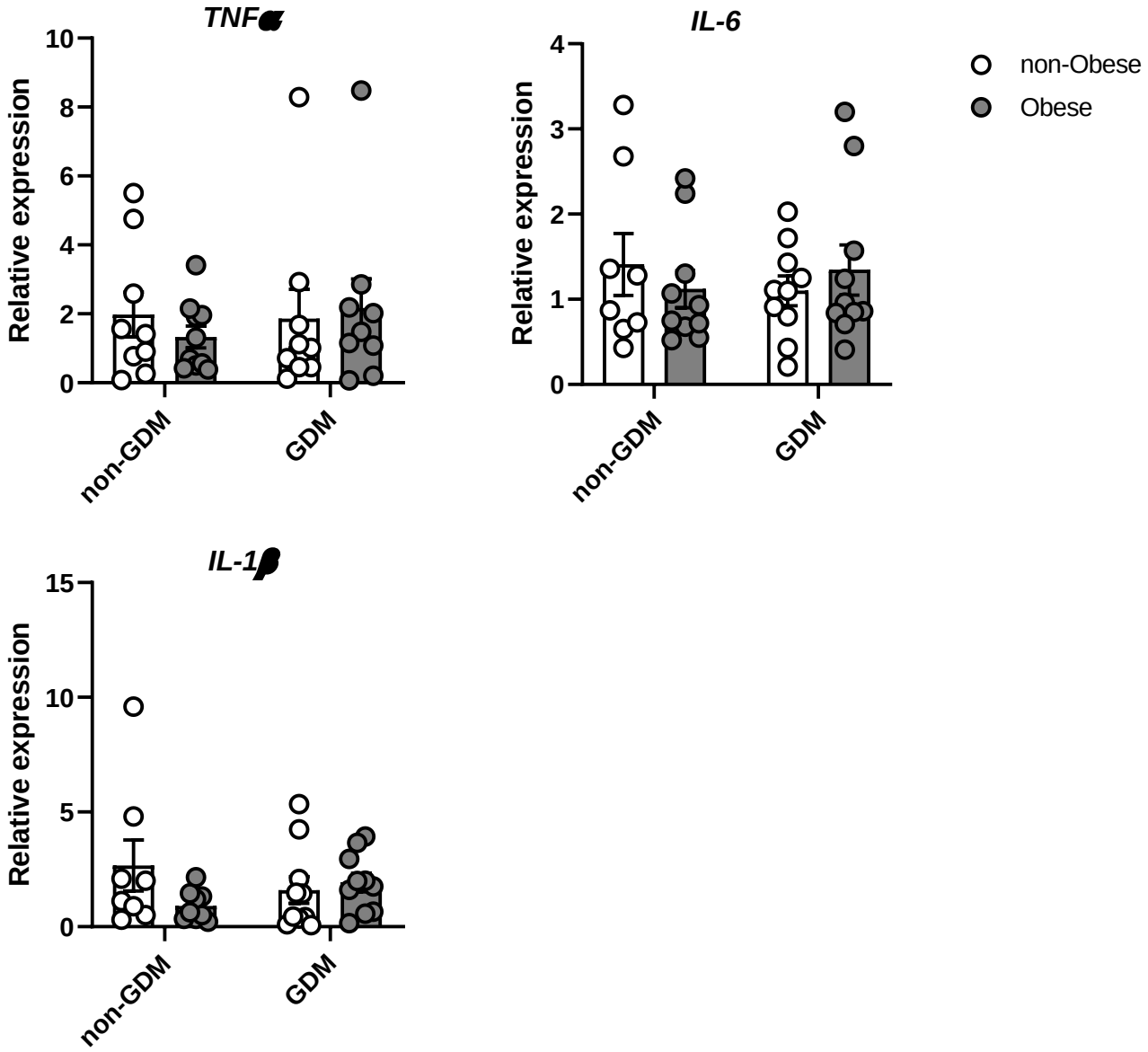


Figure 3.2. Relative gene expression of pro-inflammatory cytokines genes (*TNFα, IL-6, IL-1β*) in placental tissue of non-GDM non-obese (n = 9), non-GDM obese (n = 10), GDM non-obese (n = 10) and GDM obese (n = 10) women. Data are shown as individual mean ± SEM of relative expression normalised to the mean of *GADPH, B2MA* and *BACTIN* Ct values. Differences were determined by two-way ANOVA and Bonferroni multiple comparison tests.

Growth factors

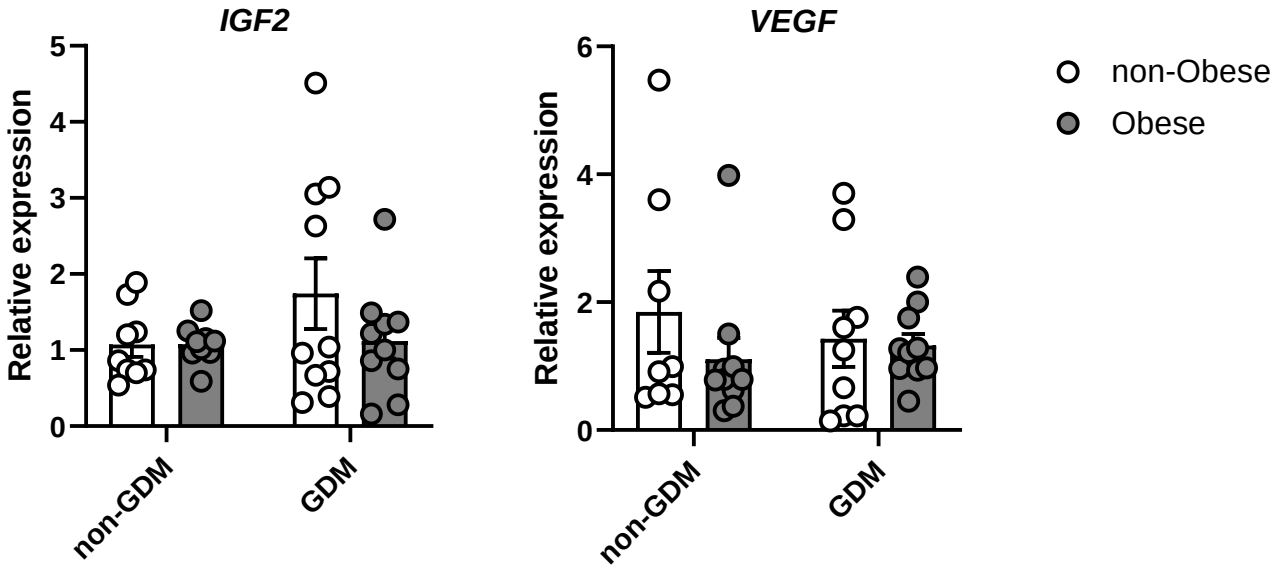


Figure 3.3. Relative gene expression of growth factors genes (*IGF2, VEGF*) in placental tissue of non-GDM non-obese (n = 9), non-GDM obese (n = 10), GDM non-obese (n = 10) and GDM obese (n = 10) women. Data are shown as individual values and mean ± SEM of relative expression normalised to the mean of *GADPH, B2MA* and *BACTIN* Ct values. Differences were determined by two-way ANOVA and Bonferroni multiple comparison tests.

Steroidogenic enzymes

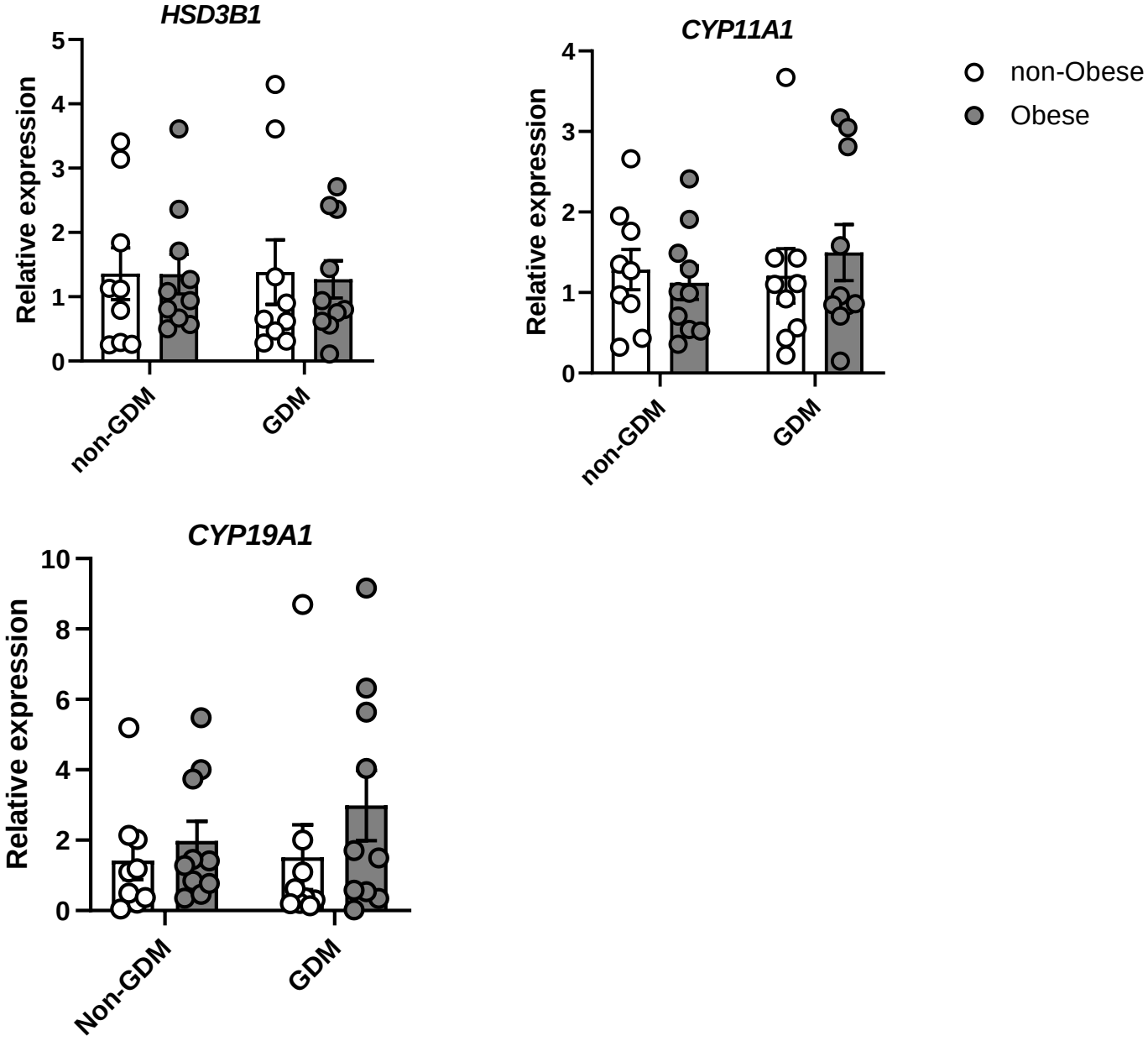


Figure 3.4. Relative gene expression of steroidogenic enzymes genes (*HSD3B1*, *CYP11A1*, *CYP19A1*) in placental tissue of non-GDM non-obese (n = 9), non-GDM obese (n = 10), GDM non-obese (n = 10) and GDM obese (n = 10) women. Data are shown as individual values and mean ± SEM of relative expression normalised to the mean of *GADPH*, *B2MA* and *BACTIN* Ct values. Differences were determined by two-way ANOVA and Bonferroni multiple comparison tests.

3.3.2 The Placental Abundance of Kisspeptin, Proinflammatory Cytokines (TNFα, IL-6) Using Western Blotting

Neither GDM nor BMI influenced placental kisspeptin protein expression (Figure 3.5). In contrast, the expression of TNFα protein was significantly influenced by GDM status, but neither by BMI nor the interaction between GDM and BMI (P_{GDM} = 0.0027, P_{BMI} = 0.9365, $P_{GDM.BMI}$ = 0.3394; Figure 3.6). Placental TNFα protein expression was lower in both GDM groups compared to the two non-GDM groups overall. There was an overall tendency for GDM to reduce placental IL-6 protein expression (P_{GDM} = 0.0634), but tendencies were not observed for BMI or the interaction of GDM and BMI (P_{BMI} = 0.1506, $P_{GDM.BMI}$ = 0.3084, respectively; Figure 3.7).

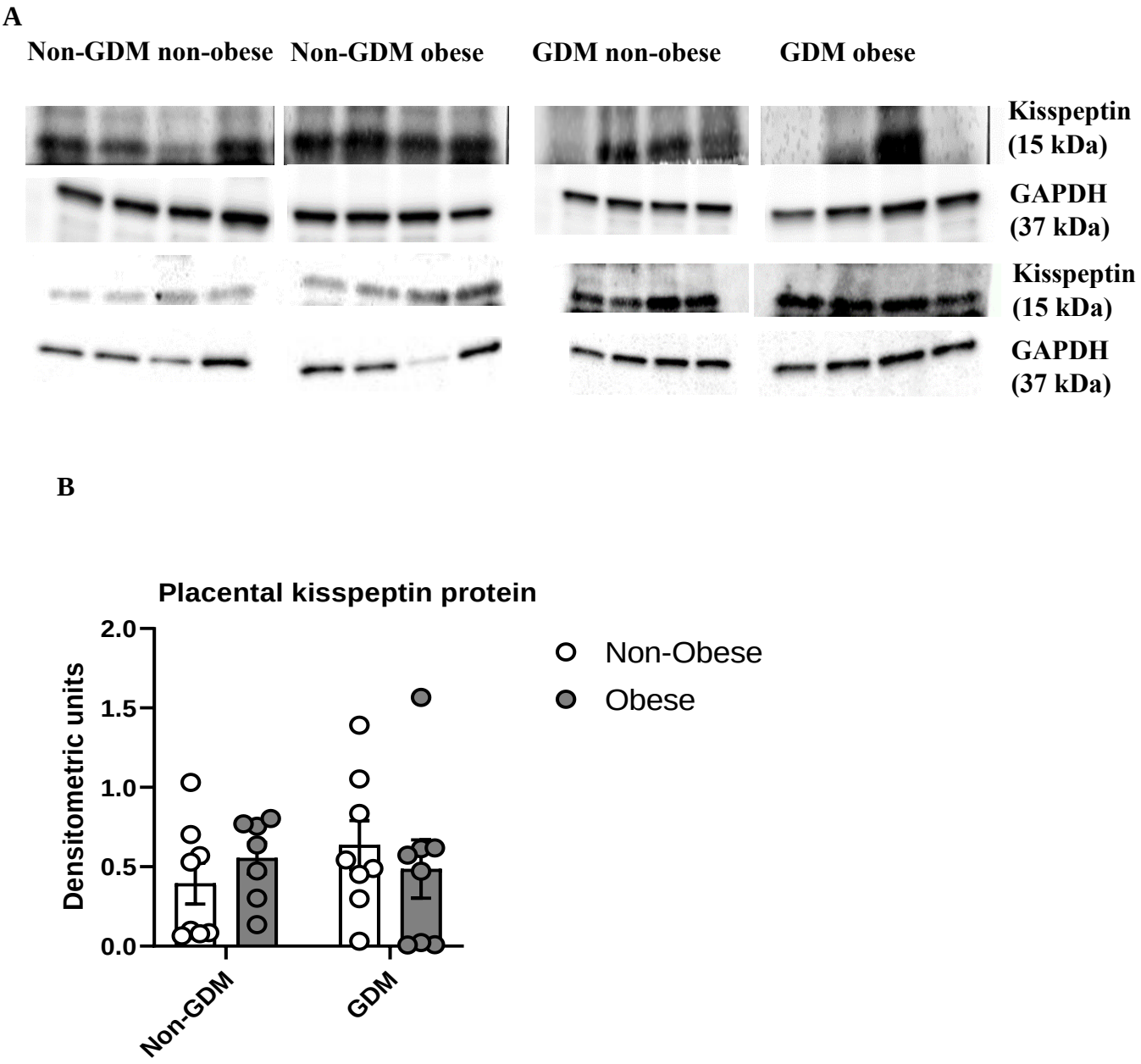
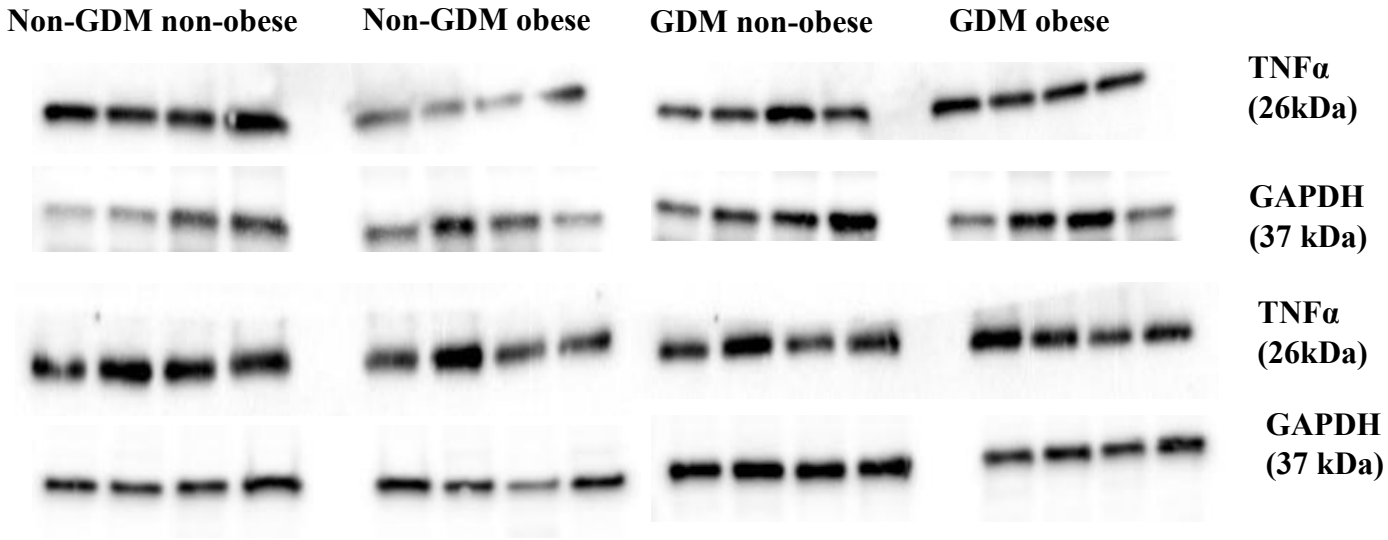


Figure 3.5 Placental kisspeptin protein expression. A. Western Blot demonstrating the expression abundance of kisspeptin protein and GAPDH proteins in placental lysates of non-GDM non-obese (n = 8), non-GDM obese (n = 8), GDM non-obese (n = 8) and GDM obese (n = 8) women. Lanes 1 and 2: first batch of experiments; Lanes 3 and 4: second batch of experiments. **B.** Corresponding densitometric analysis of kisspeptin and GAPDH in placental lysates. Data are shown as individual densitometric units and mean ± SEM of kisspeptin protein expression normalized to GADPH. Differences were determined by two-way ANOVA and Bonferroni multiple comparison tests.

A



B

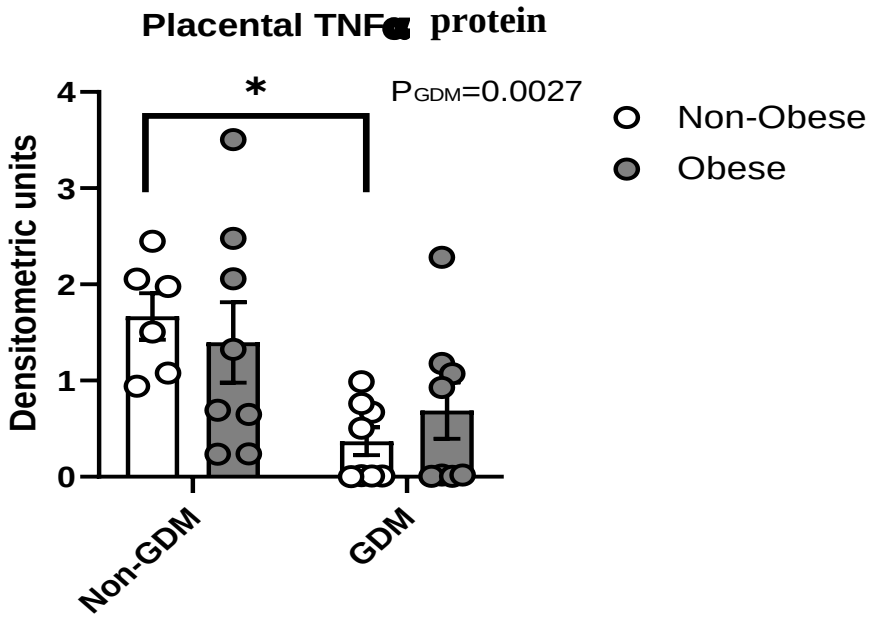
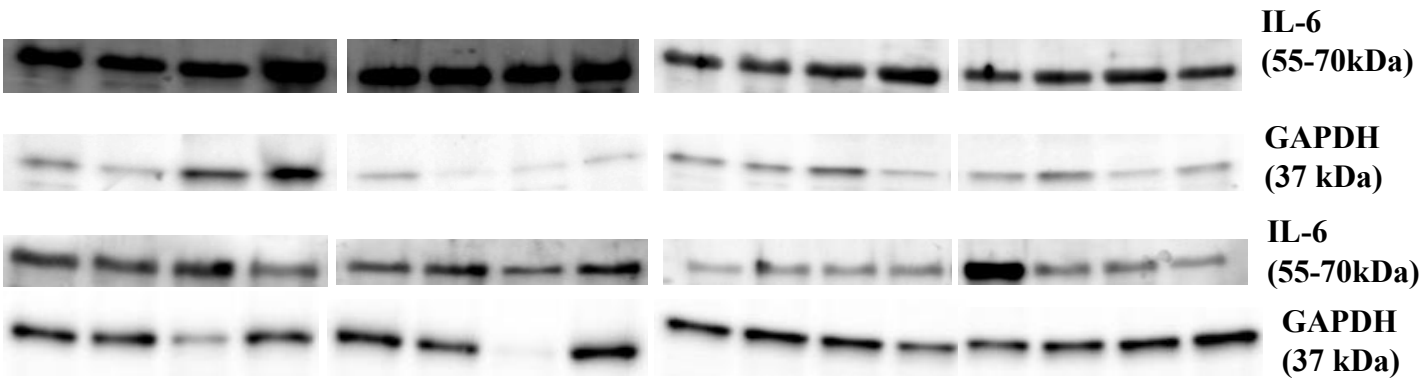


Figure 3.6 Placental TNFα protein expression. A. Western Blot demonstrating the expression of TNFα protein and GAPDH in placental lysates of non-GDM non-obese (n = 8), non-GDM obese (n = 8), GDM non-obese (n = 8) and GDM obese (n = 8) women. Lanes 1 and 2: first batch of experiments; Lanes 3 and 4: second batch of experiments. **B.** Corresponding densitometric analysis of TNFα and GAPDH in placental lysates.

*: significant difference between non-GDM non-obese and non-GDM obese cohorts. P_{GDM}: Significant effect of GDM on TNFα protein expression in the four cohorts. P <0.05 is statistically significant.

A



B

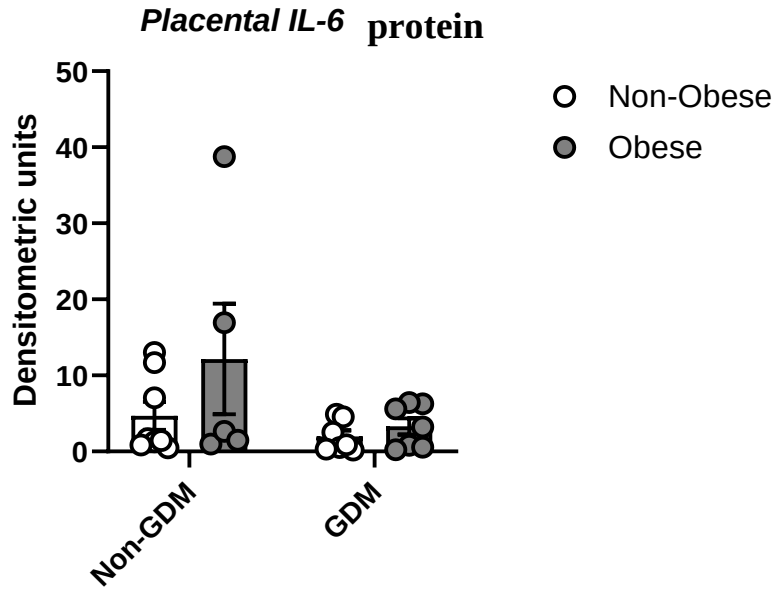


Figure 3.7 Placental IL-6 protein expression. A. Western Blot demonstrating the expression of IL-6 protein and GAPDH in placental lysates of non-GDM non-obese (n = 8), non-GDM obese (n = 8), GDM non-obese (n = 8) and GDM obese (n = 8) women. Lanes 1 and 2: first batch of experiments; Lanes 3 and 4: second batch of experiments. **B.** Corresponding densitometric analysis of IL-6 and GAPDH in placental lysates.

3.3.3 The Expression of Placental Kisspeptin, KISS1R and Proinflammatory Cytokines (TNFα, IL-6) Using Immunohistochemistry

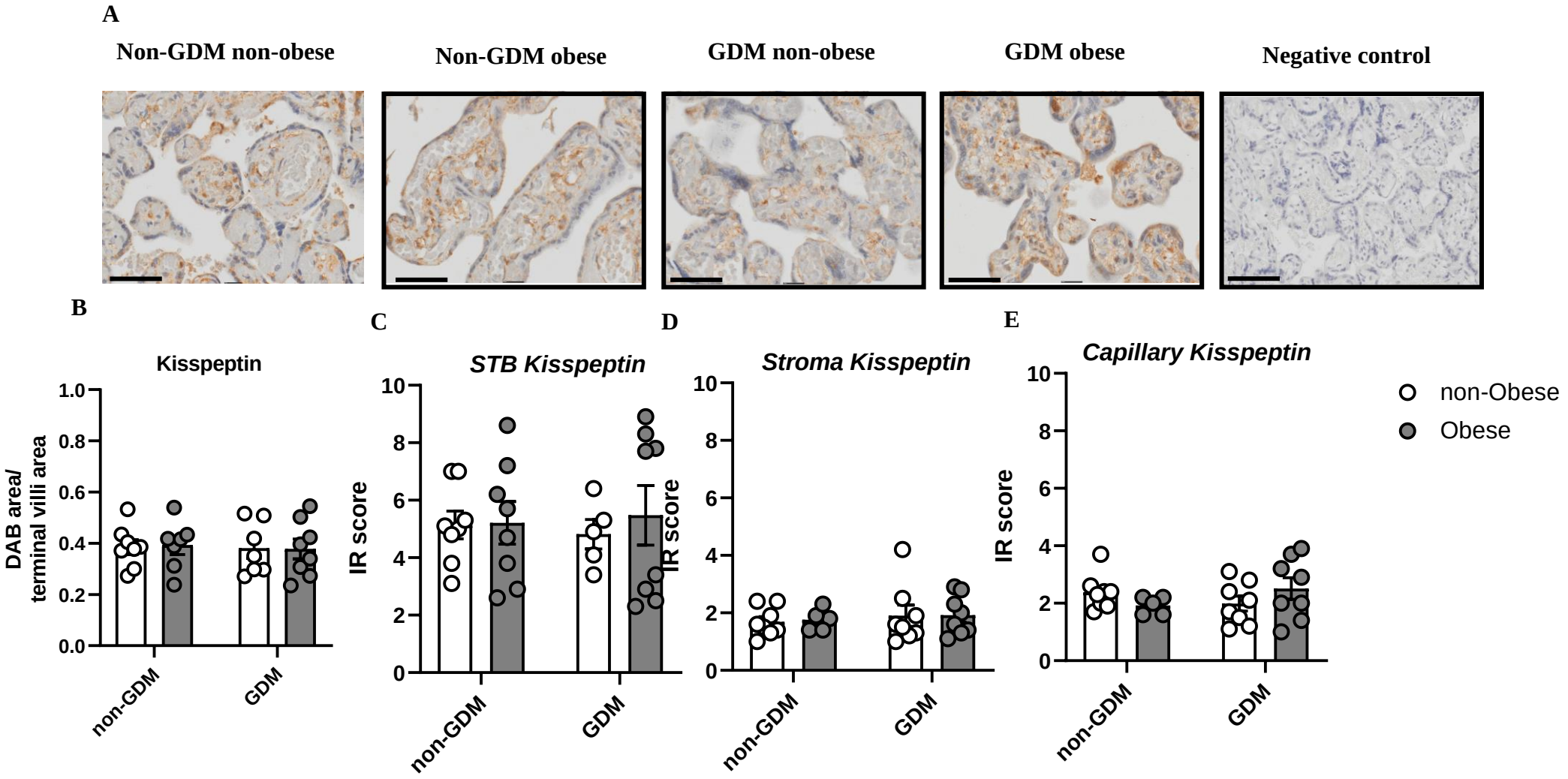
3.3.3.1 Placental Kisspeptin Protein Expression: The overall expression of kisspeptin protein by the placenta was not influenced by either GDM or BMI (Figures 3.8 A-B). Immunoreactivity (IR) score analysis revealed that kisspeptin was most abundant in the syncytiotrophoblast of placental terminal villi (Figures 3.8 C-E).

3.3.3.2 Placental KISS1R Protein Expression: KISS1R protein expression in the placenta did not differ significantly by GDM or BMI status or between the four groups in pairwise analyses (Figures 3.9 A-B). For all groups, KISS1R protein-positive immunoreactivity was greatest in the villous syncytiotrophoblast, compared with the stroma and capillaries (Figures 3.9 C-E).

3.3.3.3 Placental TNFα Protein Expression: BMI significantly influenced placental TNFα protein abundance with higher expression in obese (non-GDM and GDM) women compared to their non-obese counterparts ($P_{BMI} = 0.0099$; Figures 3.10 A-B). Pairwise comparisons revealed TNFα protein abundance was ~37% higher in GDM obese compared to GDM non-obese women (0.6912± 0.0445 vs 0.5061± 0.0321 respectively, $p = 0.0457$). There was also higher TNFα protein positive immunoreactivity in the villous syncytiotrophoblasts of obese women with or without GDM compared to their non-obese counterparts ($P_{BMI} = 0.0048$). Across all groups, the greatest expression of TNFα was in villous syncytiotrophoblast compared to the stroma and foetal capillaries (Figures 3.10 C-E).

3.3.3.4 Placental IL-6 Protein Expression: There was a significant interaction of GDM and BMI on the expression of IL-6 protein by the placenta, although pairwise comparisons failed

to identify significant differences between groups ($P_{GDM.BMI}$ = 0.0320; Figures 3.11 A-B). IR score analysis revealed that obesity overall reduced the expression of IL-6 in both the villous stroma and capillaries (P_{BMI} = 0.0028 and 0.00340, respectively). Moreover, pairwise comparisons revealed that the GDM obese group had significantly lower immunoreactivity in the villous stroma than its non-obese group (p = 0.0448; Figures 3.11 C-E).


A
Non-GDM non-obese
Non-GDM obese
GDM non-obese
GDM obese
Negative control
B
Kisspeptin
DAB area/ terminal villi area
1.0
0.8
0.6
0.4
0.2
0.0
non-GDM
GDM
C
STB Kisspeptin
IR score
10
8
6
4
2
0
non-GDM
GDM
D
Stroma Kisspeptin
IR score
10
8
6
4
2
0
non-GDM
GDM
E
Capillary Kisspeptin
IR score
10
8
6
4
2
0
non-GDM
GDM
non-Obese
Obese

F

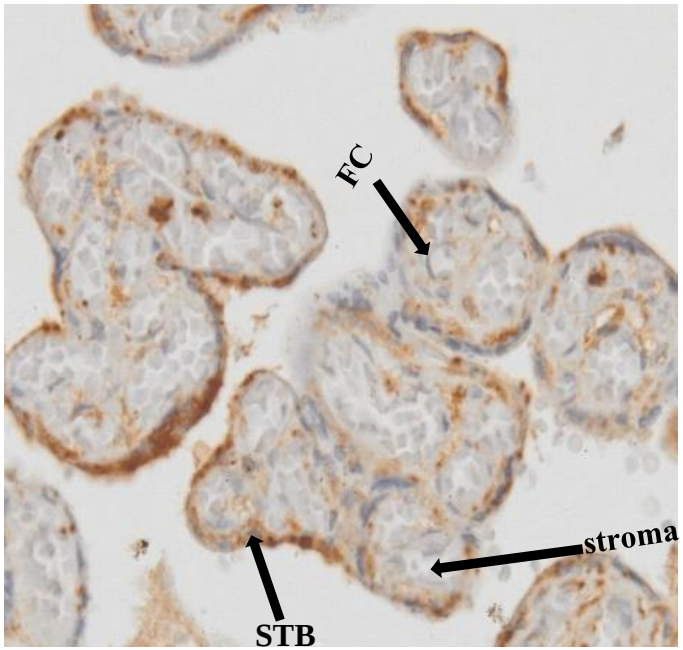
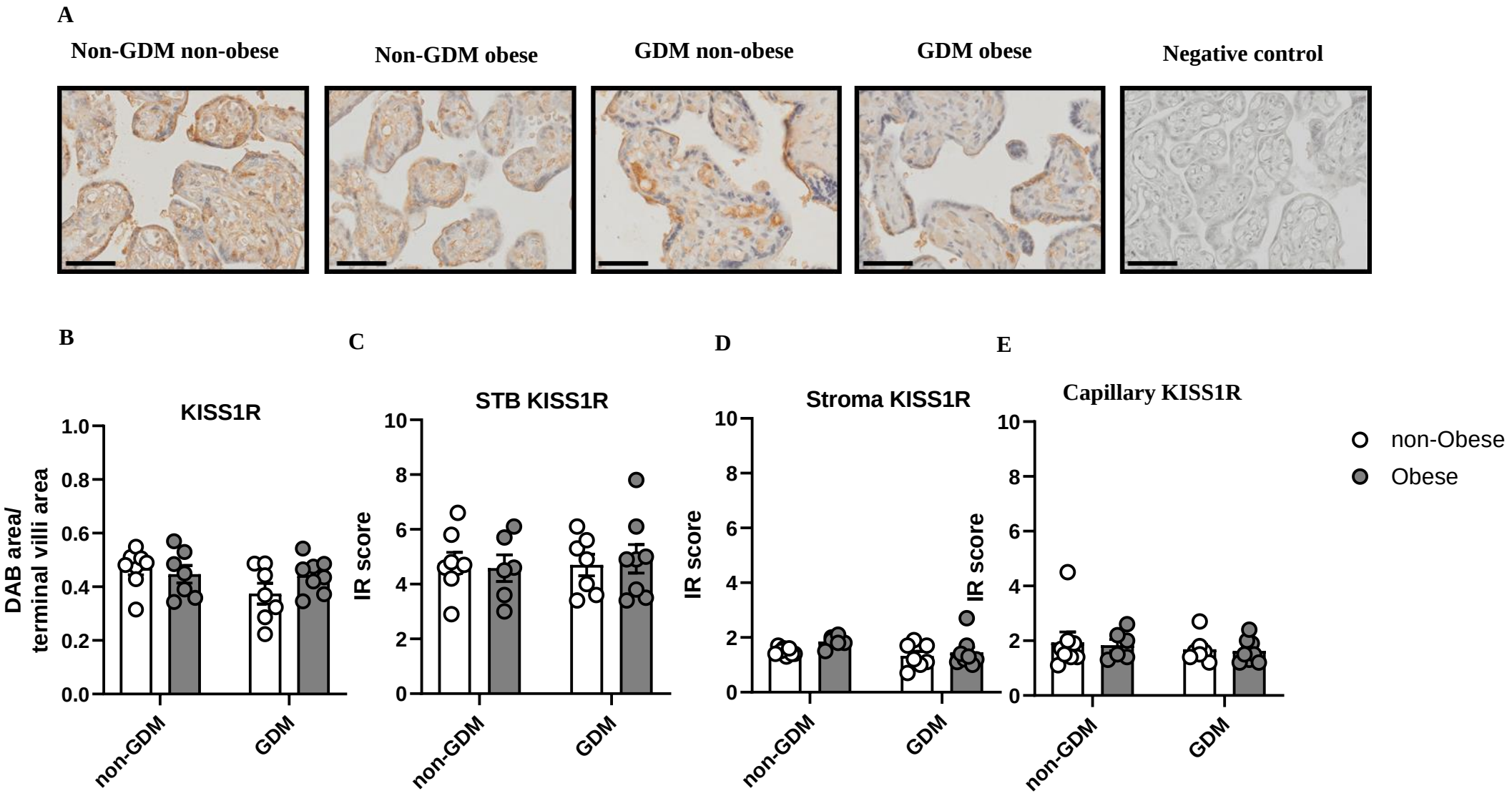


Figure 3.8. Placental kisspeptin expression. A. Immunohistochemistry analysis showing representative images of placental kisspeptin expression in non-GDM non-obese, non-GDM obese, GDM non-obese, GDM obese (n = 8 per group) women and representative negative control (no primary antibody). **Scale bar:** 50 μm. **B.** Graph representation of immunohistochemical labelling for placental kisspeptin (the percentage of DAB-area representing all terminal villi area). **C-E.** Semiquantitative analysis (IR score) of kisspeptin DAB staining area and intensity in terminal villus syncytiotrophoblast (STB), stroma and foetal capillary of the placenta in all groups. **F.** Representative graph used for placental kisspeptin IR score analysis in syncytiotrophoblast (STB), stroma and foetal capillary (FC). Data are shown as individual values and mean ± SEM of kisspeptin protein and IR score. *: $p < 0.05$, statistical differences were determined by two-way ANOVA and Bonferroni multiple comparison test.


A
Non-GDM non-obese
Non-GDM obese
GDM non-obese
GDM obese
Negative control
B
KISS1R
DAB area/ terminal villi area
1.0
0.8
0.6
0.4
0.2
0.0
non-GDM
GDM
C
STB KISS1R
IR score
10
8
6
4
2
0
non-GDM
GDM
D
Stroma KISS1R
IR score
10
8
6
4
2
0
non-GDM
GDM
E
Capillary KISS1R
IR score
10
8
6
4
2
0
non-GDM
GDM
non-Obese
Obese

F

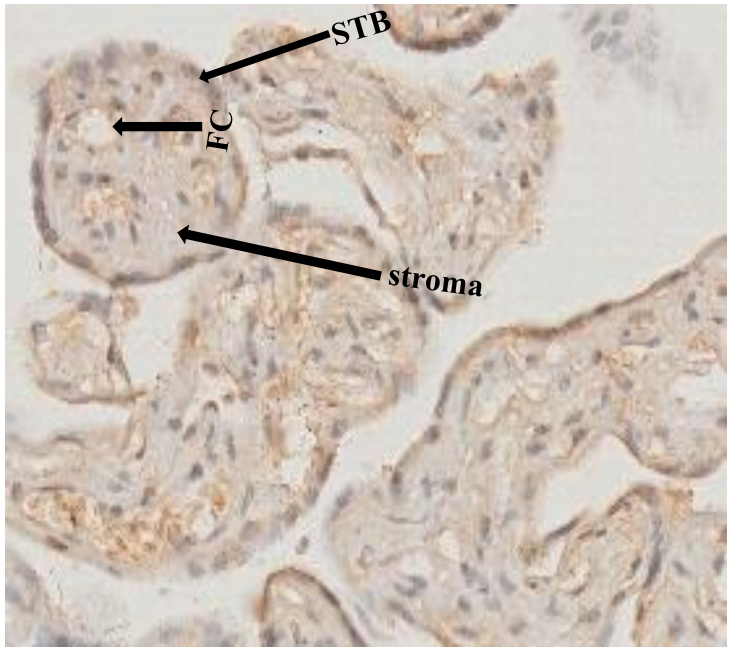
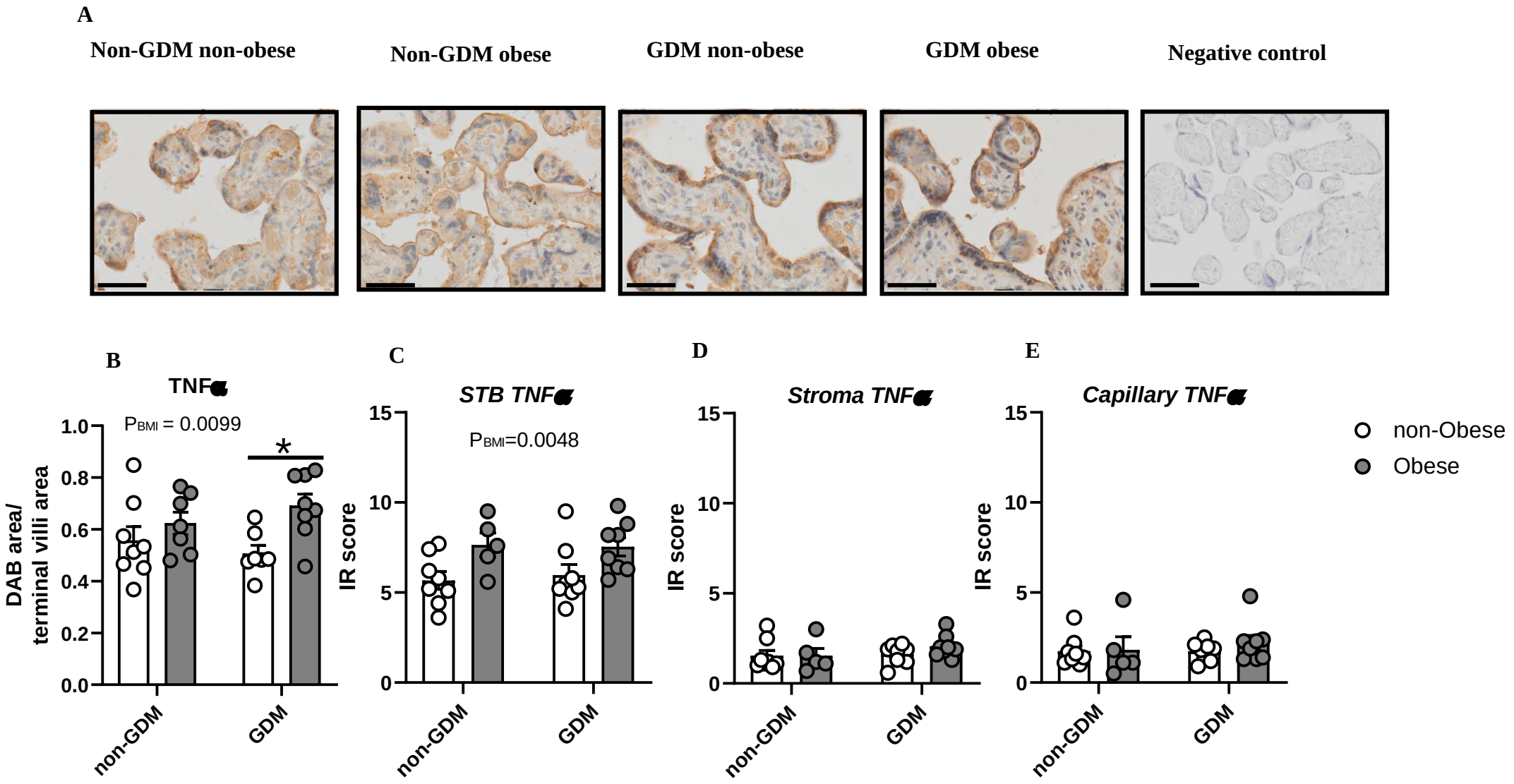


Figure 3.9. Placental KISS1R expression. A. Immunohistochemistry analysis showing representative images of placental KISS1R expression in non-GDM non-obese, non-GDM obese, GDM non-obese, GDM obese (n = 8 per group) women and representative negative control (no primary antibody). **Scale bar:** 50 μm. **B.** Graph representation of immunohistochemical labelling for placental KISS1R (the percentage of DAB-area representing all terminal villi area). **C-E.** Semiquantitative analysis (IR score) of KISS1R DAB staining area and intensity in terminal villus syncytiotrophoblast (STB), stroma and foetal capillary of the placenta in all groups. **F.** A representative graph used for placental KISS1R IR score analysis in syncytiotrophoblast (STB), stroma and foetal capillary (FC). Data are shown as individual values and mean ± SEM of KISS1R protein expression and IR score. *: $p < 0.05$, statistical differences were determined by two-way ANOVA and Bonferroni multiple comparison test.


A
Non-GDM non-obese
Non-GDM obese
GDM non-obese
GDM obese
Negative control
B
TNFα
PBMI = 0.0099
*
DAB area/ terminal villi area
1.0
0.8
0.6
0.4
0.2
0.0
non-GDM
GDM
C
STB TNFα
PBMI=0.0048
IR score
15
10
5
0
D
Stroma TNFα
E
Capillary TNFα
non-Obese
Obese

F

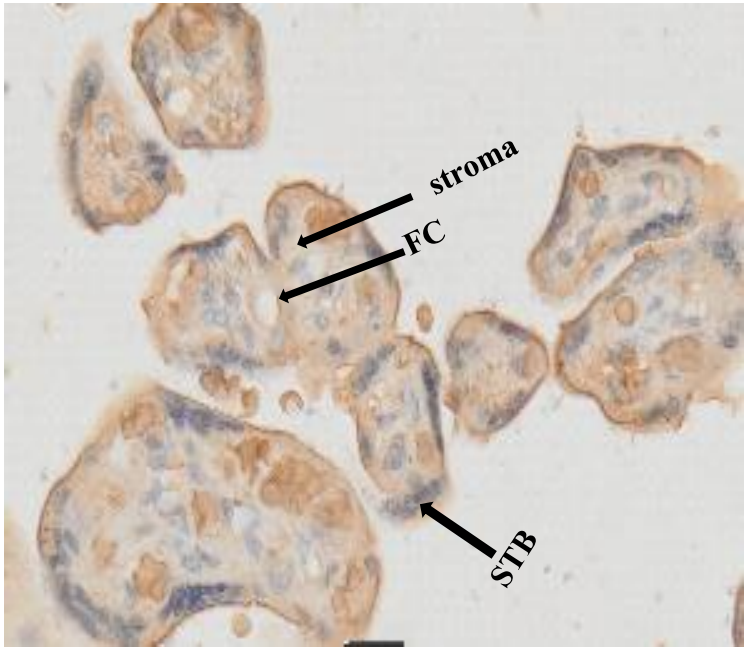
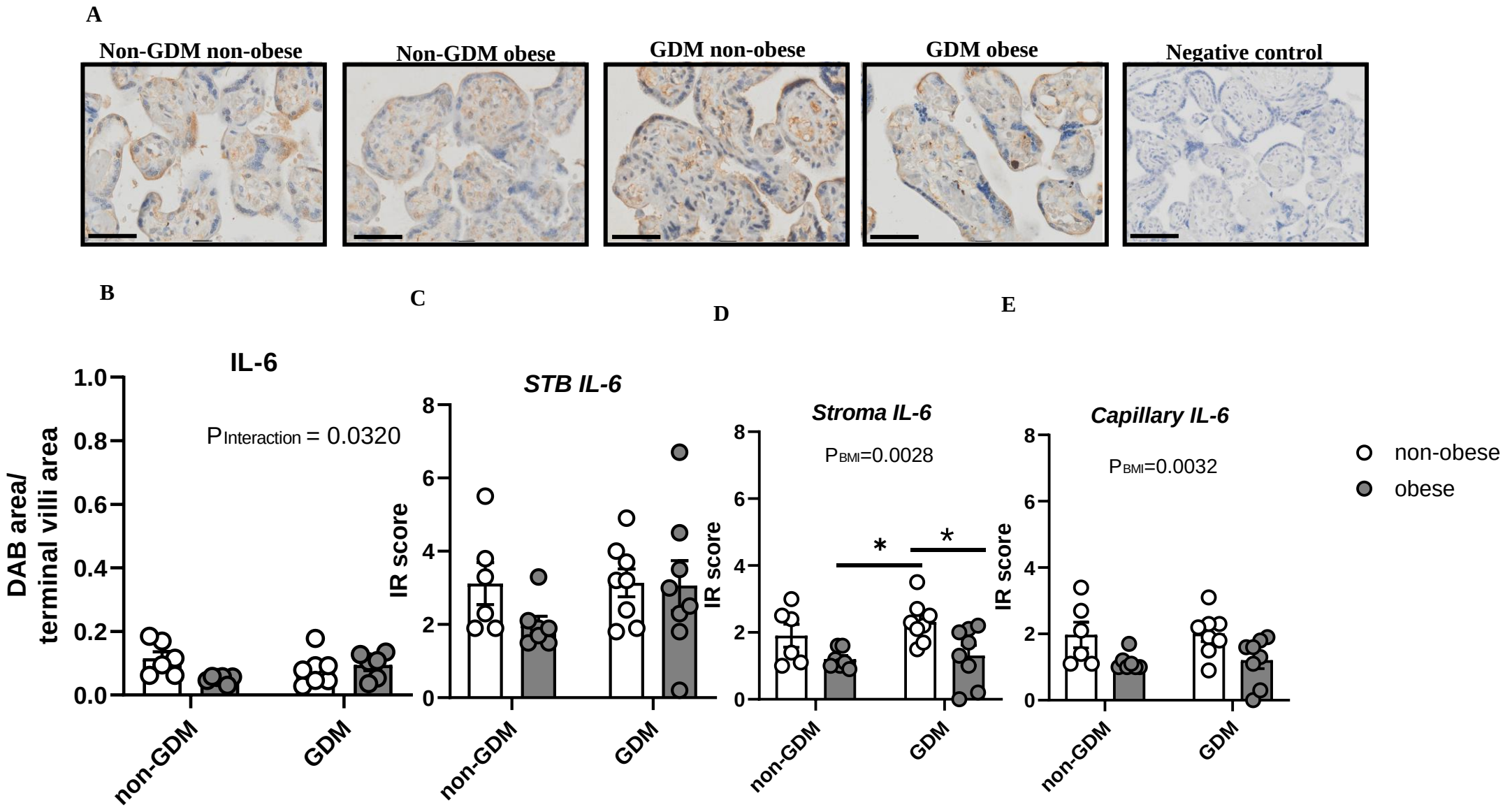


Figure 3.10. Placental TNFα expression. A. Immunohistochemistry analysis showing representative images of placental TNFα expression of non-GDM non-obese, non-GDM obese, GDM non-obese, GDM obese (n = 8 per group) women and representative negative control (no primary antibody). **Scale bar:** 50 μm. **B.** Graph representation of immunohistochemical labelling for placental TNFα (the percentage of DAB-area representing all terminal villi area). **C-E.** Semiquantitative analysis (IR score) of TNFα DAB staining area and intensity in terminal villus syncytiotrophoblast (STB), stroma and foetal capillary of the placenta in all groups. **F.** Representative graph used for placental TNFα IR score analysis in syncytiotrophoblast (STB), stroma, foetal capillary (FC). Data are shown as individual values and mean ± SEM of TNFα protein expression and IR score. *: $p < 0.05$, statistical differences were determined by two-way ANOVA and Bonferroni multiple comparison test.


A
Non-GDM non-obese
Non-GDM obese
GDM non-obese
GDM obese
Negative control
B
IL-6
PInteraction = 0.0320
DAB area/ terminal villi area
C
STB IL-6
IR score
D
Stroma IL-6
PBMI=0.0028
E
Capillary IL-6
PBMI=0.0032
non-GDM
GDM
non-obese
obese

F

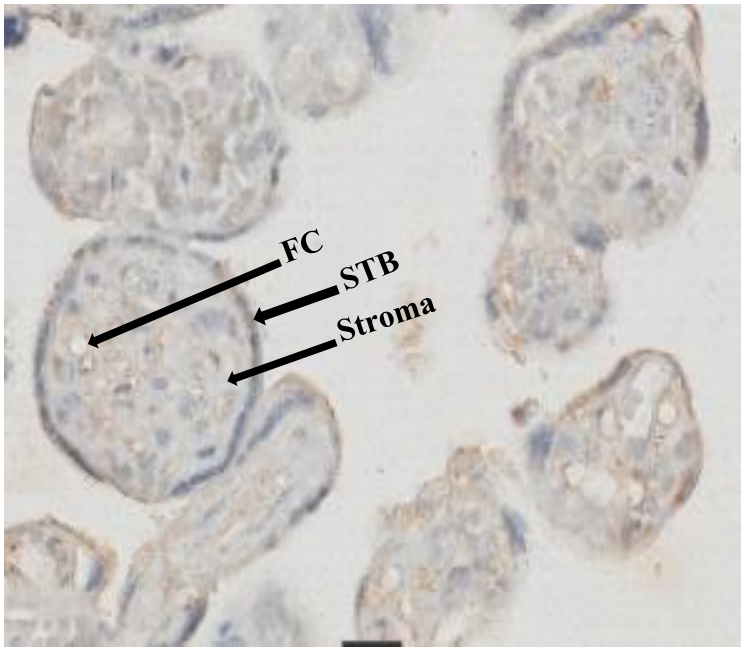


Figure 3.11. Placental IL-6 expression. A. Immunohistochemistry analysis showing representative images of placental IL-6 expression of non-GDM non-obese, non-GDM obese, GDM non-obese, GDM obese (n = 8 per group) women and representative negative control (no primary antibody). **Scale bar:** 50 μm. **B.** Graphic representation of immunohistochemical labelling for placental IL-6 (the percentage of DAB-area representing all terminal villi area). **C-E.** Semiquantitative analysis (IR score) of IL-6 DAB staining area and intensity in terminal villus syncytiotrophoblast (STB), stroma and foetal capillary of the placenta in all groups. **F.** Representative graph used for placental IL-6 IR score analysis in syncytiotrophoblast (STB), stroma, foetal capillary (FC). Data are shown as individual values and mean ± SEM of IL-6 protein expression and IR score. *: $p < 0.05$, statistical differences were determined by two-way ANOVA and Bonferroni multiple comparison test.

3.4 Maternal and Cord Concentrations of Kisspeptin and Proinflammatory Cytokines

The circulatory kisspeptin and proinflammatory cytokine levels in the maternal and cord sera of non-GDM (non-obese, obese) and GDM (non-obese, obese) pregnancies were determined using an ELISA.

3.4.1 Kisspeptin Concentration: Neither GDM nor BMI or their interaction influenced maternal and cord serum kisspeptin levels (Table 3.4).

3.4.2 TNFα Concentration: GDM alone had a significant influence on maternal serum TNFα levels in the four study groups (P_{GDM} = <0.0001, P_{BMI} = 0.3226, $P_{GDM.BMI}$ = 0.7223). However, there was no statistically significant difference in the maternal serum TNFα concentration between non-obese and obese non-GDM and non-obese and obese GDM women on pairwise analysis. Furthermore, there was no effect of GDM, BMI, or their interaction on the mean cord serum TNFα concentrations (Table 3.4).

3.4.3 IL-6 Concentration: Maternal and cord serum IL-6 levels were not influenced by BMI or GDM independently or in combination (Table 3.4).

Table 3.4. Maternal and cord serum kisspeptin and proinflammatory cytokine concentrations in non-GDM (non-obese, obese) and GDM (non-obese, obese)

	Non-GDM Non-obese Mean±SEM	Non-GDM Obese Mean±SEM	GDM Non-obese Mean±SEM	GDM Obese Mean±SEM	P value interaction	P value GDM	P value BMI
Maternal kisspeptin concentration (ng/ml)	0.79±0.10	0.86±0.06	0.83±0.10	0.91±0.06	0.9242	0.5763	0.3360
Cord kisspeptin concentration (ng/ml)	0.40±0.15	0.36±0.07	0.53±0.10	0.43±0.05	0.7475	0.2633	0.4535
Maternal TNFα concentration (pg/ml)	7.26±0.56	6.55±0.51	4.47±0.57	4.14±0.42	0.7223	**<0.0001**	0.3226
Cord TNFα concentration (pg/ml)	7.84±1.20	8.37±0.60	11.21±2.56	8.05±0.93	0.3399	0.2475	0.1420
Maternal IL-6 concentration (pg/ml)	38.84±4.98	35.10±7.24	21.97±7.07	34.54±7.27	0.3805	0.3491	0.6345
Cord IL-6 concentration (pg/ml)	5.65±1.18	4.75±1.47	8.40±2.99	6.30±1.55	0.7214	0.4588	0.3772

3.5 Correlations Between Placental Kisspeptin and Proinflammatory Cytokine Genes and Protein and their Respective Maternal and Cord Concentrations

There were no significant correlations between placental *kisspeptin*, *TNFα*, and *IL-6* genes and their respective maternal and cord concentrations among the four groups (Figure 3.12 A).

In the non-GDM obese group, placental kisspeptin protein abundance showed a significant negative correlation with the maternal serum kisspeptin concentrations ($r = -0.9512$, $p = 0.0035$), while in the GDM obese group, the negative correlation missed statistical significance. Meanwhile, in both non-obese groups, placental kisspeptin protein was positively correlated with maternal serum kisspeptin concentrations, with a near statistical significance in the GDM non-obese group. Whereas there were non-significant positive correlations between placental protein and maternal serum TNFα in the obese non-GDM and both GDM groups. Similarly, in the obese non-GDM and GDM groups, there were non-significant positive correlations between placental protein and maternal serum IL-6 levels (Figure 3.12 B).

In GDM non-obese group, placental kisspeptin and TNFα protein abundances showed near statistical tendencies to be correlated positively with the cord serum kisspeptin and TNFα concentrations, respectively. Whereas placental IL-6 protein abundance showed a significant positive correlation with the cord serum IL-6 concentrations ($r = 0.9015$, $p = 0.0055$; Figure 3.12 B).

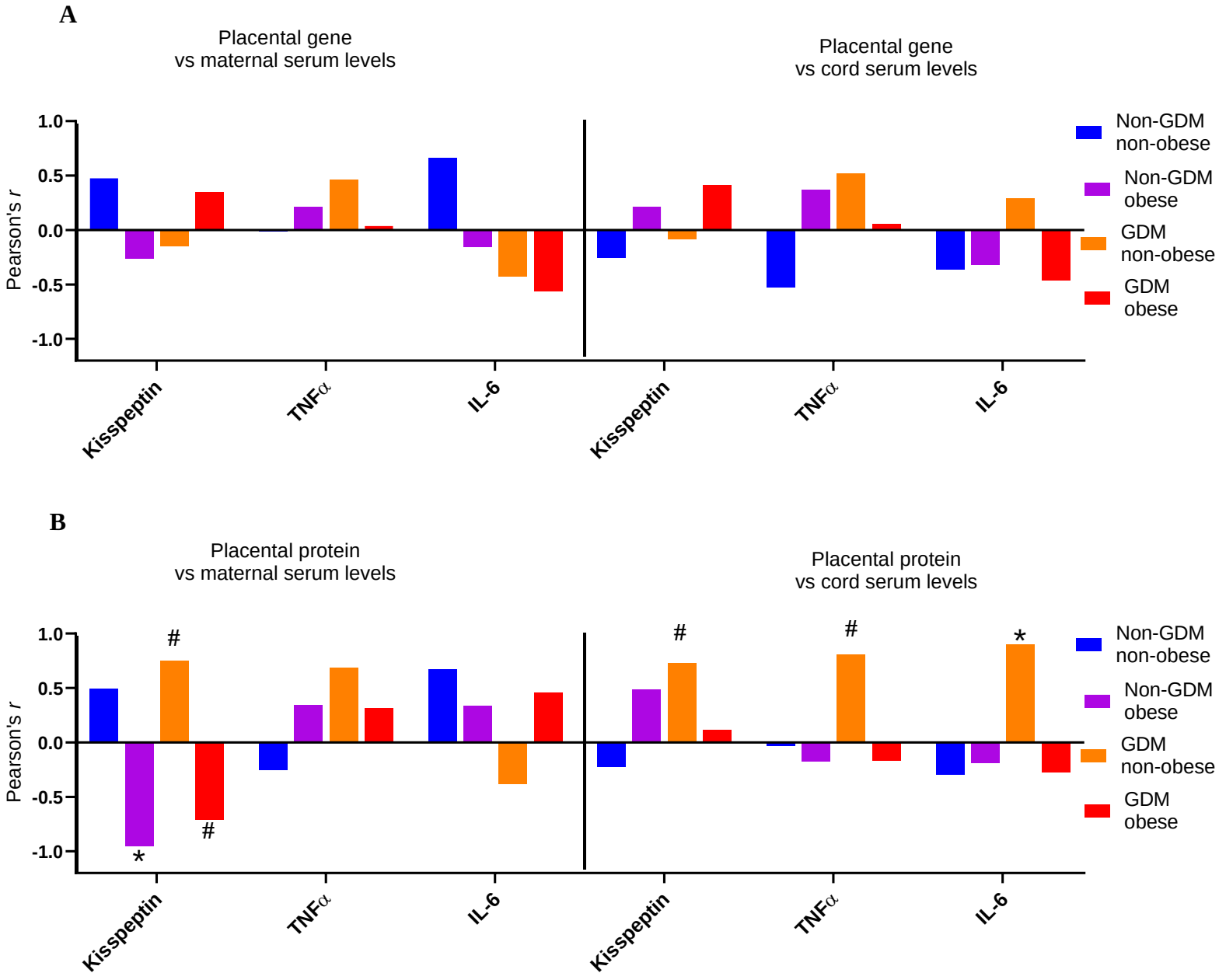


Figure 3.12. Correlation analysis for placental kisspeptin, TNFα and IL-6 gene and protein and their respective maternal and cord serum concentrations A. Placental gene levels and maternal and cord serum concentrations of kisspeptin, TNFα and IL-6 **B.** Placental protein levels and maternal and cord serum concentrations of kisspeptin, TNFα and IL-6**.** Pearson's coefficient (r) indicates the strength of relationship between data in each group. Non-GDM non-obese = blue, non-GDM obese = purple, GDM non-obese = orange, GDM obese = red. Sample size: placental protein data n = 6-8, maternal and cord serum (ELISA) data n = 11-18. *: $p < 0.05$, correlation is statistically significant, #: correlation is near statistical significance.

3.6 The Placental Villous Morphology Including Villi Maturation, Vascularity and the Surface Area for Exchange

The placental structure was analysed using stereology to determine the villous morphology, including villi maturation, vascularity and the surface area for exchange in the four groups of pregnant women (Table 3.5.). For the stem villus area, there was an overall significant interaction between GDM and BMI ($P_{GDM.BMI}$ = 0.0161), but no observed difference by either BMI or GDM category. There was a significant influence of BMI (but not GDM or interaction between GDM and BMI) on terminal villi area (P_{BMI} = 0.0008, P_{GDM} = 0.6070, $P_{GDM.BMI}$ = 0.7081), whereby both obese (non-GDM and GDM) women had a lower terminal villi area compared to their respective non-obese counterparts. Interestingly, both GDM and BMI independently and in combination influenced the intervillous space area (P_{GDM} = 0.0010, P_{BMI} = 0.0001, $P_{GDM.BMI}$ = 0.0099, respectively). The intervillous space area was greater in obese women compared to their non-obese (non-GDM and GDM) counterparts. There was no effect of GDM, BMI, or their interaction on the area of fibrosis, villous syncytiotrophoblast, stromal or foetal capillary area across the study populations (Table 3.5).

There was a significant effect of BMI on the terminal villi volume (P_{BMI} = 0.0016), which was significantly lower in obese non-GDM and GDM compared to their non-obese counterparts (Table 3.5). Intervillous space volume was influenced significantly by only GDM; it was significantly less in GDM versus non-GDM (P_{GDM} = 0.0308, P_{BMI} = 0.076, $P_{GDM.BMI}$ = 0.1047). There was a tendency for GDM to influence villous fibrosis volume, with GDM women showing higher villous fibrosis volume compared to non-GDM counterparts, although this did not achieve statistical significance (P_{GDM} = 0.0876). BMI consistently had a significant influence on the villous syncytiotrophoblast, stroma, and foetal capillary volume, but there were no significant differences by pairwise comparisons within their respective groups (P_{BMI} = 0.0069, P_{BMI} = 0.0278, P_{BMI} = 0.0083, respectively).

There was no effect of GDM, BMI or their interaction on the interhaemal membrane barrier thickness and surface density of maternal blood space (MBS), but there were differences in surface density of foetal capillaries and surface area of MBS between groups. The surface density of foetal capillaries was significantly affected by obesity, and overall, it was lower in obese compared to non-obese women (P_{BMI} = 0.0472), as was the surface area of MBS (P_{BMI} = 0.0008). In pairwise analysis, obese non-GDM and GDM had a lower surface area of MBS than their respective non-obese counterparts (p = 0.0224 and p = 0.0087, respectively) (Table 3.5). Similarly, the surface area of foetal capillaries was lower in the obese compared to non-obese women (P_{BMI} = 0.0018) (Table 3.5).

Obesity lowered the gradients for oxygen diffusion at the maternal-foetal interphase. BMI but not GDM or their interaction significantly influenced the theoretical diffusion capacity of the study groups (P_{BMI} = <0.0001), with it being significantly lower in both groups of obese (non-GDM and GDM) women compared to their respective non-obese counterparts (p = 0.0089 and p = 0.0230, respectively) (Table 3.5). Similarly, BMI but not GDM or the interaction had a significant influence on the specific diffusion capacity of the study groups (P_{BMI} = 0.0003) with a lower specific diffusion capacity in obese non-GDM and GDM compared to their respective non-obese (non-GDM and GDM) counterparts (p = 0.0438 and p = 0.0333) (Table 3.5).

Table 3.5 Stereological analyses of placental morphology and function in non-GDM (non-obese, obese) and GDM (non-obese, obese)

	Non-GDM Non-obese (n=14)	Non-GDM Obese (n=19)	GDM Non-obese (n=15)	GDM Obese (n=23)	P value interaction	P value GDM	P value BMI
Stem villi area (cm^2)	0.103 ± 0.017	0.083 ± 0.007	0,081 ± 0.012	0.130 ± 0.015	**0.0161**	0.3373	0.2941
Intermediate villi area (cm^2)	0.095 ± 0.011	0.069 ± 0.006	0.093 ± 0.006	0.087 ± 0.010	0.2524	0.3852	0.0768
Terminal Villi area (cm^2)	0.465 ± 0.026	0.376 ± 0.019	0.489 ± 0.017	0.380 ± 0.036@	0.7081	0.6070	**0.0008**
Intervillous space area (cm^2)	0.301 ± 0.026	0.429 ± 0.020*	0.285 ± 0.010	0.314 ± 0.012@	**0.0099**	**0.0010**	**0.0001**
Fibrosis area (cm^2)	0.035 ± 0.007	0.045 ± 0.009	0.051 ± 0.008	0.089 ± 0.029	0.4308	0.0927	0.1790
STB area (cm^2)	0.364 ± 0.015	0.371 ± 0.011	0.339 ± 0.014	0.378 ± 0.018	0.2994	0.5309	0.1288
Capillary area (cm^2)	0.261 ± 0.023	0.204 ± 0.022	0.274 ± 0.034	0.250 ± 0.032	0.5563	0.3088	0.1627
Stroma area (cm^2)	0.375 ± 0.023	0.429 ± 0.021	0.384 ± 0.030	0.373 ± 0.020	0.1865	0.3309	0.3835
Stem villi (cm^3)	66.23 ± 11.97	51.13 ± 5.84	56.18 ± 8.83	85.71 ± 16.87	0.0725	0.3146	0.5517
Intermediate villi (cm^3)	57.53 ± 6.20	45.76 ± 5.23	62.33 ± 3.86	57.43 ± 9.21	0.6041	0.2202	0.2153
Terminal Villi (cm^3)	312.33 ± 41.83	237.20 ± 9.30	335.72 ± 19.74	229.62 ± 2.70@	0.5513	0.7604	**0.0016**
Intervillous space (cm^3)	209.20 ± 27.90	287.41 ± 30.67	195.83 ± 11.11	199.52 ± 17.03	0.1047	**0.0308**	0.076
Fibrosis (cm^3)	20.46 ± 3.81	28. 76 ± 5.20	35.72 ± 5.90	62.31 ± 24.28	0.5121	0.0876	0.2159
STB (cm^3)	114.39 ± 16.24	87.31 ± 3.95	114.40 ± 8.64	84.65 ± 6.36	0.8912	0.8926	**0.0069**
Capillary (cm^3)	83.05 ± 12.84	48.07 ± 5.48	93.77 ± 13.70	60.68 ± 12.59	0.9376	0.3368	**0.0083**
Stroma (cm^3)	114.90 ± 18.56	101.81 ± 8.43	127.55 ± 11.75	84.29 ± 7.56	0.2231	0.8420	**0.0278**

Barrier thickness (µm)	3.01 ± 0.15	3.48 ± 0.13	3.06 ± 0.22	3.01 ± 0.12	0.1142	0.2035	0.2062
Surface density MBS (cm^2/cm^3)	658.68 ± 30.31	687.55 ± 22.95	693.77 ± 28.65	662.42 ± 36.59	0.3340	0.8720	0.9681
Surface density FC (cm^2/cm^3)	435.20 ± 40.07	343.78 ± 31.71	491.67 ± 54.68	389.22 ± 50.54	0.9066	0.2834	**0.0472**
Surface area MBS (m^2)	24.34 ± 2.86	19.44 ± 0.78*	27.52 ± 1.60	18.61 ± 2.21$^{@}$	0.2956	0.4874	**0.0008**
Surface area FC (m^2)	16.10 ± 2.25	9.70 ± 0.92	19.48 ± 2.31	11.61 ± 2.21	0.7226	0.2088	**0.0018**
Theoretical diffusion capacity (cm^2/min/kPA)	119.17 ± 16.66	72-14 ± 5.14*	136.01 ± 9.26	86.76 ± 6.61$^{@}$	0.9138	0.1334	**<0.0001**
Specific diffusion capacity (cm^2/min/kPA/g)	37.87 ± 5.96	22.24 ± 1.50*	40.92 ± 3.73	25.74 ± 2.16$^{@}$	0.9524	0.3812	**0.0003**

Data are represented as mean ± SEM. The differences between groups were determined using two-way ANOVA and Bonferroni's multiple comparisons test.

*: significant difference comparing non-GDM non-obese vs non-GDM obese.

$^{@}$: significant difference when comparing GDM non-obese vs GDM obese.

Cm2

Abbreviations. FC: foetal capillaries, MBS: maternal blood space, STB: syncytiotrophoblast.

$P < 0.05$ is considered statistically significant.

3.7 The Expression of Kisspeptin Protein in Placental Syncytiotrophoblast Extracellular Vesicles from Women with GDM

3.7.1 Clinical Characteristics of Study Participants for Study 5

Six syncytiotrophoblast extracellular vesicles (STBEV) archival samples (GDM: n =3, non-GDM: n =3) were used for western blotting in a preliminary study conducted at the University of Oxford, United Kingdom.

As seen in Table 3.6, the mean age of GDM and non-GDM subjects from whose STBEV samples were used for experiments was not different ($p = 0.4763$; Table 3.6). The GDM subjects had 62% and 74% heavier mean booking weight and BMI, respectively, than the non-GDM ($p = 0.0486$ and $p = 0.0078$ respectively). The mean SBP and DBP did not differ significantly between the two groups. Birth weight tended to be heavier in GDM than non-GDM women ($p = 0.067$; Table 3.6).

Four placental lysate samples, each from GDM and non-GDM, were also utilised to determine kisspeptin protein expression. Their clinical characteristics are summarised in Table 3.6 and showed that maternal age, booking weight, gestational age and infant birth weight did not differ statistically between the two groups.

Table 3.6. Clinical characteristics of study subjects with and without GDM

	Non-GDM	**GDM**		**Non-GDM**	**GDM**	
Samples type	STBEV n = 3	STBEV n = 3		Placental lysates n = 4	Placental lysates n = 4	
	Mean ± SEM	Mean ± SEM	P value	Mean±SEM	Mean ± SEM	P value
Maternal age	33.67±3.71	28.67±5.18	0.4763	31.00±4.00	33.67±3.38	0.6375
Maternal weight at booking (kg)	63.40±1.6	102.0±9.24	0.0486	67.60±4.60	80.67±8.97	0.3585
Maternal BMI at booking (kg/m^2)	23.33±0.88	39.67 ± 3.18	0.0078	22.00±0.00	29.67±3.28	NA
Gestational age at delivery (weeks)	38.33±0.67	38.67 ± 0.33	0.3897	39.50±0.50	38.00±1.00	0.3456
Systolic blood pressure (mmHg)	129.0±9.00	146.0±12.06	0.3897	-	145.3±12.45	-
Diastolic blood pressure (mmHg)	78.50±3.50	88.33±1.67	0.0621	-	81.67±4.41	-
Birth weight (kg)	3.17±2.53	4.36±4.09	0.0679	3.52±1.95	3.59±4.93	0.9307
Treatment:						
Diet (n)		2			2	
Metformin (n)		-			1	
Metformin + Insulin (n)		1			-	
Insulin (n)		-			-	

STBEV- syncytiotrophoblast extracellular vesicles

$p < 0.05$ - statistically significant

NA - not available

3.7.2 Determination of Kisspeptin Protein Expression in STBEV and Placental Lysates by Western Blotting

There was no significant difference in placental lysate kisspeptin protein abundance nor in the abundance of kisspeptin protein in STBEV samples between GDM and non-GDM women (Figure 3.13). However, the mean value was >50% lower in the GDM compared to the non-GDM women ($p = 0.0986$; Figure 3.13).

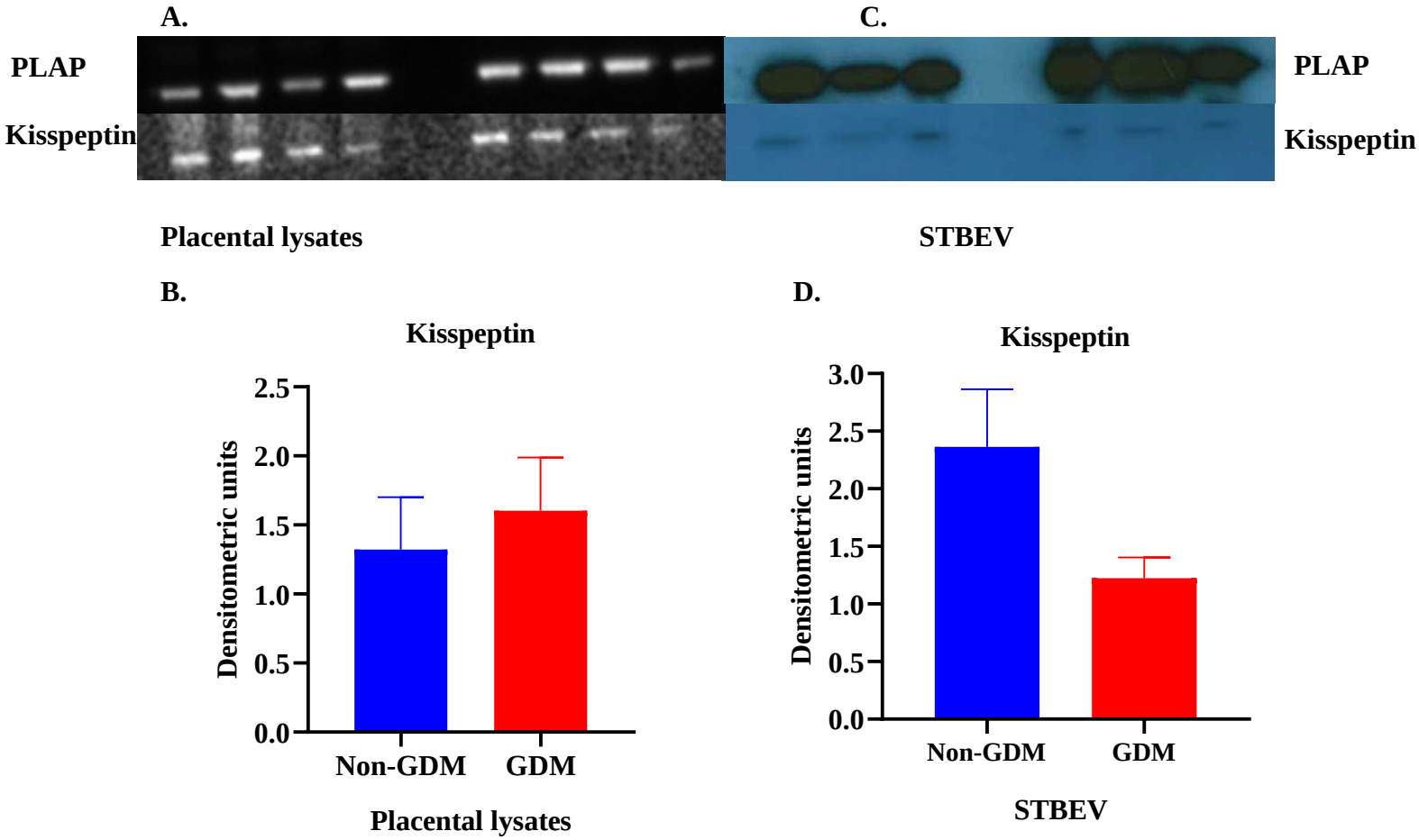


Figure 3.13 Kisspeptin expression in placental lysates and STBEV

A. Western blot of kisspeptin protein expression in placental lysates of GDM and non-GDM pregnant women. PLAP expression was used as a loading control and to confirm syncytiotrophoblast content. **B.** Densitometric analysis of kisspeptin expression in the placental lysates of GDM and non-GDM. **C.** Western blot of kisspeptin protein expression in placental STBEV of GDM and non-GDM pregnant women. PLAP expression was used as a loading control to confirm syncytiotrophoblast origin. **D.** Densitometric analysis of kisspeptin expression in the STBEV of GDM and non-GDM.

3.7.3 Determination of Kisspeptin Co-Expression with Placental Alkaline Phosphatase (PLAP) on STBEV by Immunoprecipitation

Kisspeptin protein and PLAP protein were both expressed in PLAP immuno-precipitated fractions confirming co-expression (Figure 3.14), but neither were expressed on the kisspeptin immuno-precipitated STBEV. This may imply that a significant proportion of kisspeptin protein is located intravesicularly or that the kisspeptin antibody is not strong enough to effectively immuno-precipitate kisspeptin specific STBEV (Figure 3.14).

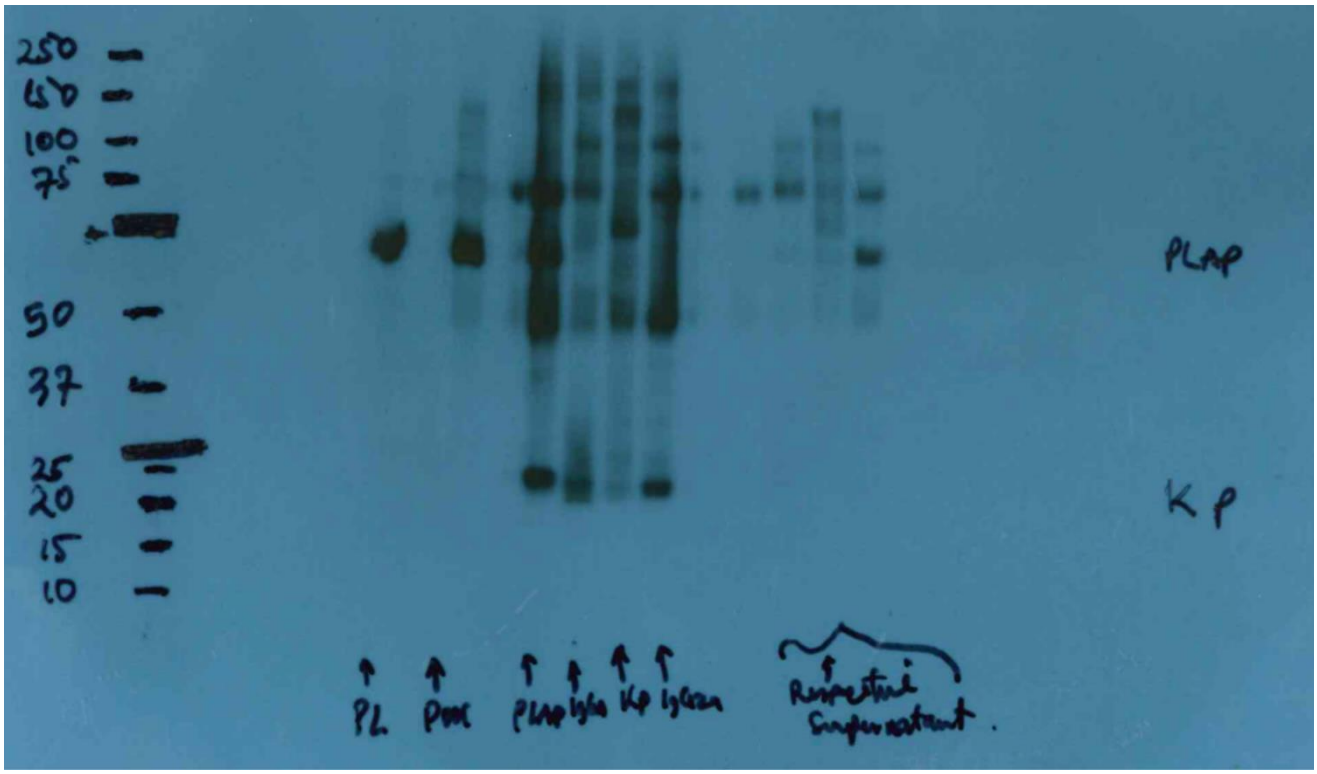


Figure 3.14. Kisspeptin and PLAP co-expression in STBEV using immunoprecipitation

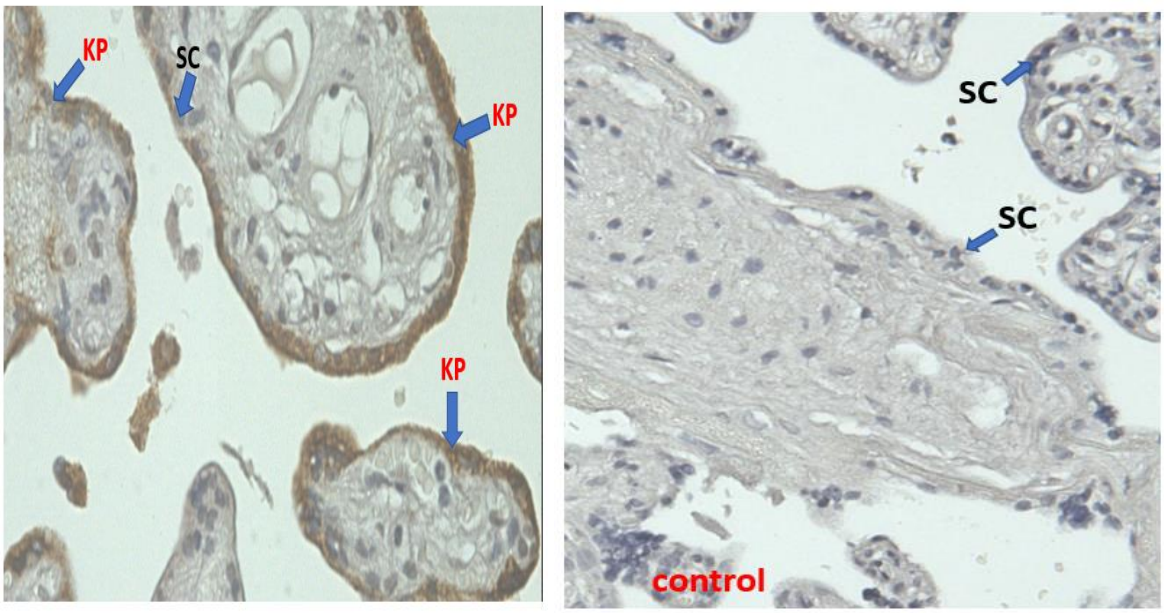
Kisspeptin and PLAP protein expression in placental lysates, pool STBEV (n = 3 samples), STBEV immunoprecipitated with anti-PLAP magnetic beads, STBEV immunoprecipitated with anti-IgGa magnetic beads, STBEV immunoprecipitated with anti-kisspeptin (KP) magnetic beads, STBEV immunoprecipitated with anti-IgG2a magnetic beads, and their respective supernatant.

PLAP: placental alkaline phosphatase

STBEV: syncytiotrophoblast extracellular vesicles

3.7.4 Validation of the Kisspeptin Antibody Used for the Determination of STBEV Kisspeptin Protein Expression in the Normal Placenta by Immunohistochemistry

To confirm the validity of the kisspeptin antibody used for the STBEV kisspeptin protein expression, immunohistochemistry was performed on normal term human placenta using an anti-kisspeptin antibody (Anti-KiSS-1 Antibody (24-Q): sc-101246). As seen in Figure 3.15, there was a significant expression of kisspeptin protein in the villous syncytiotrophoblast of a healthy pregnancy placental tissue, while no kisspeptin expression was identified in the negative control placental tissue. This confirms that syncytiotrophoblast is the predominant site for kisspeptin expression and that the kisspeptin antibody used for the STBEV kisspeptin protein expression was working.



A. Anti-kisspeptin **B. Negative control**

Figure 3.15. Kisspeptin expression in the syncytiotrophoblast of the normal placenta.

A. Kisspeptin expression is predominantly present at the syncytiotrophoblast of a normal term placenta (red arrow, left panel). **B.** Negative controls were performed without primary antibody incubation showing the absence of kisspeptin expression in the syncytiotrophoblasts (blue arrow, right panel). KP = kisspeptin, SC = syncytiotrophoblasts.

3.8 Correlations Between Placental Expression of Kisspeptin, Proinflammatory Markers and Placental Villous Morphology and Maternal and Neonatal Parameters

We investigated potential correlations between placental genes and proteins of kisspeptin, TNFα and IL-6 levels and placental villous morphology and maternal and neonatal parameters of the four groups of women

The placental *kisspeptin* gene expression showed a near statistical tendency to be negatively correlated with FPG and infant birth weight in GDM obese and non-GDM non-obese groups, respectively ($p = 0.0638$, $p = 0.0808$, respectively). Furthermore, there were significant negative correlations between placental *kisspeptin* gene expression and volume of villous syncytiotrophoblasts and theoretical diffusion capacity (TDC) in the two non-obese groups (non-GDM non-obese and GDM-non-obese groups ($p = 0.0299$, $p = 0.0147$, respectively). Whereas placental *kisspeptin* gene expression showed near statistical tendencies to be significantly negatively correlated with volumes of terminal villi and villous stroma in the non-GDM non-obese group ($p = 0.0701$, $p = 0.0935$, respectively). Similarly, in both non-obese and obese GDM groups, placental *kisspeptin* gene expression was inversely correlated with specific diffusion capacity with a near statistical significance ($p = 0.0854$, $p = 0.0590$, respectively) (Figures 3.16 and 3.17).

In the non-GDM non-obese and GDM obese groups, placental kisspeptin protein abundance was significantly negatively correlated with maternal systolic blood pressure (SBP) ($p = 0.0431$, $p = 0.0444$, respectively). Meanwhile, in the GDM non-obese group, placental kisspeptin protein had a significant negative correlation with maternal BMI at booking ($p = 0.0213$). Furthermore, placental kisspeptin protein abundance in the GDM obese group was significantly positively correlated with infant ponderal index ($p = 0.0045$). Moreover, placental

kisspeptin protein abundance was negatively correlated with stem villi and intervillous space volumes with a near statistical significance in the non-GDM obese group ($p = 0.0643$, $p = 0.0961$, respectively). Also, in the non-GDM non-obese group, placental kisspeptin protein abundance showed near statistical tendencies to be negatively correlated with intervillous space, surface area of MBS, surface area of foetal capillary (FC) and TDC ($p = 0.0583$, $p = 0.0652$, $p = 0.0755$, respectively; Figures 3.18 and 3.19).

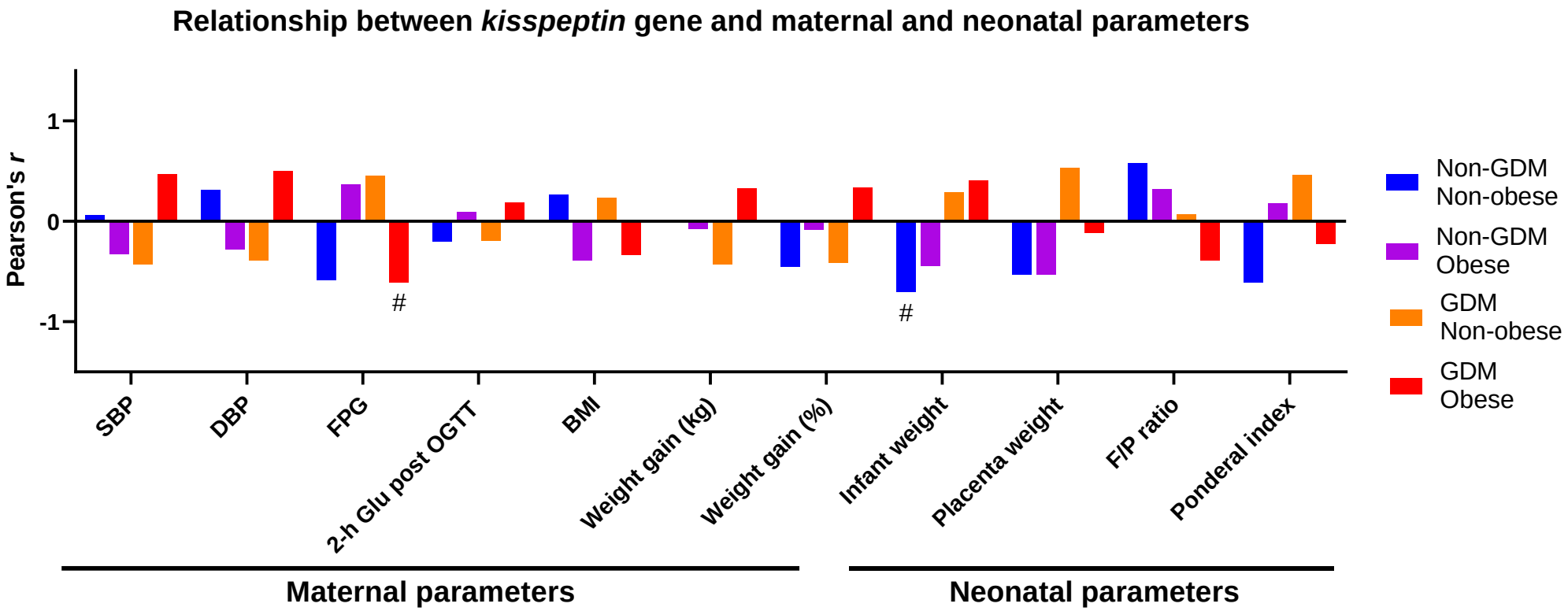


Figure 3.16. Placental *kisspeptin* gene correlation with maternal and neonatal parameters. Correlation analysis was performed between placental *kisspeptin* gene and maternal and neonatal parameters. Pearson's coefficient (r) was plotted to indicate strength of relationship between data, and analysis was performed on each group separately. Non-GDM non-obese = blue, non-GDM obese = purple, GDM non-obese = orange, GDM obese = red. Sample size (n) were between 8-10 for all groups. #: correlation is near statistical significance. **Abbreviations.** SBP: Systolic blood pressure, DBP: diastolic blood pressure, FPG: fasting plasma glucose, BMI: body mass index, 2-h Glu post OGTT: 2-hour glucose post oral glucose tolerance test, F/P: foetoplacental.

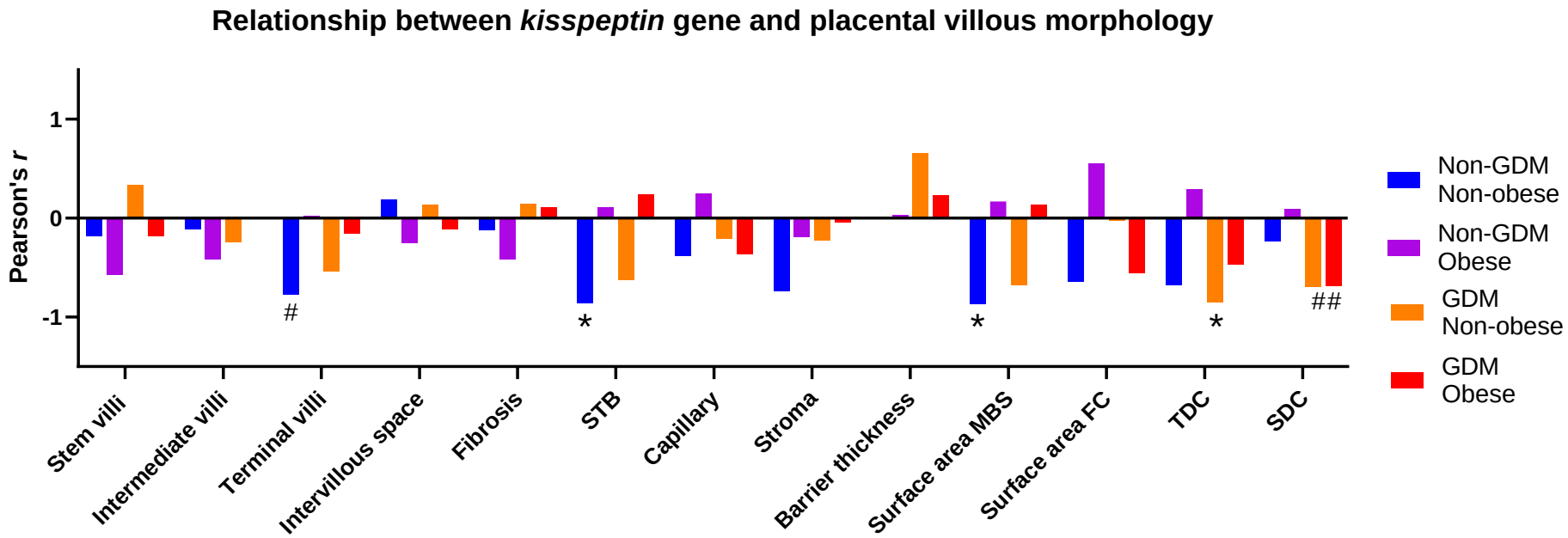


Figure 3.17. Placental *kisspeptin* gene correlation with placental villous morphology. Correlation analysis was performed between placental *kisspeptin* gene and placental villous morphology. Pearson's coefficient (r) was plotted to indicate strength of relationship between data, and analysis was performed on each group separately. Non-GDM non-obese = blue, non-GDM obese = purple, GDM non-obese = orange, GDM obese = red. Sample size (n) were between 8-10 for all groups. *: $p < 0.05$, correlation is statistically significant, #: correlation is near statistical significance. Abbreviations. FC: foetal capillaries, MBS: maternal blood space, STB: syncytiotrophoblast, TDC: theoretical diffusion capacity, SDC: specific diffusion capacity

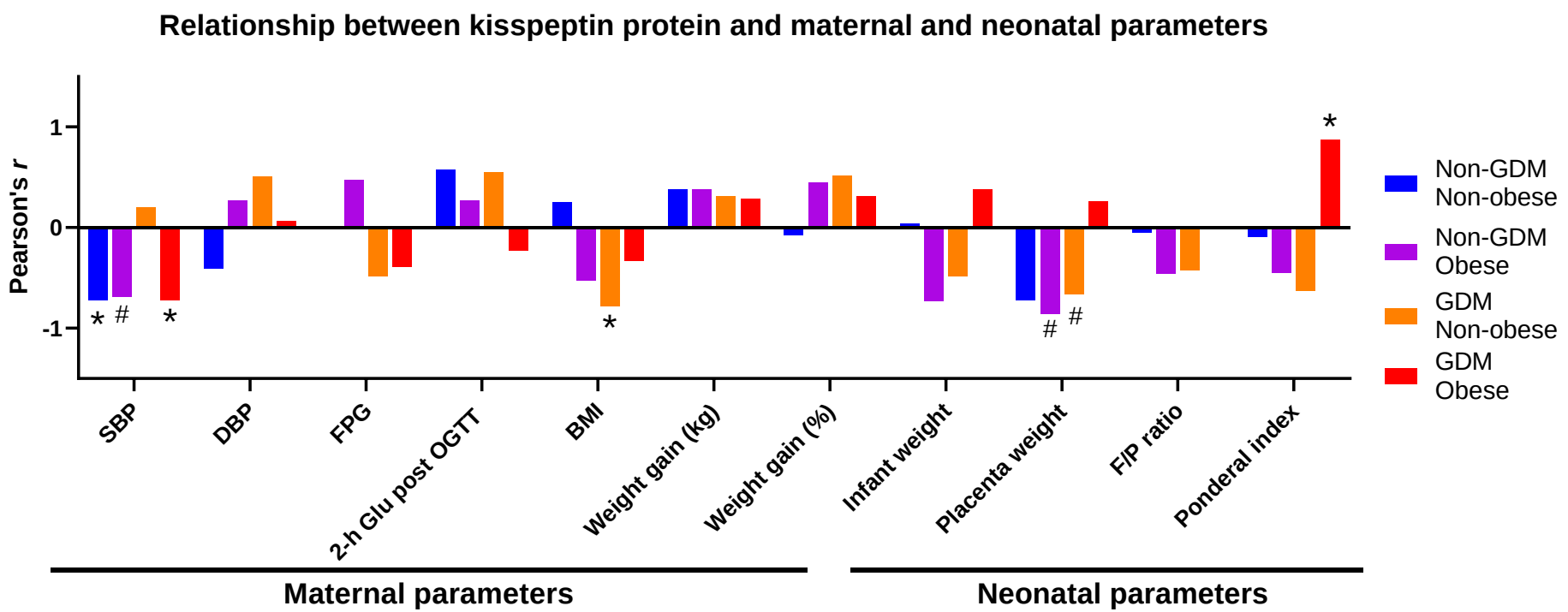


Figure 3.18. Placental kisspeptin protein correlation with maternal and neonatal parameters. Correlation analysis was performed between placental kisspeptin protein and maternal and neonatal parameters. Pearson's coefficient (r) was plotted to indicate strength of relationship between data, and analysis was performed on each group separately. Non-GDM non-obese = blue, non-GDM obese = purple, GDM non-obese = orange, GDM obese = red. Sample size (n) were between 8-10 for all groups. *: $p < 0.05$, correlation is statistically significant, #: correlation is near statistical significance. Abbreviations. SBP: Systolic blood pressure, DBP: diastolic blood pressure, FPG: fasting plasma glucose, BMI: body mass index, 2-h Glu post OGTT: 2-hour glucose post oral glucose tolerance test, F/P: foetoplacental.

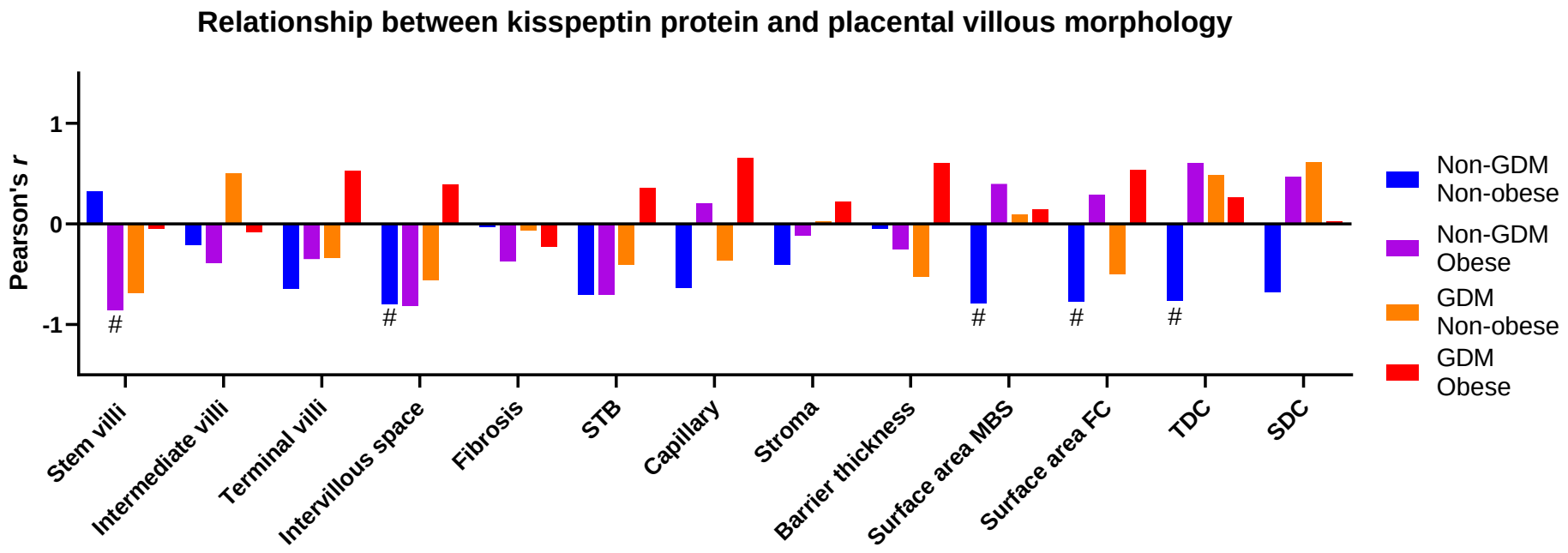


Figure 3.19. Placental kisspeptin protein correlation with placental villous morphology. Correlation analysis was performed between placental kisspeptin protein and placental villous morphology. Pearson's coefficient (r) was plotted to indicate strength of relationship between data, and analysis was performed on each group separately. Non-GDM non-obese = blue, non-GDM obese = purple, GDM non-obese = orange, GDM obese = red. Sample size (n) were between 8-10 for all groups. *: $p < 0.05$, correlation is statistically significant, $^{\#}$: correlation is near statistical significance. Abbreviations. FC: foetal capillaries, MBS: maternal blood space, STB: syncytiotrophoblast, TDC: theoretical diffusion capacity, SDC: specific diffusion capacity.

There were some statistically significant correlations between the TNFα gene and maternal diastolic blood pressure (DBP), TDC and specific diffusion capacity (SDC). Placental TNFα gene expression was positively correlated with DBP in the non-GDM obese group ($p = 0.0487$) and negatively with TDC in the GDM non-obese group ($p = 0.0182$)., The negative correlation between placental TNFα gene expression and SDC was close to significance in the GDM non-obese group ($p = 0.0652$) (Figures 3.20 and 21).

Placental TNFα protein abundance was negatively correlated with 2-hour glucose post-OGTT and placenta weight in obese and non-obese non-GDM groups, respectively ($p = 0.0066$, $p = 0.0413$, respectively). There was also a significant negative correlation between placental TNFα protein abundance and infant ponderal index in GDM non-obese group ($p = 0.0053$). In the non-GDM non-obese group, placental TNFα abundance was negatively correlated with the surface areas of MBS and FC ($p = 0.0143$, $p = 0.04$) but showed a near statistical significance to be negatively correlated with villous foetal capillary. There was also a near statistically significant tendency for a positive correlation between placental TNFα protein abundance and volume of terminal villi and foetal capillary in the GDM obese group. However, in the other three groups, namely non-obese and obese non-GDM and GDM non-obese groups, placental TNFα protein abundance was negatively correlated with the volume of terminal villi, villous syncytiotrophoblast, and villous stroma, but this relationship was not statistically significant (Figures 3.22 and 23).

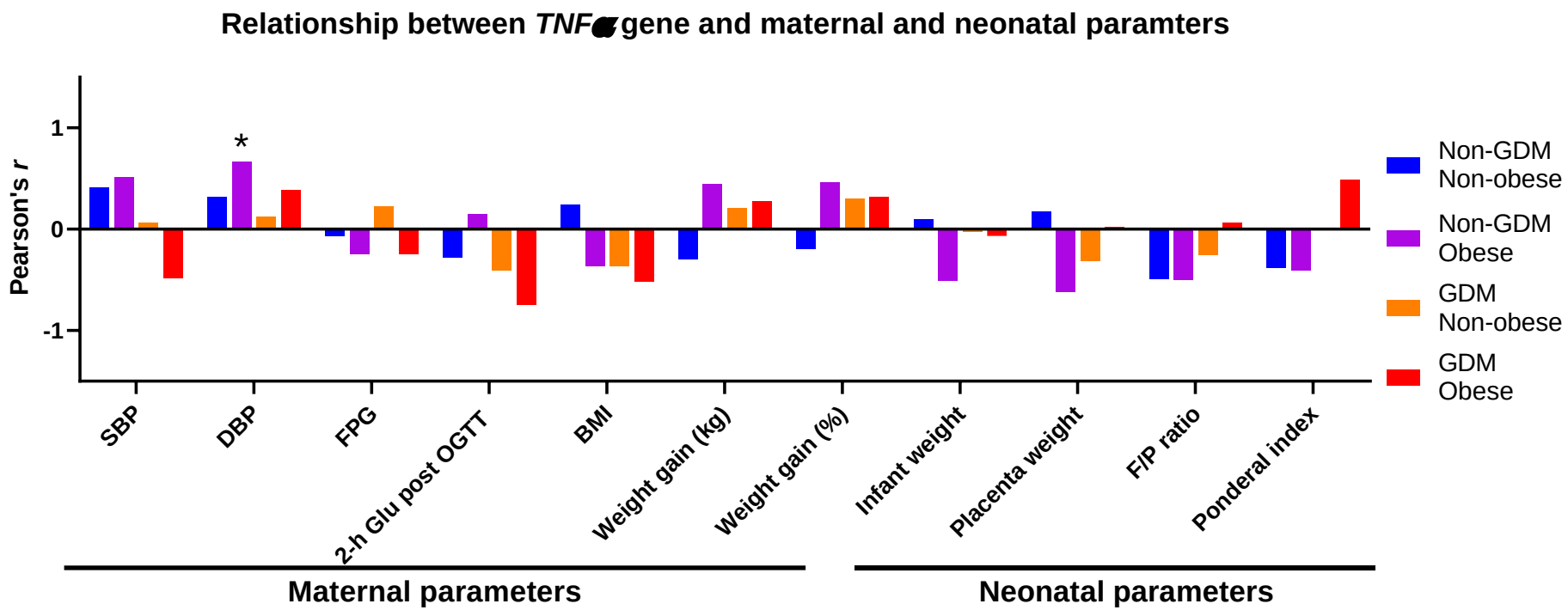


Figure 3.20. Placental *TNFα* gene correlation with maternal and neonatal parameters. Correlation analysis was performed between placental *TNFα* gene and maternal and neonatal parameters. Pearson's coefficient (r) was plotted to indicate strength of relationship between data, and analysis was performed on each group separately. Non-GDM non-obese = blue, non-GDM obese = purple, GDM non-obese = orange, GDM obese = red. Sample size (n) were between 8-10 for all groups. *: $p < 0.05$, correlation is statistically significant, $^{\#}$: correlation is near statistical significance. Abbreviations. SBP: Systolic blood pressure, DBP: diastolic blood pressure, FPG: fasting plasma glucose, BMI: body mass index, 2-h Glu post OGTT: 2-hour glucose post oral glucose tolerance test, F/P: foetoplacental.

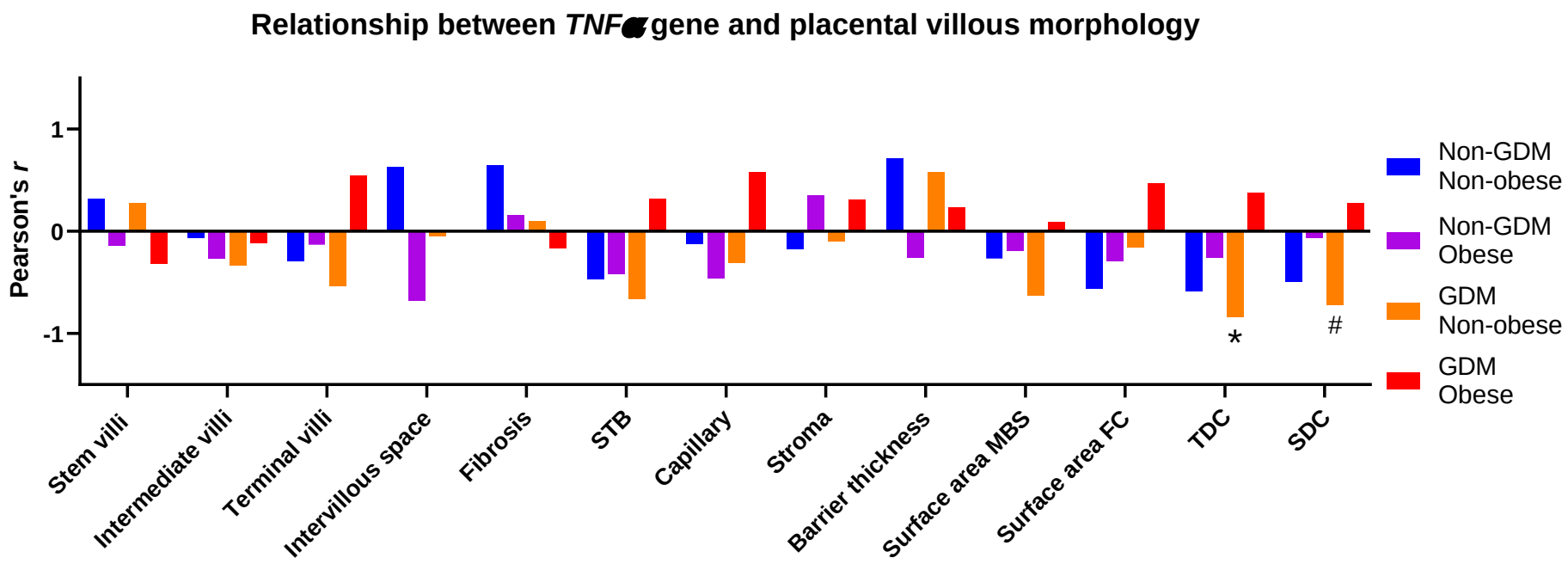


Figure 3.21. Placental *TNFα* gene correlation with placental villous morphology. Correlation analysis was performed between placental *TNFα* gene and placental villous morphology. Pearson's coefficient (r) was plotted to indicate strength of relationship between data, and analysis was performed on each group separately. Non-GDM non-obese = blue, non-GDM obese = purple, GDM non-obese = orange, GDM obese = red. Sample size (n) were between 8-10 for all groups. *: $p < 0.05$, correlation is statistically significant, $^{\#}$: correlation is near statistical significance. Abbreviations. FC: foetal capillaries, MBS: maternal blood space, STB: syncytiotrophoblast, TDC: theoretical diffusion capacity, SDC: specific diffusion capacity.

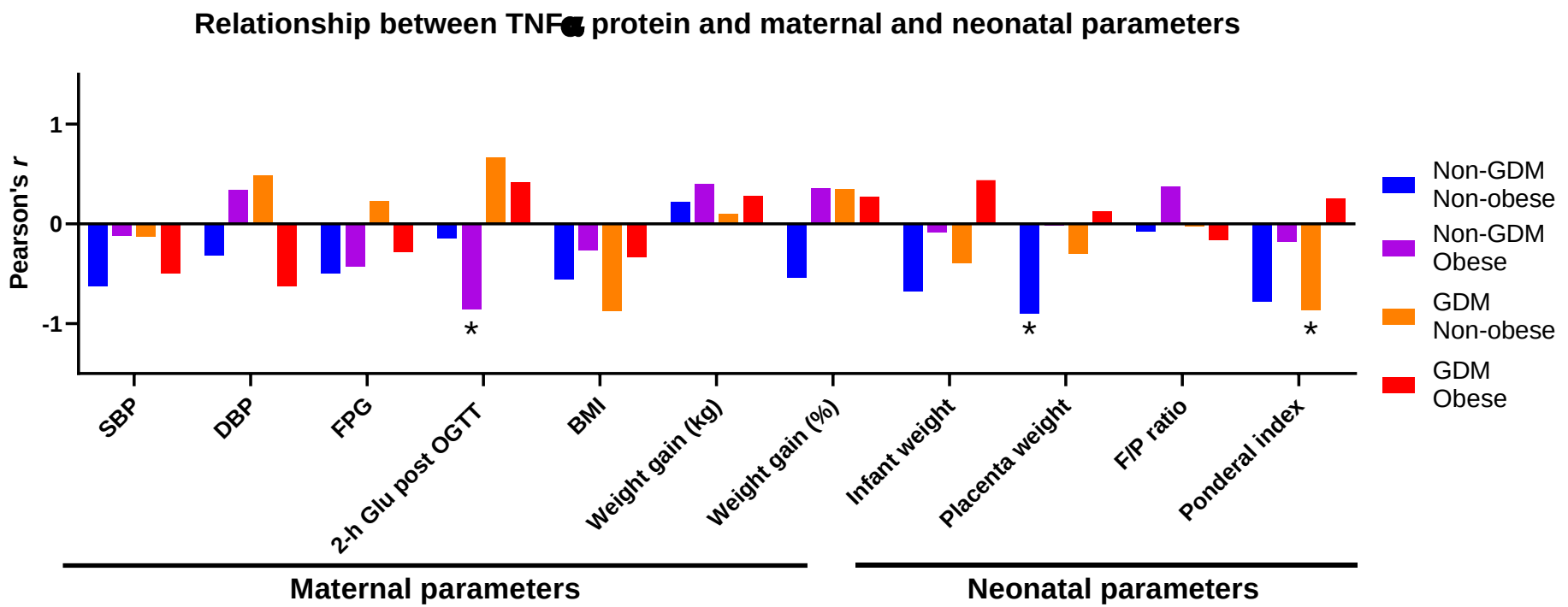


Figure 3.22. Placental TNFα protein correlation with maternal and neonatal parameters. Correlation analysis was performed between placental TNFα protein and maternal and neonatal parameters. Pearson's coefficient (r) was plotted to indicate strength of relationship between data, and analysis was performed on each group separately. Non-GDM non-obese = blue, non-GDM obese = purple, GDM non-obese = orange, GDM obese = red. Sample size (n) = 8 for all groups. *: $p < 0.05$, correlation is statistically significant. Abbreviations. SBP: Systolic blood pressure, DBP: diastolic blood pressure, FPG: fasting plasma glucose, BMI: body mass index, 2-h Glu post OGTT: 2-hour glucose post oral glucose tolerance test, F/P: foetoplacental.

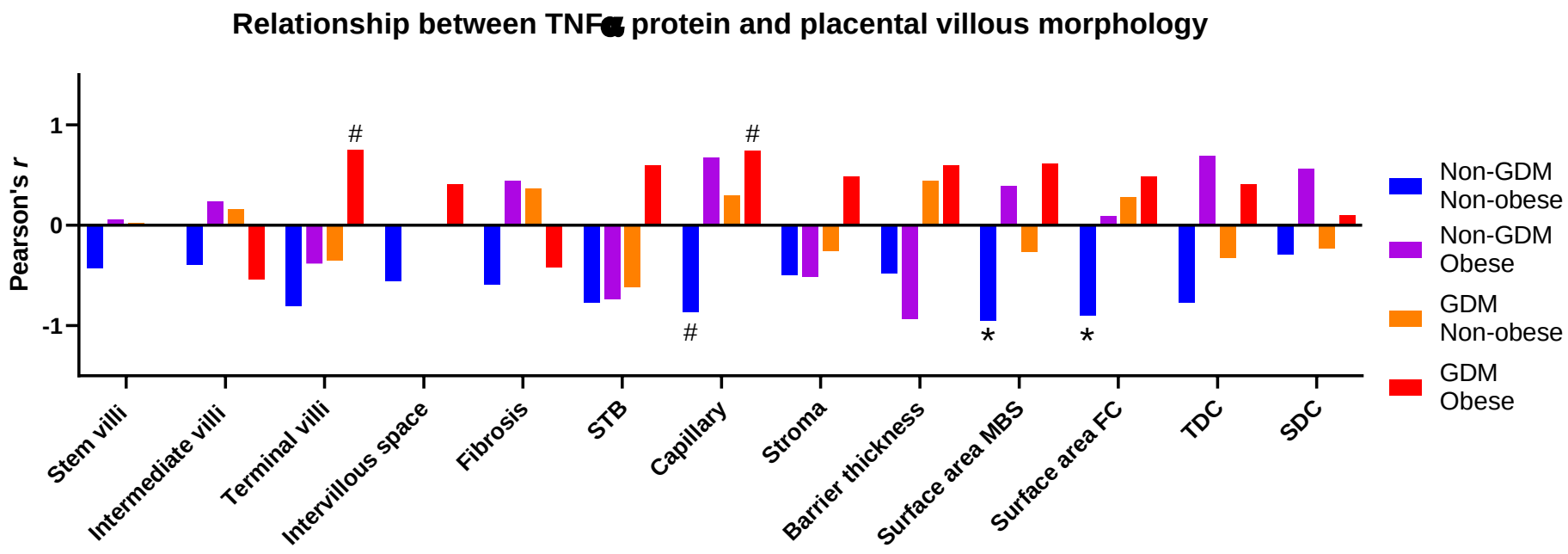


Figure 3.23. Placental TNFα protein correlation with placental villous morphology. Correlation analysis was performed between placental TNFα protein and placental villous morphology. Pearson's coefficient (r) was plotted to indicate strength of relationship between data, and analysis was performed on each group separately. Non-GDM non-obese = blue, non-GDM obese = purple, GDM non-obese = orange, GDM obese = red. Sample size (n) = 8 for all groups. *: $p < 0.05$, correlation is statistically significant, $^{\#}$: correlation is near statistical significance. Abbreviations. FC: foetal capillaries, MBS: maternal blood space, STB: syncytiotrophoblast, TDC: theoretical diffusion capacity, SDC: specific diffusion capacity.

Placental *IL-6* gene expression was significantly positively correlated with 2-hour glucose post-OGTT and infant ponderal index in obese non-GDM women ($p = 0.028$, $p = 0.045$, respectively). However, there was a significant negative correlation between placental *IL-6* gene expression and maternal percentage weight gain ($p = 0.0183$) and volume of intermediate villi ($p = 0.0075$) in non-obese non-GDM and non-obese GDM, respectively. There was a near statistically significant correlation between placental *IL-6* gene and infant birth weight, both foetal-placental ratio and volume of intermediate villi in non-GDM non-obese and GDM obese women, respectively (Figures 3.24 and 3.25).

In the non-GDM non-obese women, there was a significant negative correlation between placental IL-6 protein abundance and SBP, DBP, surface area of FC and TDC ($p = 0.0352$, $p = 0.0422$, $p = 0.0459$, respectively). In GDM obese women, there was a statistically significant positive correlation between placental IL-6 protein abundance and DBP ($p = 0.0258$). However, there were no significant correlations between placental IL-6 protein abundance and maternal weight gain, infant birth weight, foetoplacental ratio, infant ponderal index, volumes of stem, intermediate, and terminal villi, as well as intervillous space, fibrosis, villous syncytiotrophoblast, foetal capillary and villous stroma in any of the groups (Figures 3.26 and 3.27).

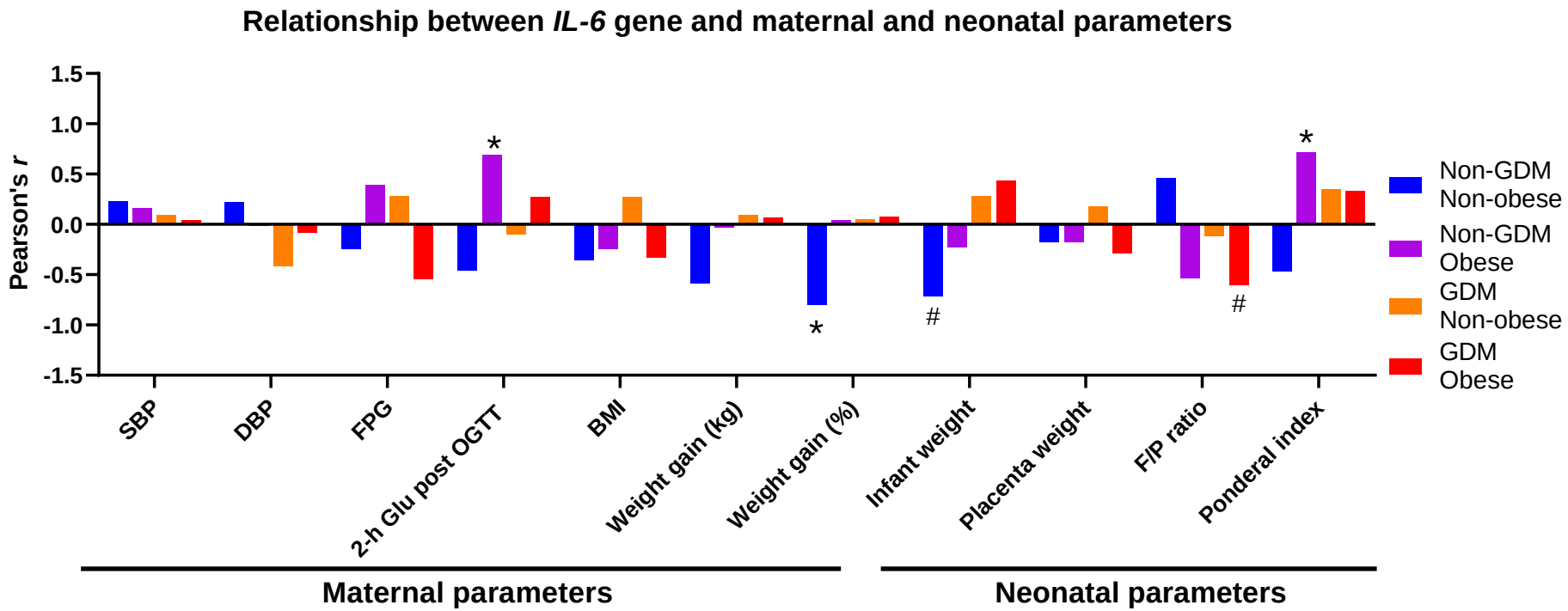


Figure 3.24. Placental *IL-6* gene correlation with maternal and neonatal parameters. Correlation analysis was performed between placental *IL-6* gene and maternal and neonatal parameters. Pearson's coefficient (r) was plotted to indicate strength of relationship between data, and analysis was performed on each group separately. Non-GDM non-obese = blue, non-GDM obese = purple, GDM non-obese = orange, GDM obese = red. Sample size (n) were between 8-10 for all groups. *: $p < 0.05$, correlation is statistically significant, #: correlation is near statistical significance. Abbreviations. SBP: Systolic blood pressure, DBP: diastolic blood pressure, FPG: fasting plasma glucose, BMI: body mass index, 2-h Glu post OGTT: 2-hour glucose post oral glucose tolerance test, F/P: foetoplacental.

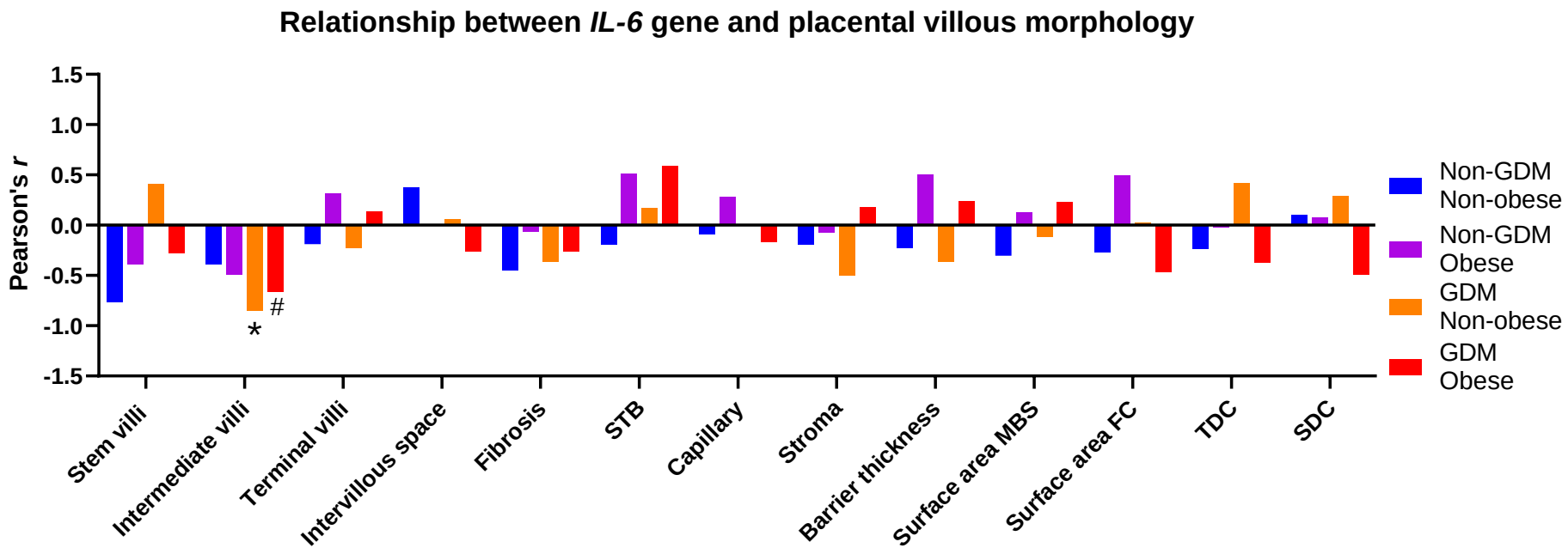


Figure 3.25. Placental *IL-6* gene correlation with placental villous morphology. Correlation analysis was performed between placental *IL-6* gene and placental villous morphology. Pearson's coefficient (r) was plotted to indicate strength of relationship between data, and analysis was performed on each group separately. Non-GDM non-obese = blue, non-GDM obese = purple, GDM non-obese = orange, GDM obese = red. Sample size (n) were between 8-10 for all groups. *: $p < 0.05$, correlation is statistically significant, $^{\#}$: correlation is near statistical significance. Abbreviations. FC: foetal capillaries, MBS: maternal blood space, STB: syncytiotrophoblast, TDC: theoretical diffusion capacity, SDC: specific diffusion capacity.

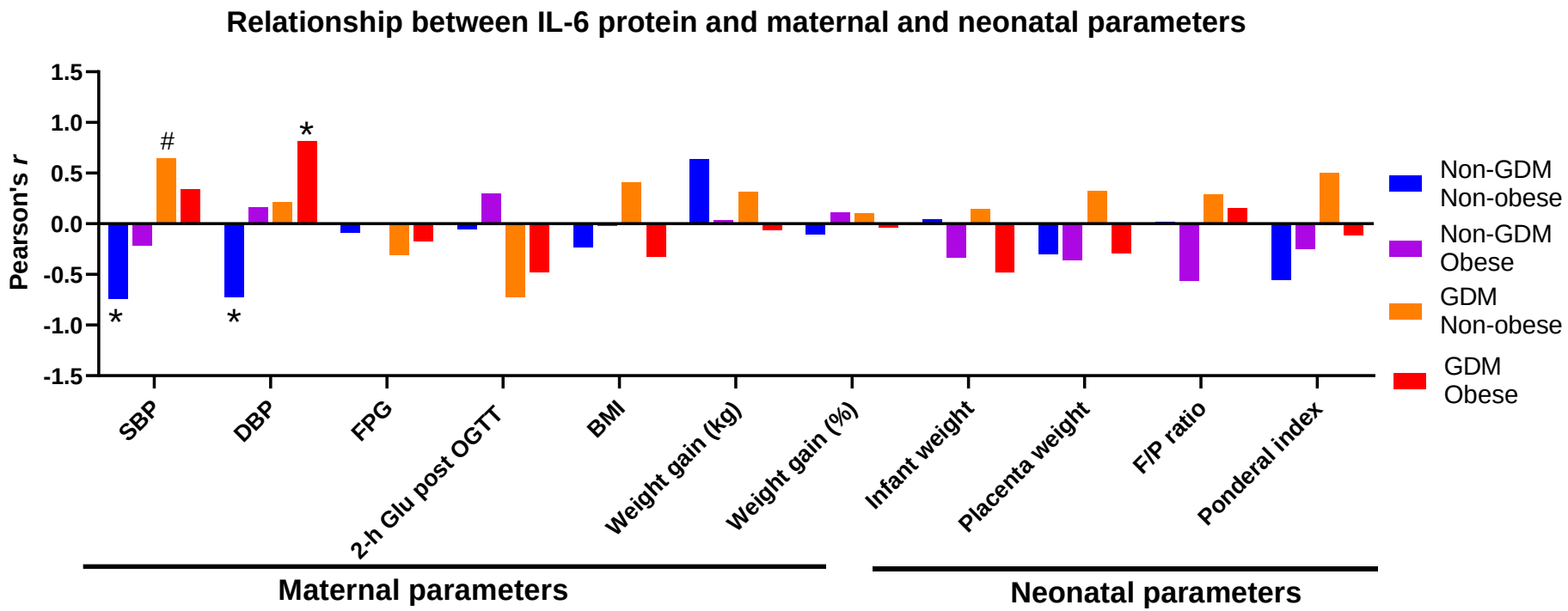


Figure 3.26. Placental IL-6 protein correlation with maternal and neonatal parameters. Correlation analysis was performed between placental IL-6 protein and maternal and neonatal parameters. Pearson's coefficient (r) was plotted to indicate strength of relationship between data, and analysis was performed on each group separately. Non-GDM non-obese = blue, non-GDM obese = purple, GDM non-obese = orange, GDM obese = red. Sample size (n) = 8 for all groups. *: $p < 0.05$, correlation is statistically significant, $^{\#}$: correlation is near statistical significance. Abbreviations. SBP: Systolic blood pressure, DBP: diastolic blood pressure, FPG: fasting plasma glucose, BMI: body mass index, 2-h Glu post OGTT: 2-hour glucose post oral glucose tolerance test, F/P: foetoplacental

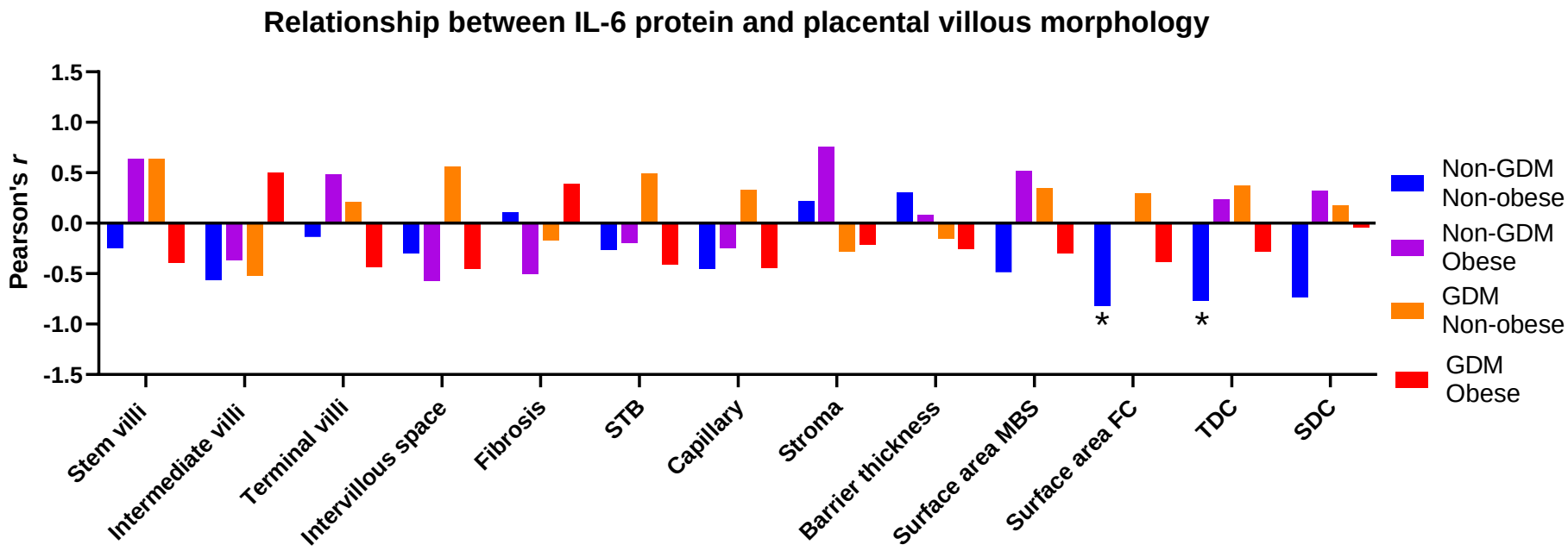


Figure 3.27. Placental IL-6 protein correlation with placental villous morphology. Correlation analysis was performed between placental IL-6 protein and placental villous morphology. Pearson's coefficient (r) was plotted to indicate strength of relationship between data, and analysis was performed on each group separately. Non-GDM non-obese = blue, non-GDM obese = purple, GDM non-obese = orange, GDM obese = red. Sample size (n) were between 8-10 for all groups. *: $p < 0.05$, correlation is statistically significant, $^{\#}$: correlation is near statistical significance. Abbreviations. FC: foetal capillaries, MBS: maternal blood space, STB: syncytiotrophoblast, TDC: theoretical diffusion capacity, SDC: specific diffusion capacity.

3.9 Correlations Between Circulatory Kisspeptin, and Proinflammatory Markers Concentrations and Placental Villous Morphology and Maternal and Neonatal Parameters

The serum maternal kisspeptin concentration was positively correlated with DBP and placenta weight and negatively correlated with infant weight, infant ponderal index, intervillous space, and villous syncytiotrophoblast. Maternal serum kisspeptin concentrations correlated positively with placenta weight in the obese non-GDM group ($p = 0.0107$). In the non-obese non-GDM group, there was a near statistically significant positive correlation with DBP ($p = 0.0699$ 0 and a negative correlation with infant weight ($p = 0.0804$). Furthermore, there was a near statistically significant negative correlation between maternal serum kisspeptin concentrations and infant ponderal index in GDM obese women ($p = 0.0721$). Also, in the non-GDM non-obese women, maternal serum kisspeptin concentrations were negatively correlated with volume of intervillous space and villous syncytiotrophoblasts, but this only reached statistical significance in its relationship with intervillous space ($p = 0.0091$) (Figures 3.28 and 3.29).

The cord serum kisspeptin concentration correlated positively with maternal DBP ($p = 0.025$) and volume of intermediate villi ($p = 0.0507$) in non-GDM non-obese women and with infant birth weight ($p = 0.0167$) in GDM obese groups, but inversely correlated with maternal BMI in the non-obese GDM group ($p = 0.002$). The cord serum kisspeptin concentration negatively correlated with the volume of terminal villi ($p = 0.0572$) and SDC ($p = 0.0257$) in obese non-GDM and obese GDM women, respectively (Figures 3.30 and 3.31).

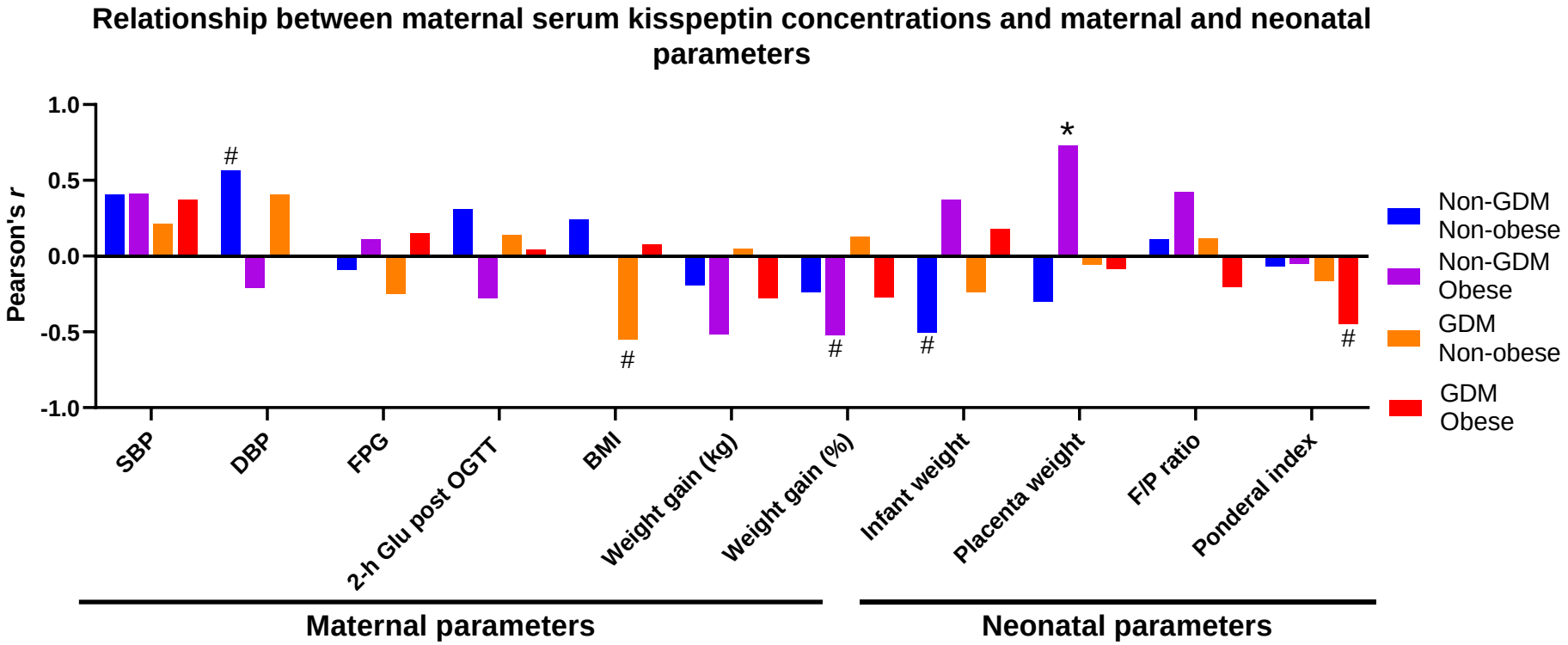


Figure 3.28. Maternal serum kisspeptin concentration correlation with maternal and neonatal parameters. Pearson's coefficient (r) was plotted to indicate strength of relationship between data, and analysis was performed on each group separately. Non-GDM non-obese = blue, non-GDM obese = purple, GDM non-obese = orange, GDM obese = red. Sample size (n) were between 18-25 for all groups. *: $p < 0.05$, correlation is statistically significant, $^{\#}$: correlation is near statistical significance. Abbreviations. SBP: Systolic blood pressure, DBP: diastolic blood pressure, FPG: fasting plasma glucose, BMI: body mass index, 2-h Glu post OGTT: 2-hour glucose post oral glucose tolerance test, F/P: foetoplacental.

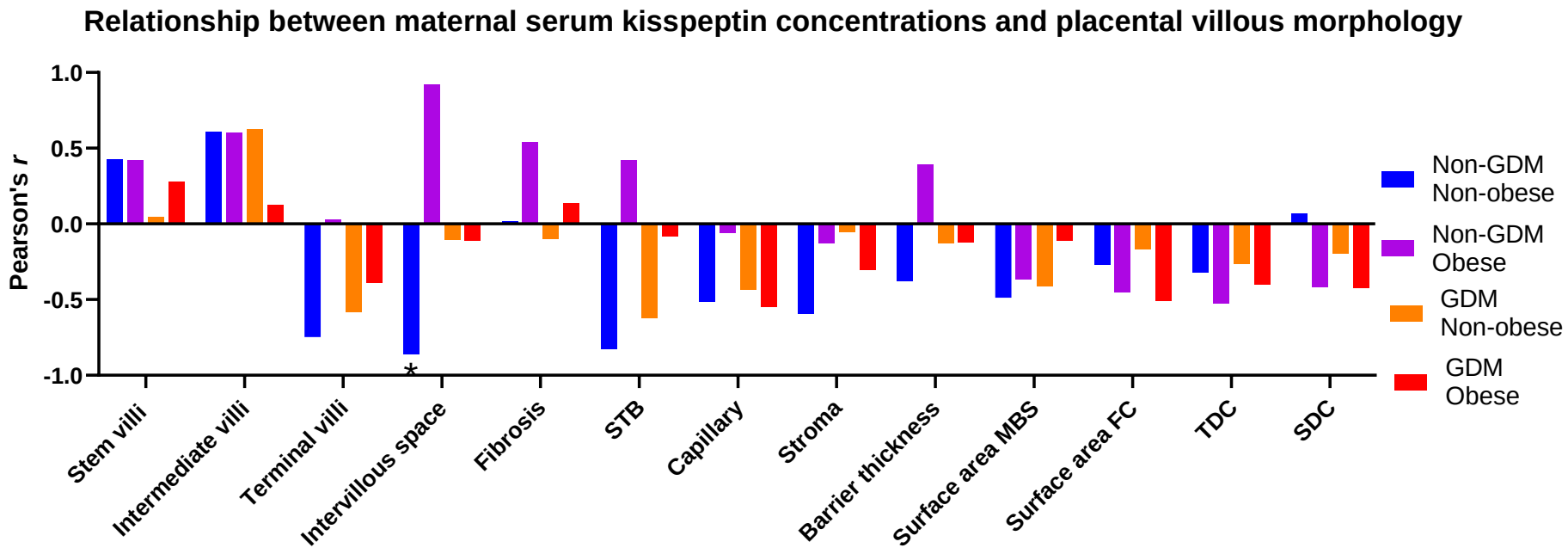


Figure 3.29. Maternal serum kisspeptin concentration correlation with placental villous morphology. Pearson's coefficient (r) was plotted to indicate strength of relationship between data, and analysis was performed on each group separately. Non-GDM non-obese = blue, non-GDM obese = purple, GDM non-obese = orange, GDM obese = red. Sample size (n) were between 18-25 for all groups. *: $p < 0.05$, correlation is statistically significant. Abbreviations. FC: foetal capillaries, MBS: maternal blood space, STB: syncytiotrophoblast, TDC: theoretical diffusion capacity, SDC: specific diffusion capacity.

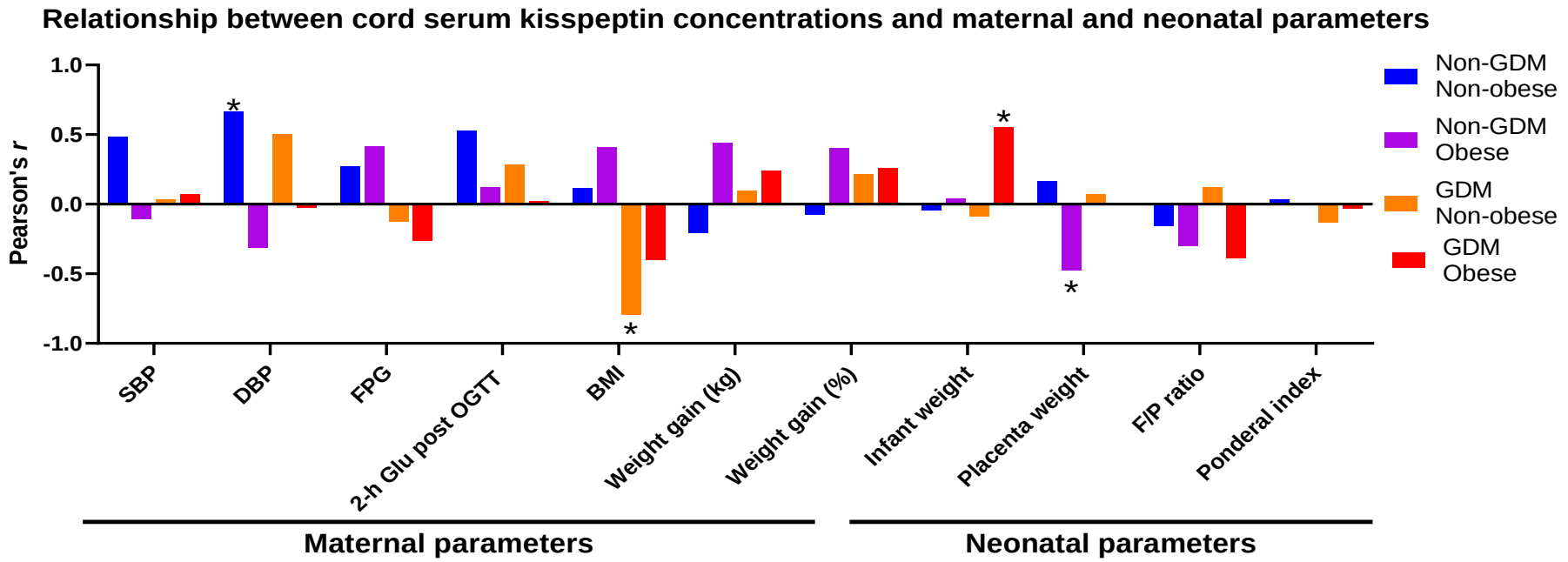


Figure 3.30. Cord serum kisspeptin concentration correlation with maternal and neonatal parameters. Pearson's coefficient (r) was plotted to indicate strength of relationship between data, and analysis was performed on each group separately. Non-GDM non-obese = blue, non-GDM obese = purple, GDM non-obese = orange, GDM obese = red. Sample size (n) were between 18-25 for all groups. *: $p < 0.05$, correlation is statistically significant, $^{\#}$: correlation is near statistical significance. Abbreviations. SBP: Systolic blood pressure, DBP: diastolic blood pressure, FPG: fasting plasma glucose, BMI: body mass index, 2-h Glu post OGTT: 2-hour glucose post oral glucose tolerance test, F/P: foetoplacental.

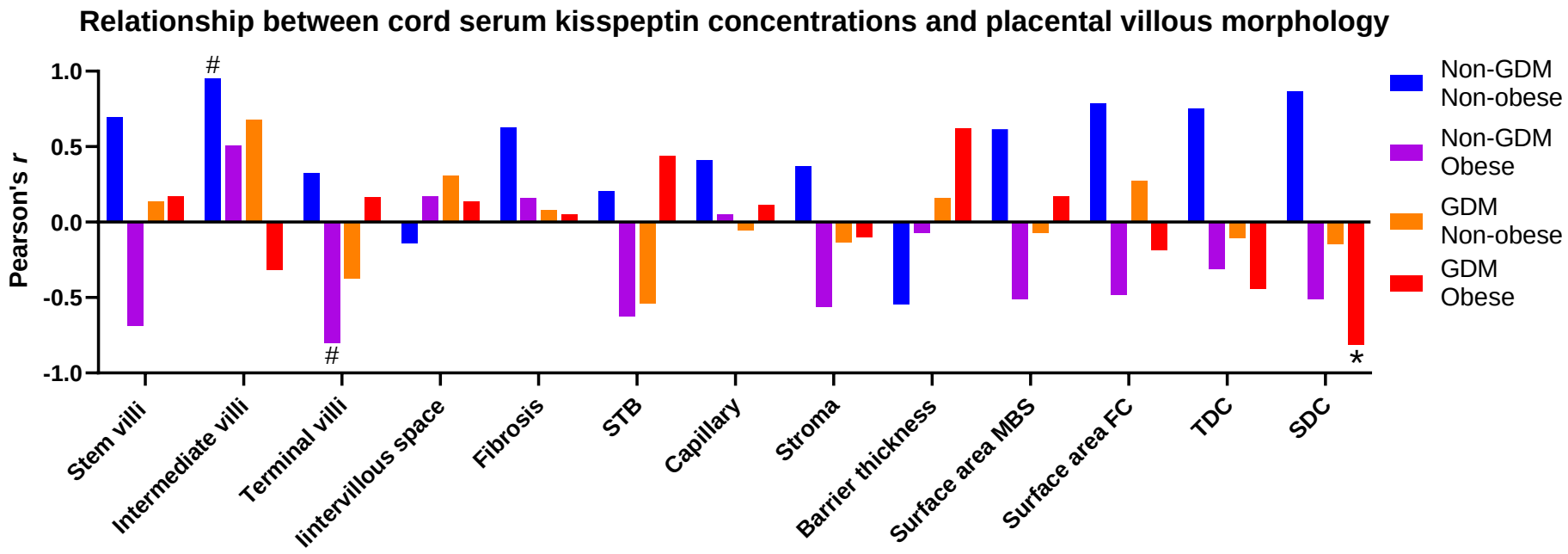


Figure 3.31. Cord serum kisspeptin concentration correlation with placental villous morphology. Pearson's coefficient (r) was plotted to indicate strength of relationship between data, and analysis was performed on each group separately. Non-GDM non-obese = blue, non-GDM obese = purple, GDM non-obese = orange, GDM obese = red. Sample size (n) were between 18-25 for all groups. *: $p < 0.05$, correlation is statistically significant, $^{\#}$: correlation is near statistical significance. Abbreviations. FC: foetal capillaries, MBS: maternal blood space, STB: syncytiotrophoblast, TDC: theoretical diffusion capacity, SDC: specific diffusion capacity

There were no significant correlations between maternal serum TNFα concentrations and maternal and neonatal parameters or placental villous morphology in the non-GDM non-obese group. In the non-GDM obese group, maternal serum TNFα concentrations correlated negatively with placenta weight, foetoplacental ratio and volume of intervillous space ($p = 0.0002$, $p = 0.0034$, $p = 0.0272$, respectively). In the obese GDM women, maternal serum TNFα concentrations were positively correlated with maternal SBP only ($p = 0.0281$) and negatively with infant ponderal index ($p = 0.0233$). (Figures 3.32 and 3.33).

Cord serum TNFα concentration was positively correlated with maternal FPG ($p = 0.0114$) and placenta weight ($p = 0.0331$) in GDM non-obese and GDM obese groups, respectively. However, it had an inverse correlation with maternal DBP ($p = 0.0123$) in GDM obese group, as well as the foetoplacental ratio in obese non-GDM women ($p = 0.0491$). Whereas there was a positive correlation between cord serum TNFα concentration and volume of intermediate villi in the GDM obese group ($p = 0.0241$) (Figures 3.34 and 3.35).

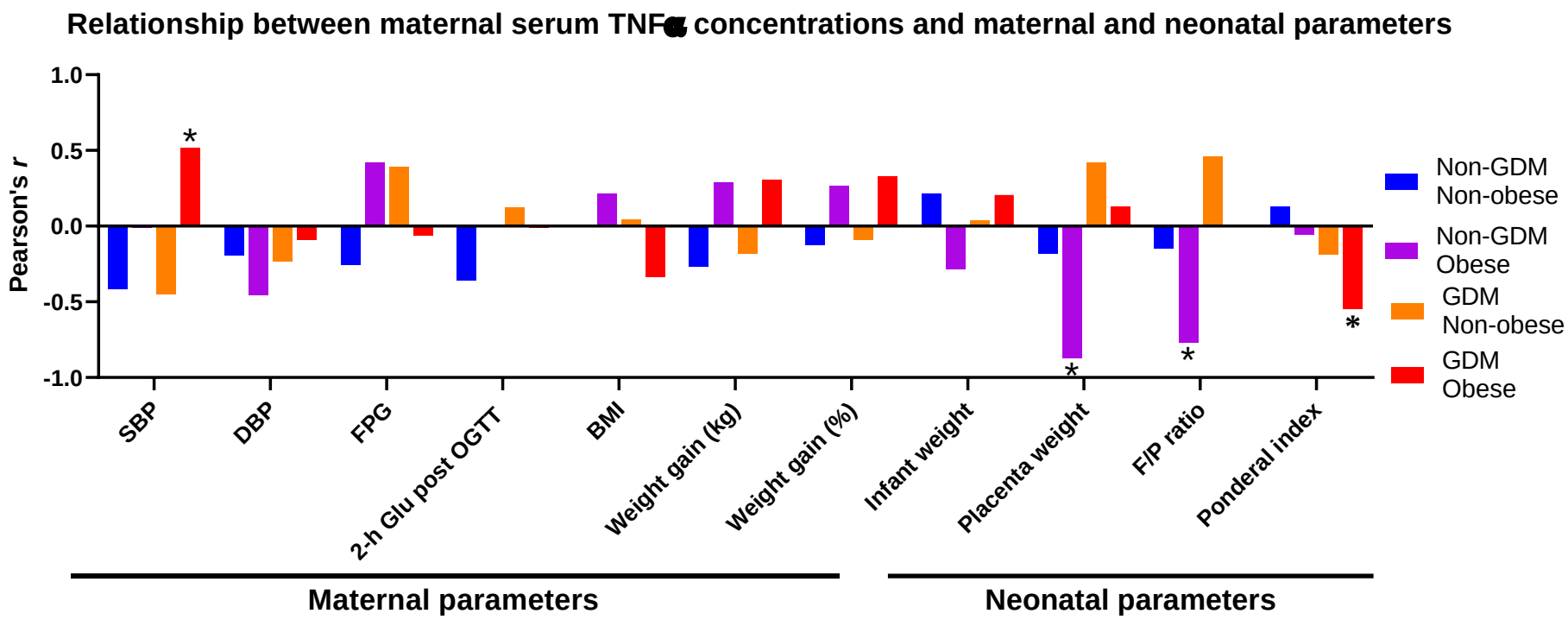


Figure 3.32. Maternal serum TNFα concentration correlation with maternal and neonatal parameters. Pearson's coefficient (r) was plotted to indicate strength of relationship between data, and analysis was performed on each group separately. Non-GDM non-obese = blue, non-GDM obese = purple, GDM non-obese = orange, GDM obese = red. Sample size (n) were between 18-25 for all groups. *: $p < 0.05$, correlation is statistically significant. Abbreviations. SBP: Systolic blood pressure, DBP: diastolic blood pressure, FPG: fasting plasma glucose, BMI: body mass index, 2-h Glu post OGTT: 2-hour glucose post oral glucose tolerance test, F/P: foetoplacental.

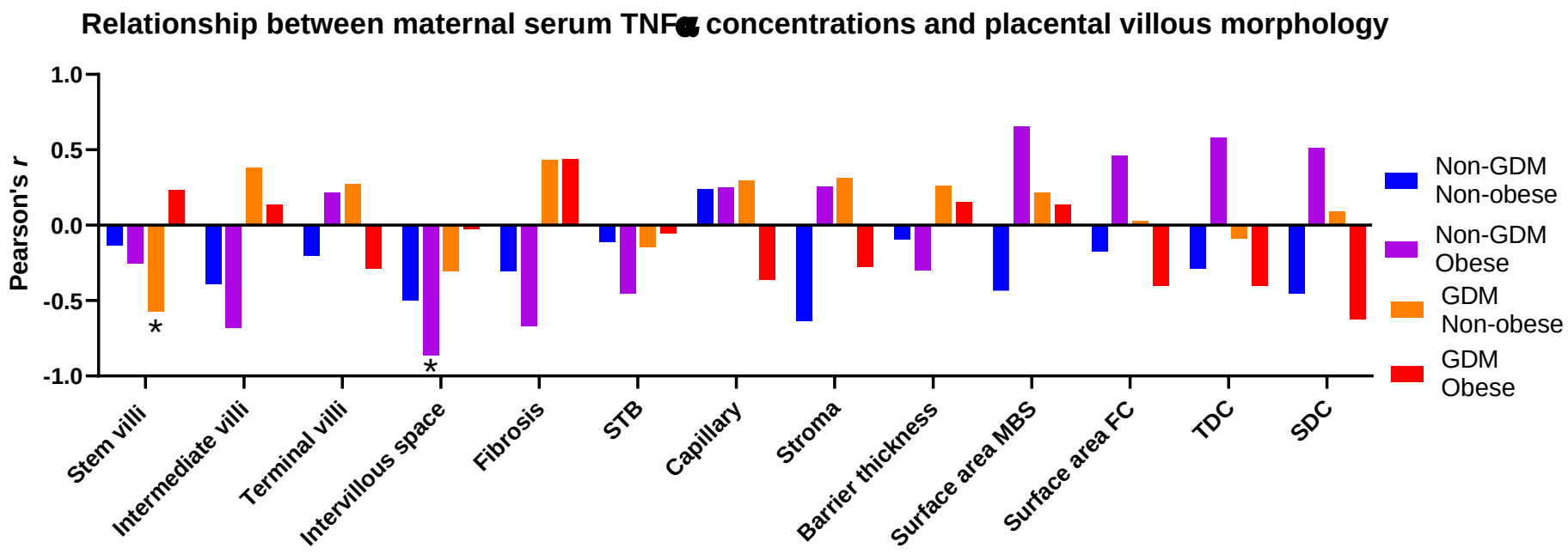


Figure 3.33. Maternal serum TNFα concentration correlation with placental villous morphology. Pearson's coefficient (r) was plotted to indicate strength of relationship between data, and analysis was performed on each group separately. Non-GDM non-obese = blue, non-GDM obese = purple, GDM non-obese = orange, GDM obese = red. Sample size (n) were between 18-25 for all groups. *: $p < 0.05$, correlation is statistically significant. Abbreviations. FC: foetal capillaries, MBS: maternal blood space, STB: syncytiotrophoblast, TDC: theoretical diffusion capacity, SDC: specific diffusion capacity.

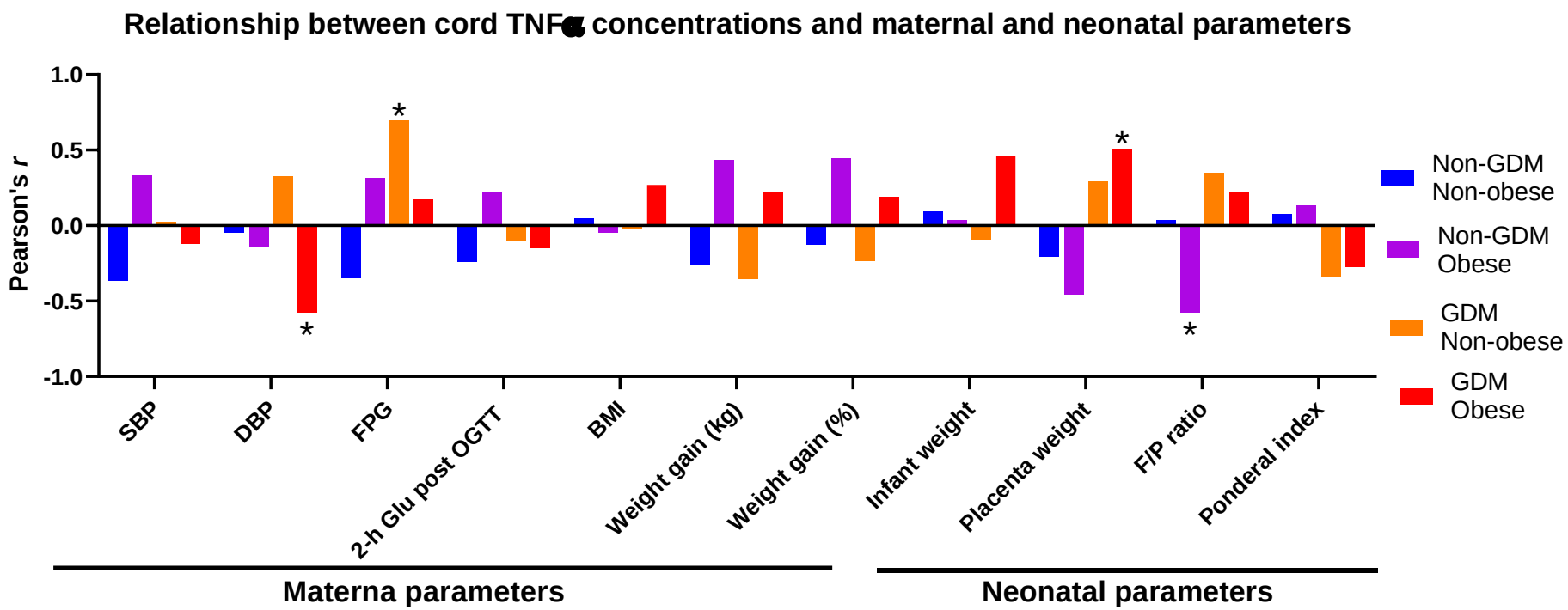


Figure 3.34. Cord serum TNFα concentration correlation with maternal and neonatal parameters. Pearson's coefficient (r) was plotted to indicate strength of relationship between data, and analysis was performed on each group separately. Non-GDM non-obese = blue, non-GDM obese = purple, GDM non-obese = orange, GDM obese = red. Sample size (n) were between 18-25 for all groups. *: $p < 0.05$, correlation is statistically significant. Abbreviations. SBP: Systolic blood pressure, DBP: diastolic blood pressure, FPG: fasting plasma glucose, BMI: body mass index, 2-h Glu post OGTT: 2-hour glucose post oral glucose tolerance test, F/P: foetoplacental.

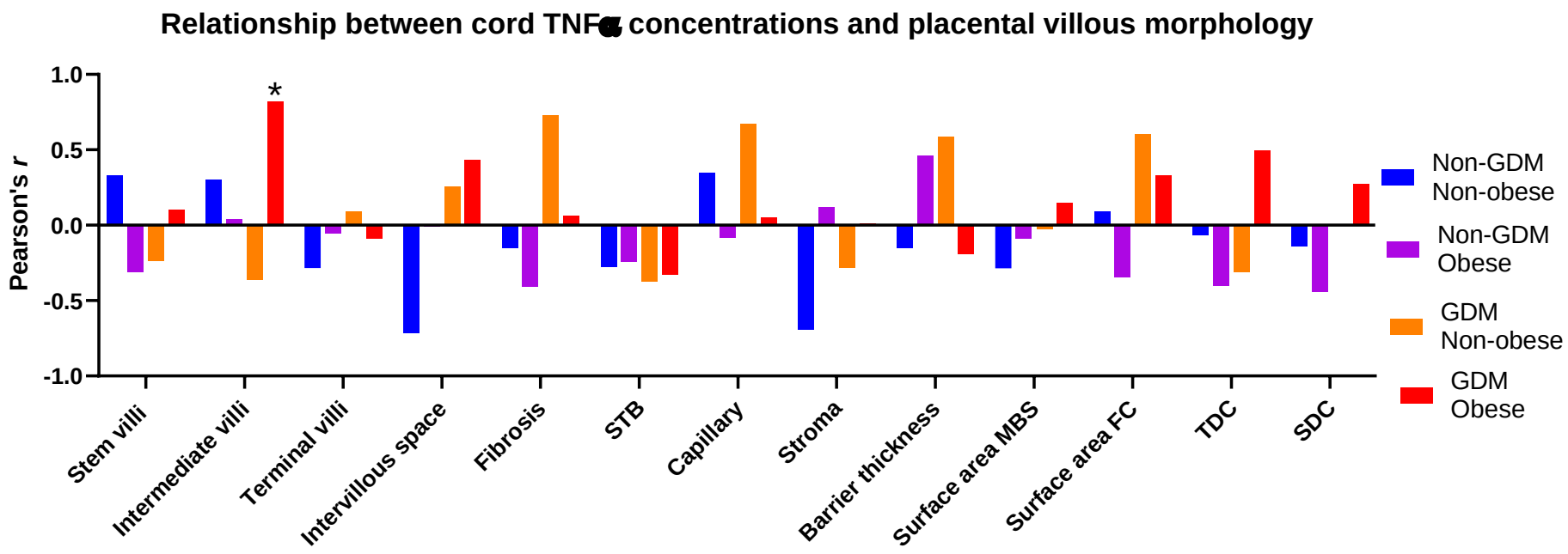


Figure 3.35. Cord serum TNFα concentration correlation with placental villous morphology. Pearson's coefficient (r) was plotted to indicate strength of relationship between data, and analysis was performed on each group separately. Non-GDM non-obese = blue, non-GDM obese = purple, GDM non-obese = orange, GDM obese = red. Sample size (n) were between 18-25 for all groups. *: $p < 0.05$, correlation is statistically significant. Abbreviations. FC: foetal capillaries, MBS: maternal blood space, STB: syncytiotrophoblast, TDC: theoretical diffusion capacity, SDC: specific diffusion capacity.

In non-obese non-GDM and GDM groups, respectively, maternal serum IL-6 concentration correlated negatively with infant birth weight and infant ponderal index ($p = 0.068$, $p = 0.0897$) at near statistical significance, but significantly correlated with foeto-placental ratio ($p = 0.039$). Also, maternal serum IL-6 concentration inversely correlated with fibrosis in non-GDM non-obese women ($p = 0.0308$); however, it correlated positively with volume of intermediate villi in the GDM non-obese group ($p = 0.0245$). Meanwhile, in the obese non-GDM women, maternal serum IL-6 concentrations correlated negatively with, TDC and SDC ($p = 0.0199$, $p = 0.0491$ respectively) (Figures 3.36 and 3.37).

In the non-GDM non-obese group, there was also a positive correlation between the cord serum IL-6 concentration and 2-hour glucose post-OGTT and surface area of FC ($p = 0.0263$, $p = 0.0392$, respectively). However, in non-GDM obese women, there was only a near statistically significant inverse correlation between cord serum IL-6 concentration and placenta weight and infant ponderal index ($p = 0.0553$, $p = 0.0772$ respectively), positive correlation with DBP in nonobese GDM ($p = 0.0722$).In GDM obese women, cord serum IL-6 concentration correlated positively with maternal FPG ($p = 0.0692$), 2-hour glucose post-OGTT ($p = 0.0532$), , intermediate villi ($p = 0.003$) and intervillous space ($p = 0.0324$) (Figures 3.38 and 3.39).

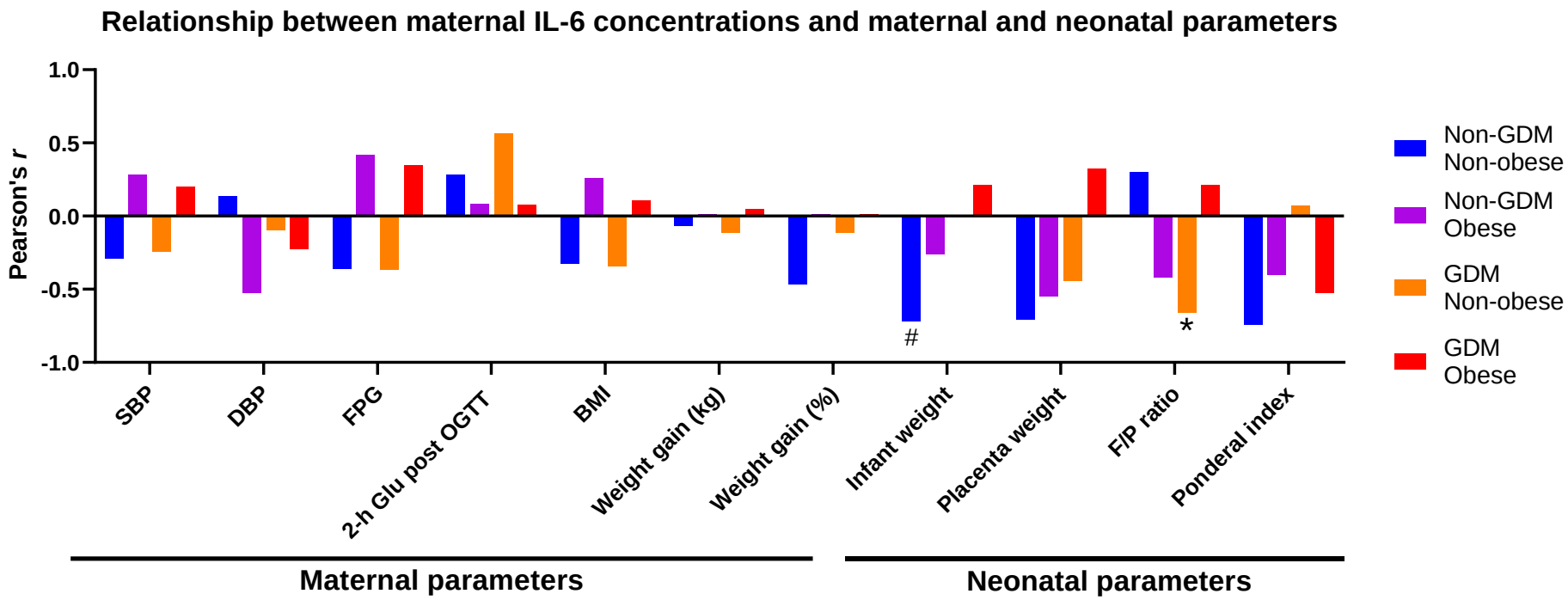


Figure 3.36. Maternal serum IL-6 concentration correlation with maternal and neonatal parameters. Pearson's coefficient (r) was plotted to indicate strength of relationship between data, and analysis was performed on each group separately. Non-GDM non-obese = blue, non-GDM obese = purple, GDM non-obese = orange, GDM obese = red. Sample size (n) were between 18-25 for all groups. *: $p < 0.05$, correlation is statistically significant, #: correlation is near statistical significance. Abbreviations. SBP: Systolic blood pressure, DBP: diastolic blood pressure, FPG: fasting plasma glucose, BMI: body mass index, 2-h Glu post OGTT: 2-hour glucose post oral glucose tolerance test, F/P: foetoplacental.

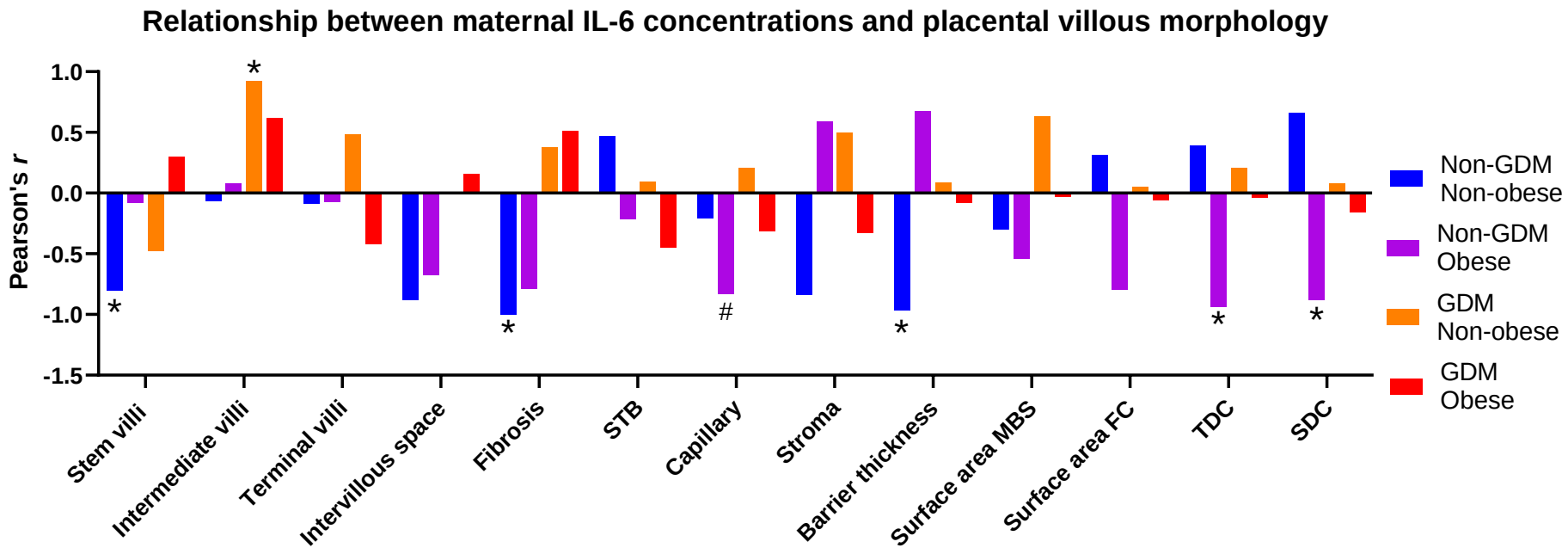


Figure 3.37. Maternal serum IL-6 concentration correlation with placental villous morphology. Pearson's coefficient (r) was plotted to indicate strength of relationship between data, and analysis was performed on each group separately. Non-GDM non-obese = blue, non-GDM obese = purple, GDM non-obese = orange, GDM obese = red. Sample size (n) were between 18-25 for all groups. *: $p < 0.05$, correlation is statistically significant, #: correlation is near statistical significance. Abbreviations. FC: foetal capillaries, MBS: maternal blood space, STB: syncytiotrophoblast, TDC: theoretical diffusion capacity, SDC: specific diffusion capacity.

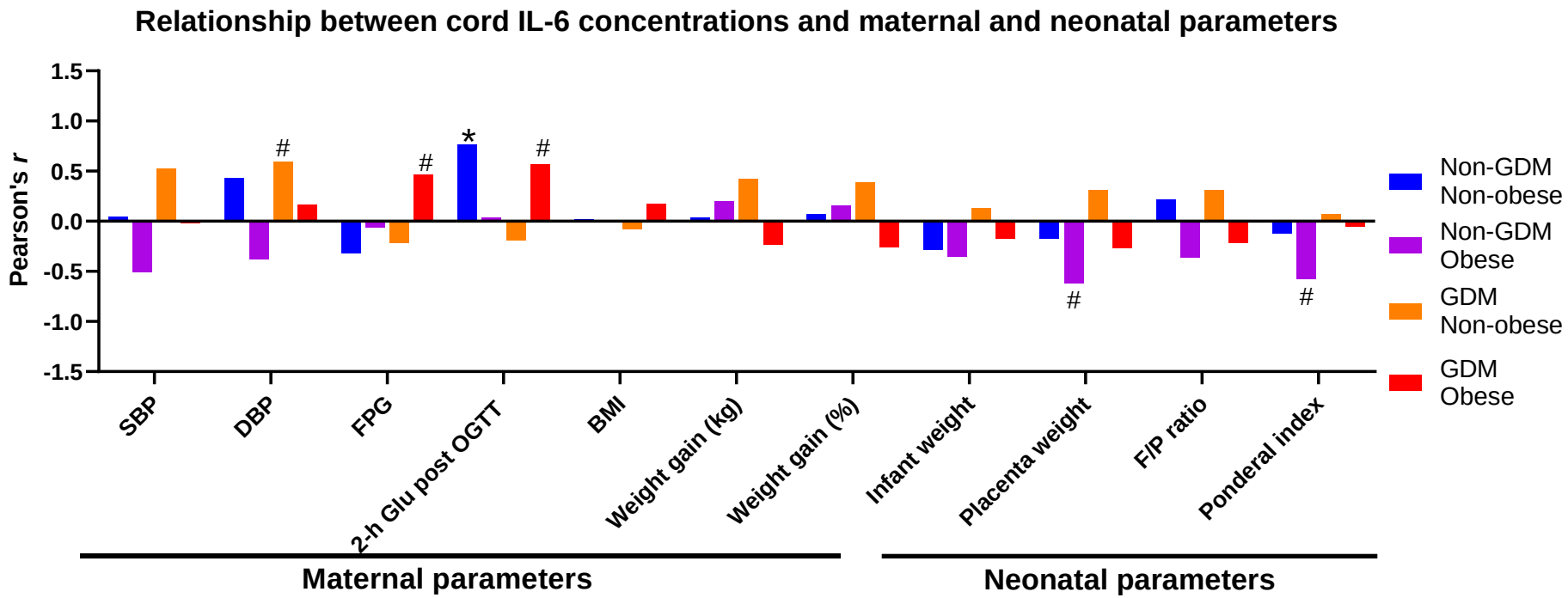


Figure 3.38. Cord serum IL-6 concentration correlation with maternal and neonatal parameters. Pearson's coefficient (r) was plotted to indicate strength of relationship between data, and analysis was performed on each group separately. Non-GDM non-obese = blue, non-GDM obese = purple, GDM non-obese = orange, GDM obese = red. Sample size (n) were between 18-25 for all groups. *: $p < 0.05$, correlation is statistically significant, $^{\#}$: correlation is near statistical significance. Abbreviations. SBP: Systolic blood pressure, DBP: diastolic blood pressure, FPG: fasting plasma glucose, BMI: body mass index, 2-h Glu post OGTT: 2-hour glucose post oral glucose tolerance test, F/P: foetoplacental.

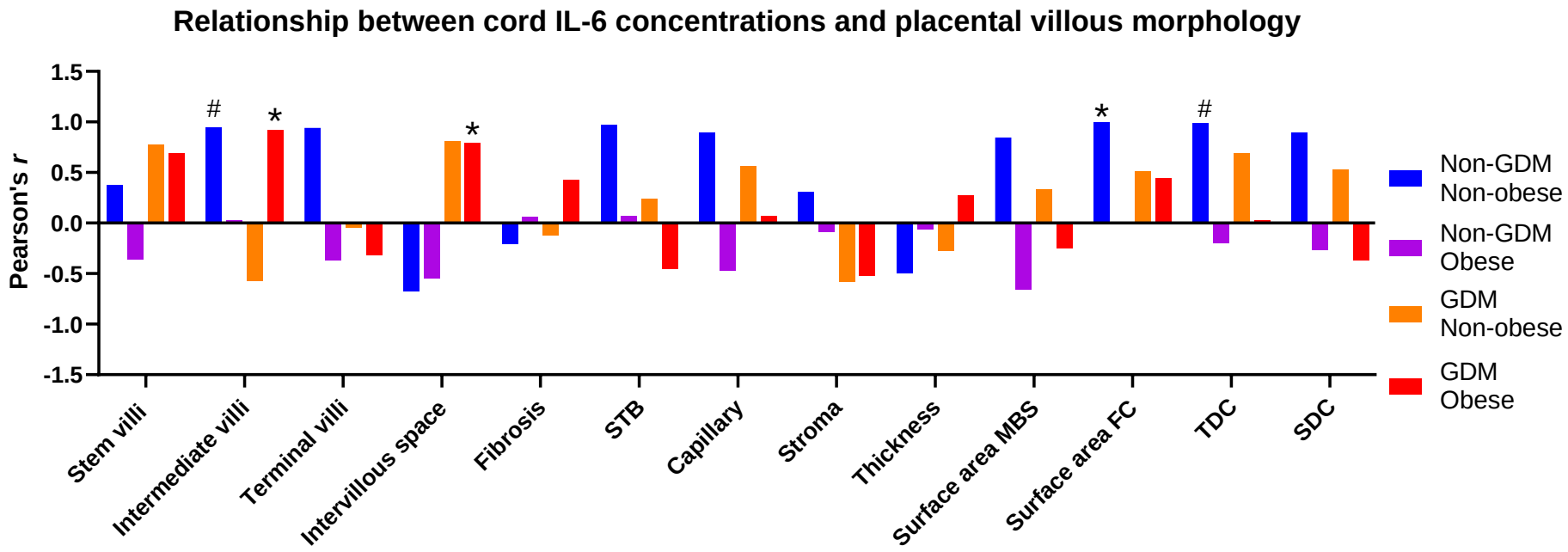


Figure 3.39. Cord serum IL-6 concentration correlation with placental villous morphology. Pearson's coefficient (r) was plotted to indicate strength of relationship between data, and analysis was performed on each group separately. Non-GDM non-obese = blue, non-GDM obese = purple, GDM non-obese = orange, GDM obese = red. Sample size (n) were between 18-25 for all groups. *: $p < 0.05$, correlation is statistically significant, #: correlation is near statistical significance. Abbreviations. FC: foetal capillaries, MBS: maternal blood space, STB: syncytiotrophoblast, TDC: theoretical diffusion capacity, SDC: specific diffusion capacity.

Having presented the results of the studies undertaken, Table 3.7 summarises some significant findings of the effects of GDM and maternal obesity on the measured placental and circulatory endocrine signals and proinflammatory factors, and placental morphology. The discussion of these results in the light of the existing literature as well as putative biological meaning follows in the next chapter.

Table 3.7. Summary of the Effects of GDM and Maternal Obesity on Measured Placental and Circulatory Endocrine Signals and Proinflammatory Cytokines, and Placental Morphology in the Study

	GDM	Maternal obesity
Placental kisspeptin (gene, protein and immunostaining) expression	↔	↔
Circulating kisspeptin levels	↔	↔
Placental *KISS1R* gene expression	↔	↓*
Placental *Leptin* gene expression	↔	↓*
Placental TNFα protein abundance	↓*	↔
Maternal circulating TNFα levels	↓*	↔
Placental TNFα protein abundance (immunostaining)	↔	↑*
TNFα staining of terminal villi syncytiotrophoblast (obese non-GDM and GDM)	↔	↑*
Placental IL-6 (gene, protein and immunostaining)	↔	↔
Maternal and cord circulating IL-6 levels	↔	↔
IL-6 staining of terminal villi stroma and foetal capillaries	↔	↓*
Intervillous space area (cm^2)	↑*	↑*
Terminal villi area and volume (cm^2/cm^3)	↓*	↓*
Intervillous space (cm^3)	↓*	↔

STB (cm^3)	⟷	↓ *
Capillary (cm^3)	⟷	↓ *
Stroma (cm^3)	⟷	↓ *
Surface density FC (cm^2/cm^3)	⟷	↓ *
Surface area MBS (m^2)	⟷	↓ *
Surface area FC (m^2)	⟷	↓ *
Theoretical diffusion capacity (cm^2/min/kPA)	⟷	↓ *
Specific diffusion capacity (cm^2/min/kPA/g)	⟷	↓ *

↑ = increase, ↓ = decrease, ⟷ = no effect

* = statistically significant ($p < 0.05$)

Abbreviations: *KISS1R*: kisspeptin 1 receptor, TNFα: Tumour necrosis factor alpha, IL-6: Interleukin 6, GDM: gestational diabetes mellitus, FC: foetal capillaries, MBS: maternal blood space, STB: syncytiotrophoblast.

The chapter commences with a summary of the main findings of the studies undertaken. This is followed by a discussion of the findings of these studies, which examined whether changes in the placental and circulatory expression of kisspeptin, and that of inflammatory cytokines, endocrine and growth factors, underlie pathophysiological and placental morphological changes associated with GDM and/or obesity and, whether these changes might have a bearing on maternal and neonatal parameters.

Our study found that maternal obesity and GDM had no effect on placental kisspeptin gene and protein expression, kisspeptin immunostaining or circulatory kisspeptin levels. Kisspeptin expression was predominantly localised in the placental villous syncytiotrophoblast compared to villous stroma and foetal capillary. However, maternal obesity reduced placental *Leptin* and *KISS1R* gene expression in the absence of GDM. Examination of the correlations between placental kisspeptin gene expression and placental morphology demonstrated a significant negative correlation between placental *kisspeptin* gene expression and volume of villous syncytiotrophoblasts and theoretical diffusion capacity in non-obese women irrespective of their GDM status. Further, placental kisspeptin protein had a significant inverse correlation with BMI and maternal systolic blood pressure in GDM women regardless of obesity, just as cord serum kisspeptin concentration correlated negatively with BMI.

With regards to the proinflammatory markers, overall, placental TNFα protein abundance (by western blotting) and maternal circulating TNFα levels were decreased in women with GDM. Despite these observations, maternal obesity significantly influenced placental TNFα protein abundance by immunostaining with a significantly higher expression in women with obesity

irrespective of GDM status. Also, TNFα staining of terminal villi syncytiotrophoblast was increased in women with obesity. Although the placental and maternal and cord IL-6 levels did not vary with groups, IL-6 staining of terminal villi stroma and vessels was reduced in women with obesity, an effect significant in those with GDM. Notably, this study showed a positive correlation between the expression of the placental *TNFα* gene and IL-6 protein and maternal diastolic blood pressure. Maternal serum TNFα and IL-6 concentrations correlated negatively with placenta weight, foetoplacental ratio and volume of intervillous space, and theoretical diffusion capacity and specific diffusion capacity, respectively. Placental stereology revealed that the placenta from women with obesity showed fewer terminal villi with fewer syncytiotrophoblast, foetal vessels and stroma, but the specific effect depended on GDM diagnosis while GDM influenced intervillous space volume by increasing it in obese groups. Maternal obesity also affected the surface areas for maternal-foetal exchange, where the surface area of maternal blood space and foetal capillary were reduced in obese women regardless of GDM status. Again, the physiological diffusion gradients for oxygen transfer at the maternal-foetal interface were significantly reduced by maternal obesity.

The next section will discuss the placental morphology stereology followed by kisspeptin and proinflammatory signalling with their respective correlations and then placental hormones, growth factors, and steroidogenic enzyme genes.

4.1 Impact of Maternal Obesity and GDM on Placental Villous Morphology, Including Villi Maturation, Vascularity and the Surface Area for Exchange

This is the first study to comprehensively interrogate the effect of maternal obesity *and* GDM on the human placental villous morphology and diffusion capacity in four groups of women: non-

GDM (non-obese, obese) and GDM (non-obese, obese). Most studies to date have only investigated pathological placental structure in the context of GDM *or* obesity. In this study, several morphological features of the placenta were affected by maternal obesity and GDM status, although overall, obesity had a much larger effect on the morphology of the placenta than GDM. With obesity, the morphological changes in the placenta suggest reduced villi maturation, vascularity, surfaces and diffusion gradients for maternal-foetal exchange of nutrients and oxygen regardless of GDM status. However, GDM appeared to increase the intervillous space volume in women with obesity. Studies have found morphological defects such as higher maternal inflammatory response, maternal and foetal neutrophilic infiltration, maternal origin villous lesions and reduced placental maturity index in the placenta of women with pregestational obesity compared to their non-obese counterparts.[283-285] It has also been previously reported that maternal obesity induces alteration in the muscularity of the villous vessels in the placenta, possibly by the effect of proinflammatory cytokines in the maternal and foetal environment.[286] Therefore, in our study cohort, the defects in placental morphology with obesity may be due to the elevated inflammatory state (TNFα) and reduced kisspeptin signalling (due to less KISS1R in the placenta) observed.

The impact of maternal obesity on the structure of the placenta would be expected to affect nutrient and oxygen exchange between the mother and the developing foetus and thus have implications for both short and long term adverse foetal outcomes. Indeed, further findings have suggested that the impacts of maternal obesity on the placental structure may have clinical implications for adverse foetal outcomes such as foetal death, stillbirth and infant death.[34, 35] Previous work has shown that obese women delivering large babies have increased placental abundance and activity of glucose (particularly GLUT1) and amino acid (including SNAT1) transporters.[287, 288] We

hypothesise that the reduction in villi formation and surface area might reflect a compensatory attempt of the placenta to prevent excessive growth of the foetus in women with obesity. This could also explain the lack of difference in birth weight and length between non-obese and obese women and especially obese women with GDM who had increased intervillous space volume (compared to non-obese with GDM). Enhanced placental nutrient transporters in women with obesity, however, could explain the finding of elevated infant ponderal index in our study, presumably due to increased nutrient supply to the foetus.

4.2 Kisspeptin Signalling

In our cohort, there was no effect of maternal obesity or GDM on placental *kisspeptin* gene expression, protein abundance, immunostaining or circulatory levels. While the effect of obesity on placental *kisspeptin* gene and/protein abundance has not been explored previously, our data are contrary to reports by Loegl et al (2017) who found a significant increase in the *kisspeptin* gene expression in trophoblasts isolated from the placenta of GDM compared to non-GDM women.[289] Loegl et al suggested that up-regulated *kisspeptin* gene may contribute to GDM-related changes in placental angiogenesis and vascular structure, although the association between kisspeptin and specific vascular regulators, like VEGF, was not examined in this work.[289] The Loegl et al study did not provide data on the BMI of the study population from whom the placenta samples were collected, so it is not known whether the difference between their findings and ours relates to study differences in maternal adiposity or the fact they studied isolated trophoblasts whilst we examined whole placenta. Previous work has shown that kisspeptin can inhibit trophoblast invasion and suppress angiogenic gene expression and new vessel formation in the placenta.[130, 147, 148, 196] For instance, the role of kisspeptin in the regulation of placental angiogenesis was demonstrated in *in vitro* studies conducted by Francis and Ramaesh et al who showed that the administration of

kisspeptin-10 reduced VEGF-A expression and inhibited new vessel formation, respectively.[148, 196] The current study showed a significant negative correlation between placental *kisspeptin* gene expression and the volume of villous syncytiotrophoblasts and theoretical diffusion capacity in non-obese women with or without GDM, which may reflect the inhibitory effect of kisspeptin on trophoblast invasion and placental development.[130]

The current study confirmed that kisspeptin protein expression is highest in the syncytiotrophoblast (compared to the stroma and foetal capillaries) of placental terminal villi.[130, 145] As the syncytiotrophoblasts are bathed in maternal blood, this spatial localisation would permit endocrine actions of placental derived kisspeptin. Germain and Sarker et al have demonstrated that maternal circulatory STBEV, which induce GSIS, rise steadily as pregnancy advances mirroring peripheral insulin resistance.[263, 264] This may indicate a possible cross-talk between circulatory STBEV and peripheral insulin resistance in GDM pathogenesis. The current study did not find any significant difference in kisspeptin protein abundance in STBEV between GDM and non-GDM women, although mean kisspeptin protein abundance in placental STBEV was >50% lower in the former compared to the latter. Kisspeptin induces insulin secretion by the pancreatic beta cells in a glucose-dependent fashion, just as healthy placenta-derived STBEV enhanced GSIS when infused into mice.[120, 121, 136, 270] Together, these data may suggest that women with GDM may have reduced GSIS secondary to lower placental STBEV kisspeptin content. Kandzija et al demonstrated that DPPIV activity, which catalyses the incretin GLP1, was greater in STBEV of GDM compared to non-GDM women.[290] Therefore, the relative roles of kisspeptin and DPP1V in STEBV on maternal GSIS requires further elucidation.

Contrary to our hypothesis, this study found neither GDM nor BMI or their interaction influenced maternal and cord serum kisspeptin concentrations. This is in keeping with data from recent studies where Arslan (2019), Abbara (2022) and Sithinamsuwan (2020) reported no differences in maternal serum kisspeptin levels in GDM compared to healthy pregnancies.[291-293] Rafique et al too found no difference in serum kisspeptin levels between normal weight and overweight/obese South Arabian women.[294] However, Abbara et al, after correcting for gestational age by using 'multiple of median' values revealed lower circulating kisspeptin concentrations in women with GDM.[291] Similarly, Bowe (2019) and Cetkovic (2012) et al reported lower maternal circulatory kisspeptin levels in GDM compared to non-GDM women.[120, 162] These conflicting results in the setting of GDM may be due to methodological variations in the specific study population, timing of sample collection during pregnancy, and assay methods including the detectable assay limits, and uncertainty of the kisspeptin isoforms quantified (such as kisspeptin 10, 13, and 54). Also, in our study cohort, pregnant women delivered between 38 and 39 weeks gestation when placental and blood samples were collected. In contrast, Bowe and Cetkovic et al collected blood samples earlier in gestation, at 24-28 weeks gestation and between 9-36 weeks (divided into first- (9-12), second- (21-25) and third- (32-36) trimesters), which may reflect the variations in kisspeptin concentrations between those and the current studies. Indeed, previous work has shown that serum kisspeptin concentration varies with maternal gestational age, and this may be related to the decline in placental function towards term.[295] Furthermore, contrary to findings that kisspeptin increases GSIS, insulin sensitivity, and pancreatic beta cell adaptation, some studies have observed an inhibitory effect of kisspeptin on GSIS.[119-121, 133, 136, 178, 179, 182] These contradictions in the role of kisspeptin in modulating GSIS may be due to its dose-dependent effect, glucose concentration used and varied experimental protocols. By implication, our study findings suggest that kisspeptin may not have a significant role in GDM development in obese or non-obese South African

pregnant women, or that we examined kisspeptin concentrations and or expression too late in gestation to pick up a change in kisspeptin that could be driving changes that manifest as GDM in later gestation. Also, kisspeptin's effect on maternal glucose homeostasis may involve alternative pathways other than GSIS, thereby requiring future investigation.

To the best of our knowledge, our study is the first to investigate the effect of maternal obesity and GDM diagnosis on foetal serum kisspeptin levels. It is also the first to investigate the relationships between the kisspeptin gene and protein expression in the placenta and maternal and cord concentrations in GDM (non-obese, obese) and non-GDM (non-obese, obese) women. This analysis revealed no significant correlations between the placental *kisspeptin* gene and maternal and cord kisspeptin concentrations among the four groups, perhaps reflecting that the placenta is not the main source of circulating maternal and cord kisspeptin at term. Meanwhile, placental kisspeptin protein abundance showed a significant negative correlation with the maternal serum kisspeptin concentrations in obese women with and without GDM. These findings may be attributable to the downregulating effect of obesity on kisspeptin production, as supported by studies that have reported an inverse correlation between plasma kisspeptin and BMI.[123, 185] Given that placental kisspeptin gene and protein levels did not vary between groups, the findings may suggest there are post-transcriptional modifications of kisspeptin in the placenta in response to maternal adiposity.

Of note, placental kisspeptin protein abundance and cord serum kisspeptin concentration correlated negatively with maternal BMI at booking in non-obese GDM women. This is in accord with previous studies that have reported an inverse relationship between plasma kisspeptin concentration and BMI.[122, 123, 185] Although Kołodziejski et al reported a negative correlation

between plasma kisspeptin and BMI in both obese and non-obese women.[122] Obesity is an established clinical marker of insulin resistance. It is also known that kiss1r knockout female mice with impaired kisspeptin signalling have decreased metabolism, energy expenditure and increased adiposity compared with wild type counterparts.[180, 296] Taken together, it can therefore be hypothesised that decreased circulatory kisspeptin concentrations in pregnancy may be associated with insulin resistance in women with obesity.

In obese GDM women, placental kisspeptin protein abundance inversely correlated with maternal systolic blood pressure. On the other hand, kisspeptin has been reported to exert a potent vasoconstrictor effect in the human coronary artery and umbilical vein.[128] Our clinical data showed maternal obesity was linked to increased systolic blood pressure. Similarly, Yen et al found a higher systolic blood pressure in overweight/obese women with GDM compared to their non-obese counterparts highlighting obesity, rather than GDM alone, may be the main modulator of maternal systolic blood pressure as well as its association with placental kisspeptin expression.[297] The putative mechanisms by which obesity might cause elevated blood pressure include overactivation of the sympathetic nervous system, stimulation of the renin-angiotensin-aldosterone system, hyperleptinaemia, dyslipidaemia, hyperinsulinaemia, obstructive sleep apnoea and renal dysfunction.[298] While there is an association between higher pre-pregnancy BMI, chronic hypertension and elevated risk of preeclampsia, obese participants in our study had no history of previous preeclampsia nor manifested with preeclampsia during pregnancy.[31, 299, 300] However, preeclampsia was an exclusion from participation in our study. Hence, future work is required to determine the effect of kisspeptin administration on blood pressure in obese GDM pregnant females in an animal model.

For the first time, this study has shown that placental kisspeptin protein abundance has a positive correlation with infant ponderal index in GDM obese women. In addition, GDM and obesity resulted in a higher infant ponderal index, suggesting that GDM and obesity may be inter-dependently associated with elaborated expression of placental factors such as kisspeptin, which may have a potential modulating effect on foetal adiposity. Furthermore, our placental morphological data showed that in obesity, GDM increased intervillous space volume, the spaces between chorionic villi that are bathed with maternal blood, which may contribute to increased nutrient transfer to the foetus leading to greater ponderal index. The ponderal index is a measure of adiposity and is calculated as weight (kg) divided by cubed height (m^3).[301] In GDM, when trying to correct glucose intolerance using metformin or insulin, the developing foetus may still receive more lipids and glucose, resulting in excess fat deposition, even when the birth weight is not affected due to increased expression of nutrient transporters.[302] Infant adiposity at birth is widely recognised as a significant predictor of obesity in childhood and metabolic syndrome later in life.[303-305] Although the ponderal index was used to measure infant adiposity in our study due to its high sensitivity, specificity, positive and negative predictive values, its accuracy and reproducibility are inconsistent. More accurate measures of adiposity, such as dual-energy x-ray absorptiometry or air displacement plethysmography (PEAPOD), would therefore be valuable for examining neonatal adiposity with the measured placental kisspeptin expression in their respective mothers.[306, 307] However, this would be challenging as it requires access to sophisticated and expensive equipment which may be available in research units but not in routine clinical services, such as those in Cape Town.

Maternal serum kisspeptin concentrations correlated positively with the placenta weight but negatively with the volume of intervillous space and villous syncytiotrophoblasts in obese non-

GDM women. Our study further showed a negative correlation between cord serum kisspeptin concentration and placental specific diffusing capacity in obese GDM women. These data suggest that kisspeptin may negatively affect placental perfusion, surface area and/or diffusion gradients for maternal-foetal exchange in specific groups of women. Cetkovic et al showed that placental dysfunction is significantly associated with decreased serum kisspeptin concentrations in the third trimester and may explain the negative relationship kisspeptin has with placental intervillous space and villous syncytiotrophoblasts.[162] Overall, these findings may suggest the role of obesity and GDM in regulating placental factors, like kisspeptin, with consequences for the placental structure and functional capacity.

Following kisspeptin-KISS1R binding, downstream intracellular signalling events ensue with the coupling of the $_{Gq}$ subunit, activation of the phospholipase C pathway and stimulation of β-arrestin for the exertion of kisspeptin's biological functions.[126, 130-132] We found that women with obesity had a lower placental expression of the *KISS1R* gene, which would be expected to reduce kisspeptin signalling capacity consistent with a previous study which showed that kiss1r knockout resulted in decreased physical activity and energy utilisation, both linked to the development of obesity.[180] Hence, there may be a relationship between altered kisspeptin-KISS1R signalling and obesity. The KISS1R protein expression in the placenta did not differ significantly with GDM or obesity status; however, KISS1R positive immunoreactivity was greatest in the villous syncytiotrophoblast compared to the stroma and foetal capillaries in all groups, consistent with the current evidence that KISS1R is mostly expressed in villous syncytiotrophoblast.[130, 145] Contrariwise, Kapustin et al recently reported a higher placental kisspeptin protein but equal KISS1R protein expression in GDM compared to non-GDM, but the impact of obesity was not examined.[167]

4.3 Proinflammatory Cytokine Signalling

During pregnancy, TNFα and IL-6 are predominantly expressed in the placenta and adipose tissues and have been demonstrated to be potent stimulators of inflammatory activities leading to insulin resistance.[308] Maternal obesity is associated with low-grade inflammation in the placenta and adipose tissue leading to the expression of pro-inflammatory cytokines (TNFα, IL-6).[309] Our study, however, found no significant effect of maternal obesity and GDM diagnosis on the expression of *TNFα*, *IL-6*, and *IL-1β* genes by the placenta. The placental *TNFα* and *IL-6* genes expression data are in keeping with the findings of Kleiblova and Allbrand et al, but not with the findings of Henig and Stirm.[243, 310-312] Heinig found a higher expression of the *TNFα* gene by the placenta in GDM than non-GDM women, while Stirm, Mrizak and Roberts et al found increased placental *IL-6* gene expression in GDM compared to healthy controls.[286, 310, 311, 313] Mrizak et al recruited GDM women who had macrosomic babies and healthy age-matched women who delivered normal-weight babies, suggesting the GDM women selected were already at high risk for enhanced placental inflammation, which possibly led to the difference in placental *IL-6* gene expression.[313] Other work by Kleiblova et al showed increased *IL-6* gene expression by maternal subcutaneous fat in GDM women compared to normal glucose tolerant women.[243, 312] The major reasons for the variations between our placental findings and those of other studies may be differences in GDM screening methods, mode of delivery, maternal morbidity selection, and foetal sex. In the study by Heinig et al, 50 g OGTT was utilised for GDM screening and may influence the glycaemic targets for diagnosis and perhaps placental and clinical outcomes.[311] Additionally, over 80% of GDM women in Heinig's study had a vaginal delivery, which is associated with increased placental and circulating cytokines, particularly TNFα, IL-6 and IL-1β,[314-316] while in our study, about 27% and 52% of GDM non-obese and GDM obese women respectively had a vaginal delivery.[311] Barke et al demonstrated that placental sex significantly influenced placental inflammatory gene expression

in women with GDM,[317] and although all four groups in our study contained both sexes, we did not have sufficient power to segregate the data by sex, and there was a higher proportion of female babies among the non-GDM groups.

While maternal obesity and GDM did not impact *TNFα* gene expression by the placenta, placental TNFα protein abundance determined by western blotting, and maternal serum TNFα levels determined by ELISA were significantly influenced by GDM status (there was no effect of obesity). At the same time, GDM and BMI had no effect on placental IL-6 protein abundance. Hara Cde et al found no difference in serum TNFα levels but increased IL-6 levels in GDM compared to non-GDM placental homogenates using flow cytometry.[240] While using an ELISA technique, Zhang et al found higher levels of TNFα and IL-6 in placenta lysates of GDM compared to non-GDM women.[239] However, in contrast to our study, some studies did not find any significant difference in circulatory TNFα concentrations between GDM and non-GDM women. A systematic review published in 2013, which involved 1982 participants from 22 studies, reported no difference in circulating TNFα concentrations in GDM compared to non-GDM.[318] However, most studies were excluded because of significant differences in selection criteria, sampling and assay methods and reporting of results.[318] Recently, Zhang et al also did not find any difference in TNFα concentrations in GDM and non-GDM pregnancies with prepregnancy BMI of 38.70 kg/m^2 and 28.70 kg/m^{2,} respectively.[239] Furthermore, Vega-Sanchez et al found no correlation between maternal adiposity and maternal and foetal plasma TNFα concentrations.[75] Meanwhile, Noureldeen and Wei et al found significantly higher TNFα circulating concentrations in GDM than in non-GDM pregnancies.[319, 320] Furthermore, Kirwan and colleagues found significantly higher maternal serum TNF-α concentrations in obese GDM compared to lean non-GDM women.[90] Another systematic review in 2014 reported higher circulating TNF-α levels in GDM

compared to their BMI-matched controls.[321] The latter studies indicated higher placental and circulatory TNFα expression in relation to GDM, suggesting that GDM rather than maternal obesity influences placental inflammation, as supported by our findings. It is not entirely clear what the cause of the discrepancies seen in the study findings, where there was a significant effect of GDM on placental TNFα protein expression in non-obese women and on circulating TNFα levels in both non-obese and obese GDM women, while immunohistochemistry analysis showing no difference in TNFα staining in any of the placental structures analysed, I, however, propose the possible influence of differing sensitivities and interferences of the antibodies used in the different techniques.

Although TNFα is described as the most potent predictor of insulin resistance and contributor to exaggerated inflammatory responses during pregnancy,[90] as previously mentioned, published studies show conflicting results on the significance of TNFα in the presence of GDM. Placental inflammation is influenced by many factors in pregnancy, such as maternal obesity, duration of labour, vaginal delivery and metformin use, which may explain the variability reported in the available literature and our study cohort's altered placental and maternal serum cytokine expression. For example, in our study, all GDM women received metformin alone or in combination with insulin to control their hyperglycaemia. Metformin is known to reduce inflammatory cytokines.[322] A study by Tizazu et al reported metformin monotherapy or metformin-gliclazide combination treatment reduced circulating TNFα levels when compared to glipizide monotherapy in diabetic patients.[322] Additionally, Hyun et al demonstrated that metformin resulted in downregulation of TNFα expression (mRNA and protein) and secretion by the visceral adipose tissue of obese mice.[323] Similarly, in a randomised control trial involving patients with type 2 diabetes, Mo et al found decreased TNF-α, IL-6 and IL-1β circulatory levels

after a year of treatment with metformin compared with baseline.[324] The possible mechanisms by which metformin downregulates inflammation include activating AMP-activated protein kinase and exerting an antioxidant effect and insulin sensitisation.[325-327] Hence we hypothesise that the downregulating effect of metformin on inflammation may provide a potential explanation for the significantly lower placental and maternal circulating TNFα levels in our GDM women. Placental inflammation, particularly induced by TNFα, has been implicated in GDM pathogenesis through induction of insulin resistance by the increasing free fatty acids, sphingomyelin, stress kinases and activation of inflammatory pathways.[86, 87] Further work is required to understand the links between placental inflammation, insulin sensitivity, GDM and GDM management with metformin.

Although there was no effect of obesity on placental *TNFα* gene expression by qPCR or TNFα protein abundance by western blotting, using immunohistochemistry, overall TNFα protein abundance was greater in obese than non-obese women, and this effect was most pronounced in those diagnosed with GDM. By performing immune-reactive (IR) score analysis, this increase in obese women was found to be localised to the syncytiotrophoblast, consistent with what has been reported in the literature.[328, 329] This finding also aligns with previous studies where Oliva, Basu and Challier et al observed increased TNFα protein expression by the placenta in women with obesity.[330-332] Other work by Hotamisligil et al has shown that TNFα protein levels are increased in adipose tissue of obese individuals.[73] Overall, our findings and that of others may be explained by the positive relationships that exist between increased adiposity, TNFα production and insulin resistance in general, as well as the correlation between TNFα levels and adiposity in pregnancy.[333]

By using immunohistochemistry, our work identified an interaction between BMI and GDM diagnosis on the expression of IL-6 protein by the placenta and that villous stroma and foetal

capillaries from women with obesity exhibited significantly lower expression of IL-6 protein. The reduction in villous stromal IL-6 protein abundance was most pronounced in obese compared to non-obese women with GDM. Neither GDM nor BMI or their interaction influenced maternal and cord IL-6 levels. Hassiakos et al reported serum IL-6 levels at 11–14 weeks of gestation were higher in GDM compared to normal pregnancies even after correcting for maternal obesity, suggesting that as the placenta is bathed in maternal blood, changes in placental IL-6 could have implications for maternal circulating levels.[237] Intriguingly, IL-6 has been demonstrated to have both pro-and anti-inflammatory effects depending on the type of receptor it binds to. When IL-6 binds to the classic IL-6 receptor, it produces an anti-inflammatory effect, and a proinflammatory response is present when it binds to the soluble IL-6 receptor.[334] It would be interesting to determine whether maternal obesity and GDM affect the expression of the traditional or the soluble IL-6 receptor by the placenta, as this may inform the mechanisms underlying the augmented expression of IL-6 by the placenta in obese, and especially obese women with GDM. Akin to our findings, Lain and Friis et al reported no significant difference in the IL-6 concentrations in obese and non-obese women at the end of pregnancy.[79, 335] However, our findings are contrary to Zhang, Morisset and Kuzmicki et al who reported higher IL-6 concentrations in GDM than non-GDM women.[78, 239, 336] As discussed earlier, the differences in the study findings may be at least in part ascribed to the anti-inflammatory effect of metformin. Another possible reason for the discrepancies is the gestational age at sample collection, with samples collected at term in our study as opposed to the second trimester in studies by Morisset and Kuzmicki et al. The role of low-grade inflammation on induction of insulin resistance and the influence of proinflammatory cytokines on impaired insulin signalling have been demonstrated. IL-6 exerts pleiotrophic effects and is associated with numerous insulin and glucose homeostasis indices, which may explain the contradictions among studies.[78, 337] Interestingly, IL-6 concentrations were about 4-7 fold higher

in maternal compared to cord serum, respectively, in GDM and non-GDM women suggesting placental inflammation may not always correlate with a foetal inflammatory response.

Notably, this study appears to be the first to investigate the relationships between proinflammatory cytokine gene and protein expression in the placenta and their respective maternal and cord concentrations in GDM and non-GDM women with or without obesity. We hypothesise that the lack of relationship between placental *TNFα* and *IL-6* genes and their respective protein expression and circulatory concentrations in the four groups of women may result from increased post-transcriptional modifications of the placental genes. Furthermore, in non-obese GDM women, there was a significant positive correlation between placental IL-6 protein abundance and cord serum IL-6 concentrations, suggesting a link between placental inflammation and the offspring's systemic inflammatory state. In addition, it possible that the increased cord blood IL-6 concentration is more likely a result of the birth process from foetal stress and hypoxia, however, there was no difference in the APGAR scores of infants in women in the four study groups. This may be supported by the demonstrated association between maternal obesity and GDM status and increased cardiovascular disease, obesity, and type 2 diabetes in the offspring.[28]

Vascular inflammation through endothelial dysfunction is implicated as one of the mechanisms underpinning the aetiopathogenesis of hypertension and cardiometabolic diseases.[338] Studies have reported increased levels of plasma TNFα in hypertension and an association between TNFα concentrations and blood pressure.[339, 340] Notably, this study found a positive correlation between placental *TNFα* gene expression and maternal diastolic blood pressure in obese non-GDM women. Conversely, there was a negative correlation between placental *TNFα* gene expression and placental diffusion capacity in non-obese GDM, just as TNFα protein abundance negatively

correlated with placenta weight and surface areas of maternal blood space and foetal capillary in non-GDM non-obese women. These findings suggest that chronic placental inflammation may result in defective placental development and function by virtue of compromised maternal-foetal diffusion capacity, independent of GDM or BMI. Favourable pregnancy outcome is dependent on adequate placenta growth and development to ensure sufficient oxygen and nutrient delivery from the mother to the developing foetus.[341] Maternal obesity is characterised by heightened inflammation and oxidative stress during pregnancy and thus may negatively influence placental development and function, supporting our findings.[342] Placental dysfunction is associated with preeclampsia and alterations in foetal growth, which is negatively impacted by the inflammatory state.[343]

The current study found that placental *IL-6* gene expression was significantly positively correlated with 2-hour glucose post-OGTT and infant ponderal index in obese non-GDM, suggesting that maternal obesity, an inflammatory state, may be associated with glucose intolerance and foetal adiposity. Interestingly, available data shows that increased IL-6 stimulates increased amino acid, free fatty acids and folate transport in the placenta via STAT3 activation leading to foetal overgrowth and increased fat deposition.[344] Furthermore, from our results, it is possible that maternal hyperglycaemia may be mediating changes in placenta IL-6 expression. On the other hand, Guillemette and Braga et al reported a positive correlation between plasma TNFα and 2 hr glucose post-OGTT and placenta weight, just as Akcan et al found no correlation between TNFα levels and infant ponderal index.[345-347] Furthermore, plasma IL-6 concentrations have previously been found to correlate positively with 2hr glucose post-OGTT.[345] Placental IL-6 gene expression was inversely correlated with maternal percentage weight gain and volume of intermediate villi in non-obese non-GDM and GDM women. Our findings imply that the IL-6 pathway may be

necessary for regulating weight gain, as well as placental development and function in non-obese pregnancies. In a systematic review, Patsalos et al showed an increase in weight following IL-6 pathway inhibition.[348] Further, proinflammatory cytokines (TNFα and IL-6) are connected with weight loss and satiety via their cachectogenic and anorexigenic effects.[349-351] Similar to the placental *TNFα* gene, placental IL-6 protein abundance had a significant positive correlation with diastolic blood pressure in obese GDM, suggesting that proinflammatory cytokines may have a role in the pathophysiology of hypertension in obese women with or without GDM. IL-6, produced by endothelial cells, macrophages and smooth muscle cells, correlated with the rise in blood pressure and is an independent risk factor for hypertension induced by angiotensin II.[352] However, our study has a limitation in that we did not measure receptors for the cytokines measured (particularly TNFα and IL-6). This is important considering the indispensability of cytokine ligand-receptor signalling for their biological function. Therefore, future investigation of placental TNFα and IL-6 expression and their respective receptors in relation to blood pressure, glycaemia, gestational weight gain and placental morphology would be of interest.

Maternal and cord serum TNFα concentrations correlated negatively with the foeto-placental weight ratio in obese non-GDM women, as well as placenta weight and volume of intervillous spaces. In obese GDM women, maternal serum TNFα concentrations correlated negatively with infant ponderal index, while cord serum TNFα concentration correlated positively with placenta weight and volume of intermediate villi. Furthermore, TNFα concentrations were positively correlated with maternal systolic blood pressure, while cord TNFα concentrations inversely correlated with maternal diastolic blood pressure in obese GDM women.[353] Proinflammatory cytokines have been shown to mediate endothelial dysfunction and renin-angiotensin-aldosterone system activation, which characterise the pathogenesis of hypertension.[298, 354] Thus, a future area

of study may be the assessment of endothelial dysfunction and renin-angiotensin angiotensin-aldosterone system in pregnancy in relation to proinflammatory cytokines in pregnancy. Also, maternal serum IL-6 concentrations correlated negatively with placental diffusion capacity (theoretical and specific) in obese non-GDM. These data confirm our finding that maternal obesity lowers surface areas and physiological gradients for nutrient and oxygen transfer from the mother to the developing foetus. Meanwhile, in non-GDM non-obese and GDM obese women, respectively, cord serum IL-6 concentration showed a positive correlation with 2-hour glucose post-OGTT and intermediate villi, and intervillous space volumes suggesting that in women with obesity and GDM, foetal inflammation may be associated with maternal glycaemia and reduced nutrient transfer. Notably, this is the first study to explore the relationship between serum TNFα and IL-6 concentrations and placental morphology.

4.4 Placental Hormones, Growth Factors and Steroidogenic Enzyme Genes

Our study showed a lack of significant effect of obesity or GDM diagnosis on placental *GHV*, *hPL1*, *hPL2* gene expression. These data are consistent with previous findings, which found no difference in placental *GHV* or *hPL* gene expression between GDM and non-GDM women.[213-216, 222, 355] However, overall, placentae from the obese GDM women had the highest expression of these particular hormones. The GH/PL family of hormones have been shown to be associated with the normal acquisition of insulin resistance in the mother during pregnancy; however, the process may be perpetuated by obesity leading to glucose intolerance and GDM.[47, 48] In contrast, Jin et al in 2018 reported a higher *hPL1* and *GHV* mRNA in insulin-treated obese GDM women, but lower *hPL1* and *GHV* mRNA expression in obese women alone.[211] In the study by Jin et al, women were categorised into three groups, namely lean (BMI: 20–25 kg/m^2), obese (BMI: 35 kg/m^2), and obese GDM (BMI: 35 kg/m^2 and GDM diagnosis) as opposed to our cohort of women where obesity was

defined as BMI ≥ 30 kg/m^2 (according to the WHO classification) and non-obese as ≤ 30 kg/m^2. The differences in maternal obesity classification may explain the disparity between our and Jin et al's findings.

There was an overall significant reduction in *Leptin* gene expression by the placenta in women with obesity. This effect was more pronounced in women not diagnosed with GDM, implying that maternal obesity had more impact on placental endocrine function than GDM status. Also, the low placental *Leptin* gene expression may explain increased ponderal index of the infant of our obese women in keeping with findings of the study by Lepercq et al.[356] However, there are conflicting data on whether placental *Leptin* expression is altered in obesity with and without GDM. Tsiotra et al in 2018 found no significant difference in placental *Leptin* mRNA levels in obese and non-obese women with and without GDM.[244] Similarly, Allbrand et al in 2015 found no significant difference in placental *Leptin* gene expression of obese and non-obese women.[312] On the other hand, Tsiotra et al demonstrated higher visceral adipose tissue *Leptin* mRNA expression and plasma leptin concentrations in obese GDM and normoglycaemic women compared to their non-obese GDM and normoglycaemic counterparts.[244] In addition, Mrizak and Lepercq reported a higher placental *Leptin* mRNA in GDM and type 1 diabetes mellitus compared to controls, although the BMI status of the study population is unknown.[313, 357]

Obesity, insulin resistance and diabetes arise in ob/ob or db/db mice with homozygous mutations in the leptin (LEP/OB) or leptin receptor (OB-R).[358, 359] Whether our obese groups of women have ab initio been leptin deficient is however unknown. Furthermore, elevated leptin levels, typically seen in the circulation of obese individuals, may result in a down-regulation of the placental *Leptin* gene expression in gestation, as leptin is purported to act in negative feedback with the

hypothalamus to inhibit food intake and regulate energy homeostasis and adiposity.[360] In future work, it will be important to measure leptin receptor expression by the placenta in our South African pregnant women.

Once again, data in the literature in relation to the impact of GDM and obesity on placental *VEGF* and *IGF2* gene expression are inconsistent. Our findings of no significant effect of obesity or GDM diagnosis on *IGF2* or *VEGF* gene expression by the placenta of the study cohorts are consistent, at least for the latter, with findings by an Australian study.[201] Lappas et al demonstrated similar placental *VEGF-A* gene expression and circulatory VEGF levels in GDM and non-GDM with or without obesity.[201] In contrast, Akarsu and Meng et al reported decreased *VEGF-A* mRNA and protein expression and higher syncytiotrophoblast and stromal VEGF immunoreactivity in women with GDM than in a control group.[199, 200] Similarly, Su et al reported significantly higher placental *IGF2* gene expression and cord IGF2 levels in GDM compared to normoglycaemic women.[207] Our study findings suggest obesity and/or GDM may have little influence on placental angiogenesis and/or IGF2 mediated trophoblast proliferation, which may be consistent with a lack of an effect on the volume of foetal capillaries as determined by stereology.

There was again no effect of obesity or GDM diagnosis on the expression of select steroidogenic enzyme genes in the placenta, suggesting similar placental production of sex steroid hormones, namely oestradiol and progesterone, in our study cohorts. These results are congruent with those of Maliqueo et al who showed no differences in the expression of *CYP11A1*, *HSD3B1* and *CYP19A1* genes between obese and control pregnant women.[361] Neither did Li et al find an association between plasma progesterone and GDM risk,[227] however, Couch and Montelongo et al reported that circulating progesterone and oestradiol are associated with increased risk of

GDM.[233, 234] Meanwhile, Lassance et al further demonstrated lower plasma oestradiol and progesterone concentrations in obese compared to lean pregnant women.[362] On the whole foetal sex has been shown to influence changes in placental gene expression, including that of steroidogenic hormones. Maliqueo et al demonstrated that obese women with female foetuses had higher placental *HSD3B1* mRNA while the obese women with male foetuses showed lower placental *CYP19A1* expression to control women.[361] Thus, increasing sample size and splitting data by infant sex would be essential in future work to determine its influence on the placental endocrine signal expression.

4.5 Strengths

This study has numerous strengths. Firstly, to the best of our knowledge, this is the first study to investigate the effect of maternal obesity and GDM on the physiological changes within the placenta (assessing the expression of multiple genes and proteins, as well as the detailed morphology of the exchange interface) and maternal and cord blood in an African population. Second, this is also the first study to demonstrate the presence of kisspeptin protein in STBEV and determine whether its abundance in GDM and non-GDM pregnancies may be altered. Thirdly, we collected information on maternal and neonatal clinical and metabolic characteristics and correlated these with the placental and circulatory parameters assessed to understand better the physiological changes seen in GDM and non-GDM women with or without obesity. In summary, this study provides a unique intersection between clinical and basic science perturbations with the potential to advance our understanding of the mechanisms underpinning GDM and foetal programming.

4.6 Limitations

Our study has some limitations. First, the sample sizes within the study sub-groups were relatively small with possible low power, which could have resulted in some near statistically significant observations. Second, as a result of the relatively small sample size, it was not possible to examine whether there may be a sex-specific effect on placental gene and protein expression along with associated morphological changes. Third, near statistical or statistically significant correlations may have occurred due to type I or II error. Finally, in our study, participants were categorised into obese and non-obese (including overweight women rather than only normal weight in the latter), which could have accounted for the variability in our findings. Additionally, variability in our data may have resulted from the different modes of delivery, impact of metformin treatment given to all GDM women and samples collection at term. The placenta at term undergoes senescence with decreased function that may result in the attenuation of potential differences in gene expression, protein expression and placental hormones in the blood which may have been present earlier in gestation.[295]

4.7 Future Research

There are some considerations for future research. The first relates to kisspeptin signalling. Tolson et al demonstrated kisspeptin signalling in the placenta was impaired in a sexually dimorphic fashion in animal studies.[180] Thus, a key area for future work will be to assess if sex is a factor modulating kisspeptin signalling in human placental samples and the mechanisms by which this may occur. Our study did not examine the relationship between plasma kisspeptin and indices for insulin secretion as Bowe et al reported[120] hence, this will be an important body of work in our future research. The downregulation of GSIS has been demonstrated in pancreatic beta cell kiss1r knock-out female mice; however, the effect of KISS1R disruption in humans is unknown.[120, 180] It

will also be interesting to investigate the mechanisms underpinning GSIS via the KISS1R modulation through ex vivo studies on placentae of our cohort. Kisspeptin has an incretin effect, and with our finding of reduced kisspeptin protein expression in STBEV of GDM women, in the context of a previous study in the same population which showed increased activity of DPPIV enzyme, encourages future work on examining the relationship between kisspeptin and DPPIV enzyme in STBEV.

Secondly, regarding proinflammatory markers, our study showed a significant relationship between proinflammatory cytokines (TNFα and IL-6) and blood pressure in women with obesity, but the mechanisms underlying this are unknown. Future work will therefore benefit from investigating the role of the sympathetic nervous system, renin-angiotensin-aldosterone system, hyperleptinaemia, dyslipidaemia, hyperinsulinaemia, and renal dysfunction in blood pressure regulation in women with pregestational obesity. We found there was altered *Leptin* gene expression in obese women, but leptin receptor expression is unknown and should be studied in future work. With the postulated effect of metformin treatment on the placental and circulatory proinflammatory cytokines in our study, further well-structured and powered research to investigate the effect of metformin on these cytokines will be undoubtedly required.

4.8 Summary

This PhD thesis has contributed significantly to the body of knowledge by demonstrating that neither maternal obesity nor GDM influences placental and circulatory kisspeptin levels, but that maternal obesity and not GDM status lowers the expression of the *KISS1R* gene, suggesting the role of obesity in altering kisspeptin-KISS1R signalling in a sample of well-matched pregnant women. The thesis has demonstrated that kisspeptin protein expression is present in the placenta-

derived STBEV and that increased placental kisspeptin gene expression is associated with decreased volume of villous syncytiotrophoblasts and theoretical diffusion capacity in non-obese women, irrespective of their GDM status. Our study further shows that placental kisspeptin protein has an inverse relationship with maternal BMI and systolic blood pressure in GDM women irrespective of obesity status. In GDM women, obesity resulted in greater localisation of TNFα protein in the syncytiotrophoblast than in the stroma and foetal capillaries. Moreover, our work shows the expression of IL-6 protein immunostaining by the placenta was influenced by the interaction between BMI and GDM diagnosis, but with the revelation that obese women tended to have lower expression of IL-6 by the stroma. Notably, this study shows a positive correlation between placental *TNFα* gene expression and IL-6 protein and maternal diastolic blood pressure, consistent with the role of inflammation and insulin resistance in the pathophysiology of hypertension. Hence, TNFα and IL-6 could be surrogate markers of diastolic hypertension in GDM women. Furthermore, in maternal obesity alone, maternal serum TNFα and IL-6 concentrations inversely correlated with placenta weight, foeto-placental ratio, volume of intervillous spaces, and theoretical and/or specific diffusion capacity. Maternal obesity seems to compromise placental maturation and affects the surface areas and diffusion gradients required for maternal-foetal nutrient and oxygen exchanges with potential clinical implications for the mother and developing foetus. In addition, maternal obesity influences *Leptin* gene expression by the placenta in women with normal glucose tolerance suggesting placental endocrine dysfunction may occur in women with obesity. Below is the schematic diagram summarising key findings and connotations for future research (Figure 4.1).

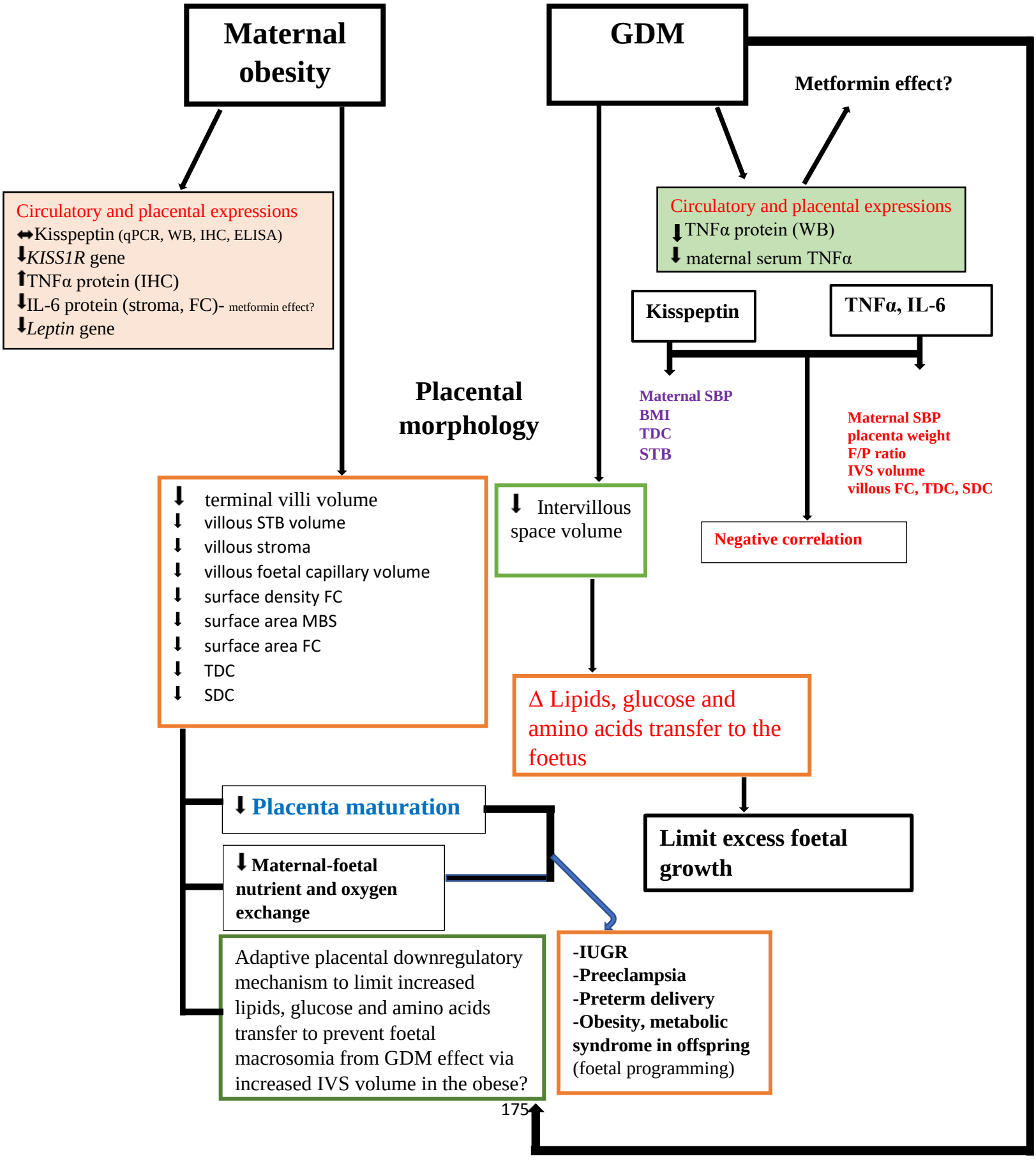

Maternal obesity
GDM
Metformin effect?
Circulatory and placental expressions
↔Kisspeptin (qPCR, WB, IHC, ELISA)
⬇*KISS1R* gene
⬆TNFα protein (IHC)
⬇IL-6 protein (stroma, FC)- metformin effect?
⬇*Leptin* gene
Circulatory and placental expressions
⬇TNFα protein (WB)
⬇ maternal serum TNFα
Kisspeptin
TNFα, IL-6
Maternal SBP
BMI
TDC
STB
Maternal SBP
placenta weight
F/P ratio
IVS volume
villous FC, TDC, SDC
Negative correlation
Placental morphology
⬇ terminal villi volume
⬇ villous STB volume
⬇ villous stroma
⬇ villous foetal capillary volume
⬇ surface density FC
⬇ surface area MBS
⬇ surface area FC
⬇ TDC
⬇ SDC
⬇ Intervillous space volume
Δ Lipids, glucose and amino acids transfer to the foetus
⬇ Placenta maturation
⬇ Maternal-foetal nutrient and oxygen exchange
Limit excess foetal growth
Adaptive placental downregulatory mechanism to limit increased lipids, glucose and amino acids transfer to prevent foetal macrosomia from GDM effect via increased IVS volume in the obese?
-IUGR
-Preeclampsia
-Preterm delivery
-Obesity, metabolic syndrome in offspring
(foetal programming)

Figure 4.1. Summary of key study findings: Abbreviations. KISS1R: kisspeptin 1 receptor, TNFα: tumour necrosis factor alpha, IL-6: interleukin 6, qPCR: quantitative polymerase chain reaction, WB: western blotting, IHC: immunohistochemistry, ELISA: enzyme-linked immunosorbent assay, FC: foetal capillaries, IVS: intervillous space, MBS: maternal blood space, STB: syncytiotrophoblast, TDC: theoretical diffusion capacity, SDC: specific diffusion capacity, IUGR: intrauterine growth restriction, BMI: body mass index, SBP: systolic blood pressure.

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Appendix 1

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room E53-46 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 406 6492
Email: sumayah.ariefdien@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/humanethics/forms

19 July 2018

HREC REF: 463/2018

Prof N Levitt
Chronic Disease Initiative for Africa
J47, J-Floor
OMB

Dear Prof Levitt

PROJECT TITLE: INTERPLAY BETWEEN PLACENTAL KISSPEPTIN AND INFLAMMATORY MARKERS, TNF-a AND IL-6, IN GESTATIONAL DIABETES MELLITUS-PhD-candidate Dr E Musa)

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee (HREC) for review.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

Approval is granted for one year until the 30 July 2019.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

We acknowledge that the student: Dr E Musa will also be involved in this study.

Please quote the HREC REF in all your correspondence.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please note that for all studies approved by the HREC, the principal investigator **must** obtain appropriate institutional approval, where necessary, before the research may occur.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE

Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938

This serves to confirm that the University of Cape Town Human Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Convention on Harmonisation Good Clinical Practice (ICH GCP), South African Good Clinical Practice Guidelines (DoH 2006), based on the Association of the British Pharmaceutical Industry Guidelines (ABPI), and Declaration of Helsinki (2013) guidelines.
The Human Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.

Appendix 2

Participants Information

Studies 1 to 4

Gestational Diabetes Mellitus: Dissecting Placental Kisspeptin Expression and its Interplay with Markers of Inflammation, Pancreatic Beta Cell Secretory Function and Insulin Resistance

Reference Number: HREC: 463/2018

Principal Investigator: Dr Ezekiel Musa

Address: Division of Endocrinology and Diabetic Medicine, Department of Medicine, and Department of Obstetrics and Gynaecology, University of Cape Town, South Africa
Contact Number and Email Address: 0783622912, 0216502034, ezemusa2000@gmail.com

Introduction

You are invited to participate in this research project. Take some time to read this information leaflet given to you as it will give you details of this study. You may feel free to ask the research doctor or assistant any questions about the research that you do not fully understand. It is important you understand clearly what this study involves and how you could be part of it. Taking part in this research project is voluntary and you may withdraw at any stage. If you do not agree to take part, it will not affect the care you receive.

Ethical Approval

This study has been approved by the Health Research Ethics Committee at the University of Cape Town and we will follow ethical guidelines and principles of the international declaration of the Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council Ethical Guidelines for Research.

What is the Purpose of the Study

This study aims to study factors that are made by the placenta to see whether they are different in people with gestational diabetes- that is diabetes that is first found during pregnancy. The placenta makes many factors that are important for a healthy pregnancy, but sometimes the

placenta makes too much or too little of these factors. This may lead to problems in pregnancy and is why we want to measure kisspeptin, insulin and some factors that are associated with inflammation.

What Procedures to be Performed

We will take a blood sample from a vein in your arm at delivery and a portion of your placenta will be collected immediately after delivery for laboratory tests. We would keep blood and placenta samples for future research in gestational diabetes. This research has been approved by the University Ethics Committee

Discomfort and Inconvenience Encounter During the Study Procedures

Blood collection may cause a bruise at the puncture site, vein swelling, bleeding or infection. These risks are highly unlikely if blood is collected by experienced personnel and only mild discomfort may happen. In the event of any incidental study-related injury, you will receive adequate treatment.

Benefits of Participating in this Study

You will not benefit directly from taking part. If you have gestational diabetes you will be offered treatment if hyperglycaemia persists after delivery as part of usual care.

Financial Arrangements

You will not receive payment to participate in this study.

Confidentiality

All information that will be highly confidential during the period of the study. Data collected will be published in scientific journals, but your identity will not be included. The University of Cape Town's Research Ethics Committee may be able to audit the study records if the need arises.

Contact Information

Kindly contact the investigator if you have any question or medical complaint using the telephone number: 0216502034. If you have questions which you do not feel you can discuss with the

research staff, please contact the University Ethics Research Committee: Professor Marc Blockman 021 4066492

Consent Form

I …… agree to participate in this research study entitled

Gestational Disease Mellitus: Dissecting Placental Kisspeptin Expression and its Interplay with Markers of Inflammation, Pancreatic Beta Cell Secretory Function and Insulin Resistance.

I have read, and I understand the provided information and have had the opportunity to ask questions. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving a reason. I understand that I will be given a copy of this consent form. I voluntarily agree to take part in this study.

I agree to have my samples stored for future research YES/NO

Participant's name: ……..(print)

Participant's signature: ……..... Date: ……..

Principal investigator's name: ……(print)

Principal investigator's signature: ……. Date: ……..

Witness's name: …….....(print)

Witness's signature: ……..... …….Date: ……..

Appendix 3

Studies 1 to 4 Questionnaire

Serial number: ……

Hospital number: ……

Date of interview: ……...

Data on socio-demography:

Date of Birth	Year	Month	Day		
Age (years)					
Address					
Contact number	Home Telephone		Mobile		
Ethnicity	Black	White	Coloured	Indians/Asians	Others
Occupation	Formal employment	Unemployed	Student	Informal employment	Other
Level of Education	Tertiary	High school	Primary	Informal	None

#Tick appropriate box

Clinical evaluation:

Past Medical History	GDM	Preeclampsia	Foetal macrosomia	Still births/Miscarriages (specify)	Premature delivery
First Degree Family History	Diabetes		Hypertension		

Gestational Age of Diagnosis of GDM (weeks)			
Results of OGTT (mmol/L)	0 hour	1 hour	2 hours
Medical therapy during pregnancy	Metformin alone	Insulin alone	Metformin and insulin
Mean fasting glucose in the last trimester (GDM ONLY) (mmol/L)			

#Tick appropriate box

Clinical measurements

Maternal weight (kg) At booking		
Maternal height (m)		
BMI (kg/m^2) At booking		
Maternal weight at delivery (kg)		
Blood pressure (mmHg)	Systolic blood pressure	Diastolic blood pressure

Pregnancy outcomes

Foetal outcomes			
Body weight (kg)			
Length (cm)			
APGAR score	0 minute	5 minutes	
Congenital malformation (specify)			
Placental weight (g)			
Maternal outcome			
Mode of delivery	Vaginal delivery	Caesarean section	Instrumental delivery

#Tick appropriate box

Laboratory Investigations:

Serum kisspeptin (ng/ml)	
Serum TNFα (pg/ml)	
Serum IL-6 (pg/ml)	

Tissue molecular studies

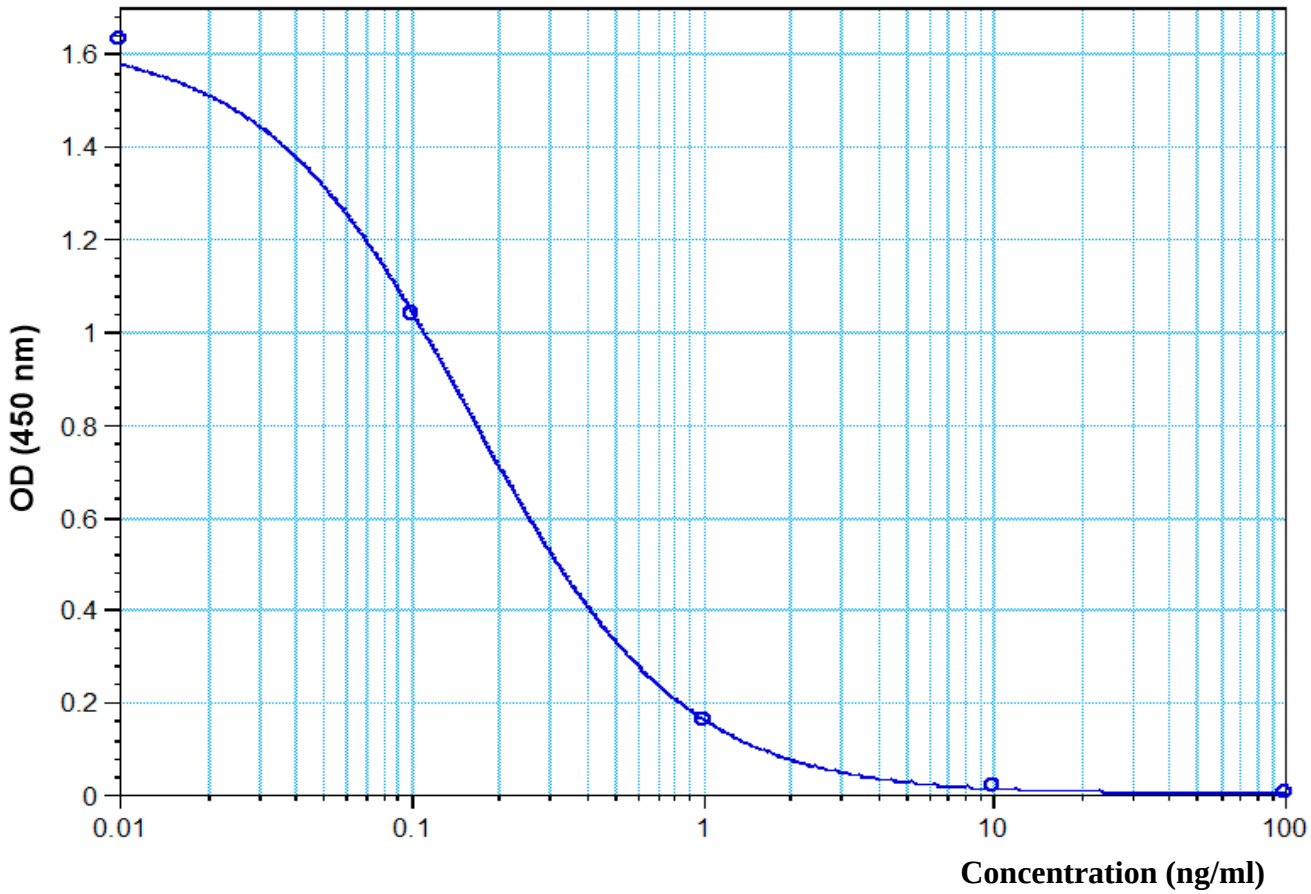
Quantitative RT-PCR	
Western blotting	
Immunohistochemistry	

Appendix 4. The concentration of primary and secondary antibodies used for Western Blotting

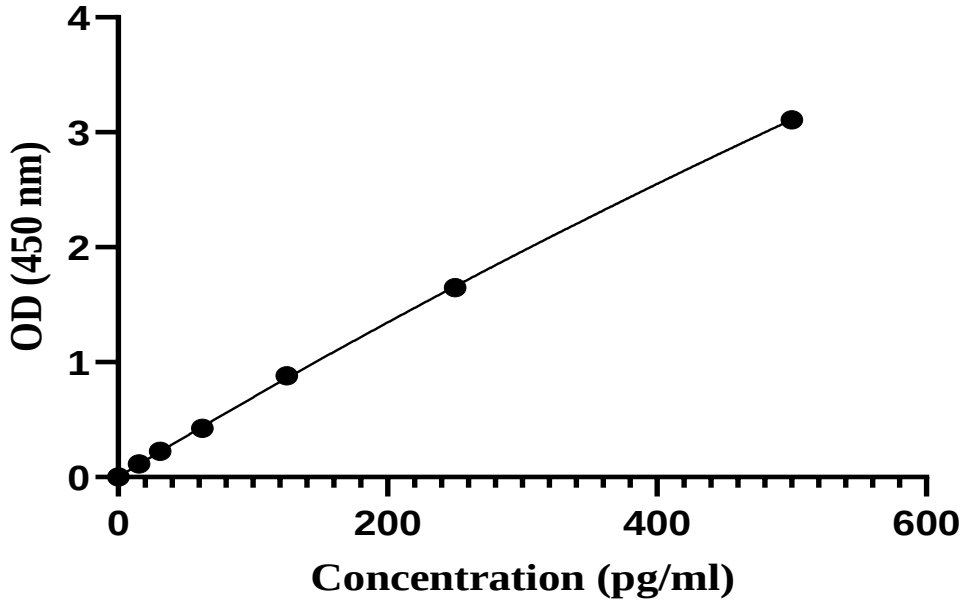
Protein	Primary antibody dilution	Secondary antibody dilution
Kisspeptin	1:500	1:8000 (goat anti-rabbit)
TNFα	1:1000	1:4000 (goat anti-mouse)
IL-6	1:800	1:4000 (goat anti-rabbit)
GAPDH	1:1000	1:4000 (anti-goat rabbit)

Appendix 5. Composition of buffers used for the western blotting

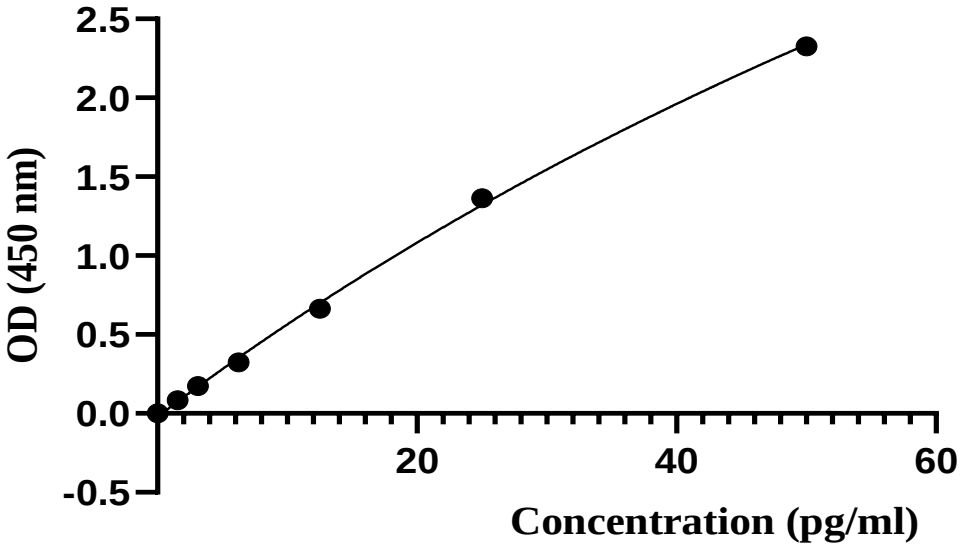
Buffers	Composition
Laemmli 4x reducing Buffer	900 µl of 4X Laemmli buffer. 100 µl of 2-β mercaptoethanol
10xTBS	Add 87.6 g of NaCl and 12.1 g of Tris to 700 of distilled water Dissolve in a bottle with a magnet. Adjust the pH to 8 Top up with filtered water to make 1L.
TBS-T	100 ml from the 10xTBS stock. 900 ml of distilled water 1 ml of Tween-20 5 g of non-fat milk in 100 ml of TBST
5% Milk TBST	
1M Tris solution	Add 121.4 g Tris to 700 ml of distilled water, dissolve it using a magnet. Bring the pH to 9.4 Top-up with water to make 1 L.
Anode 1 buffer	150 mL of Tris 1M pH 9.4. 100 mL of methanol. Top up to 500 ml with distilled water
Anode 2 buffer	12.5 ml of Tris 1M pH 9.4 100 ml of methanol. Top-up to 500 ml with distilled water
Cathode buffer	12.5 ml of Tris 1M pH 9.4 100 ml of methanol. 2.6 g of α-aminohexanoic acid (6- amninocaproic acid)
Stripping	15 g glycine 1 g SDS 10 ml Tween 20 Dissolve in 800 ml distilled water Adjust pH to 2.2 Bring volume up to 1 L with distilled water



Appendix 6. Human kisspeptin ELISA standard curve: This shows a standard curve of known kisspeptin concentrations in ng/ml as a function of optical density (OD) at 450 nm determined using a human KiSS-1 (112-121) amide / Kisspeptin-10 / Metastin (45-54) ELISA. The goodness of fit (straight line) R^2 value for the standard curve was 0.9960.



Appendix 7. Human TNFα ELISA Standard Curve: This shows a standard curve of known TNFα concentrations in pg/ml as a function of optical density (OD) at 450 nm determined using a human TNFα ELISA. The goodness of fit (straight line) R^2 value for the standard curve was 0.9999.



Appendix 8. Human IL-6 ELISA Standard Curve: This shows a standard curve of known IL-6 concentrations in pg/ml as a function of optical density (OD) at 450 nm determined using a human IL-6 ELISA. The goodness of fit (straight line) R^2 value for the standard curve was 0.9989.

Appendix 9

Reproductive Sciences
https://doi.org/10.1007/s43032-020-00437-7

REVIEW

Kisspeptins and Glucose Homeostasis in Pregnancy: Implications for Gestational Diabetes Mellitus—a Review Article

Ezekiel Musa[1] · Mushi Matjila[2] · Naomi S. Levitt[1]

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Abstract
Gestational diabetes mellitus (GDM) is becoming an increasingly common complication of pregnancy with the global rise of obesity. The precise pathophysiological mechanisms underpinning GDM are yet to be fully elucidated. Kisspeptin, a peptide encoded by the *KISS1* gene, is mainly expressed by placental syncytiotrophoblasts during pregnancy. It is an essential ligand for kisspeptin 1 receptor (KISS1R), which is expressed by both the villous and invasive extravillous cytotrophoblast cells. Circulatory kisspeptins rise dramatically in the second and third trimester of pregnancy coinciding with the period of peak insulin resistance. Kisspeptins stimulate glucose-dependent insulin secretion and decreased plasma levels inversely correlate with markers of insulin resistance. Additionally, kisspeptins play a critical role in the regulation of appetite, energy utilisation and glucose homeostasis. GDM pregnancies have been associated with low circulatory kisspeptins, despite higher placental kisspeptin and KISS1R expression. This review evaluates the role of kisspeptin in insulin secretion, resistance and regulation of appetite as well as its implications in GDM.

Keywords Kisspeptin · Glucose homeostasis · Gestational diabetes mellitus · Insulin secretion · Insulin resistance

Introduction

Gestational diabetes mellitus (GDM) complicates up to 17% of pregnancies globally and 1 in 4 pregnancies in South Africa [1, 2]. It is associated with both short- and long-term adverse pregnancy outcomes for the mother and baby [3, 4]. For the mother, GDM is an established risk factor for the future development of type 2 diabetes, cardiovascular disease and metabolic syndrome. As a consequence of placental programming, the potential long-term complications for the fetus are similar [3, 5]. A state of insulin resistance characterizes the later stages of normal pregnancies, thought to be induced by the effects of placental hormones such as progesterone, oestrogen, human placental lactogen and cortisol [6]. GDM, however, develops if there is inadequate pancreatic beta-cell insulin secretory response to overcome the peripheral insulin-resistant state [7, 8].

KISS1, the tumour suppressor gene encodes kisspeptin [9] and has four bioactive derivatives, namely kisspeptin 10, 13, 14 and 54. The initial discovery of kisspeptin involved the suppression of malignant melanoma in athymic nude mice [10]. Beyond tumour suppression, kisspeptins are essential in the regulation of pubertal onset and reproduction, as inactivating mutations of the kisspeptin receptor gene (in both humans and mice) results in idiopathic hypogonadotropic hypogonadism [9, 11–13]. Additionally, kisspeptins play key regulatory roles in embryo implantation, trophoblast invasion and early placental development, all indispensable components of early gestation [14, 15]. More recently, there is growing evidence that kisspeptins are important in glucose homeostasis. Kisspeptins induce glucose-stimulated insulin secretion in the pancreas as well as regulation of insulin sensitivity [16–20].

This narrative review begins with an overview of the biology of kisspeptin. It focusses on kisspeptin's role in pregnancy with particular emphasis on its effect on glucose homeostasis, insulin secretion and resistance, including its potential implications for the pathophysiology of GDM.

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Kisspeptins and KISS1R Signalling

Alternative splicing of the *KISS1* gene results in four bioactive kisspeptins (kisspeptin-10, 13, 14 and 54), products of proteolytic cleavage of the 145 amino acid peptide (kisspeptin-145) (Fig. 1) [21]. The four peptides have a common C-terminal sequence and similar affinity to their receptor, KISS1R. Kisspeptins and KISS1R are both expressed in the brain, pancreas and placenta, as well as other tissues including the adrenal glands, kidneys, adipose tissue, liver, small intestine and coronary arteries [9, 22–25]. Upon ligand-receptor binding, the downstream intracellular signalling events include the coupling of $_{Gq}$ subunit with activation of the phospholipase C pathway and stimulation of β-arrestin, thus mediating essential biological functions related to kisspeptin [14, 21, 26, 27].

Kisspeptin and KISS1R in Pregnancy

Syncytiotrophoblasts are the predominant source of placental *KISS1* mRNA and protein expression and to a lesser degree, the cytotrophoblasts [14, 28, 29]. *KISS1R* mRNA and protein expression occurs in syncytiotrophoblasts, although reports regarding their expression in cytotrophoblasts remain conflicting [14, 28, 30]. The highest placental *KISS1* mRNA expression occurs in the first trimester, suggesting plausible involvement in the control of trophoblast invasion in a period when the depth of invasion is critical. Limited trophoblast invasion is associated with disorders of defective deep implantation such as preeclampsia and intrauterine growth restriction [31–33].

What ultimately regulates placental kisspeptin expression is not entirely clear. However, a ligand-receptor negative feedback mechanism, along with downregulation of *KISS1R* expression in syncytiotrophoblasts, influences this process [34].

Plasma kisspeptin levels markedly rise in the second and third trimester of pregnancy, reaching approximately 7000 times higher concentrations in the latter, compared to the first trimester. This significant surge in circulatory kisspeptin levels in the third trimester is likely the result of greater placental expression with increasing placental mass, as pregnancy advances [35, 36]. Given the placental source of kisspeptin expression, plasma kisspeptin concentrations unsurprisingly fall to pre-pregnancy levels within 5 days post-partum [35].

Normal placentation and successful pregnancies can occur in the absence of a functional maternal kisspeptin-receptor coupling and signalling system. *Kiss1* mutant mice still

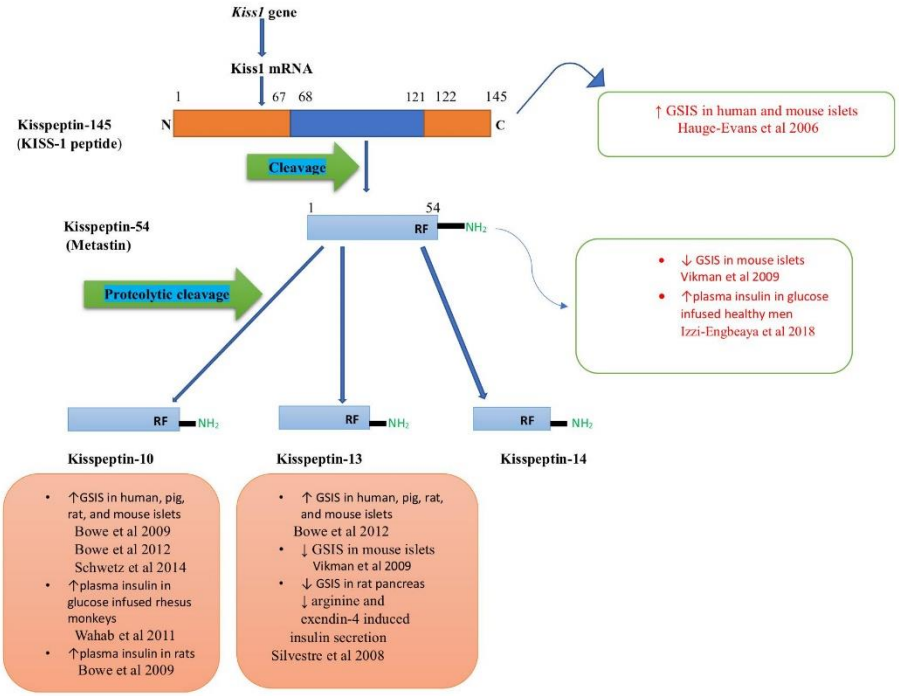

Fig. 1 Kisspeptin bioactive forms and their putative roles on insulin secretion

manifest normal placental structure and function [37], and women with *KISS1R* loss of function mutations have demonstrated successful pregnancies [38]. This highlights the importance of other role-players required for adequate placental function, and more so, maternal genetic aberrations are not always phenotypically expressed in the placenta, as the placental genomic constitution is distinct from that of the mother.

There is a correlation between lower circulatory kisspeptin levels and disorders of defective deep placentation such as miscarriage, intrauterine growth restriction and preeclampsia [33, 39–42]. In preeclampsia, cross-sectional studies report low circulatory kisspeptin levels, despite elevations in placental expression [42–44]. Similarly, in GDM, lower plasma kisspeptin levels and increased placental kisspeptin expression have been described [17, 41, 45].

The demonstration of kisspeptin and KISS1R expression in the pancreas strongly suggests their putative function in the regulation of pancreatic hormonal secretion.

Role of Kisspeptins in Pancreatic Insulin Secretory Function

The initial discovery of the expression of kisspeptin and its receptor in the pancreatic β and α cells led to an exploration of their potential role in insulin secretion [23]. As seen in Table 1 and Fig. 1, data from numerous in vivo and in vitro studies [17, 23, 46, 48] suggest that kisspeptin is important in insulin secretion via glucose dependency and that different kisspeptins may play varying physiological roles in glucose homeostasis. Also, there may be sexual dimorphism in the effect of kisspeptins on glycaemia and metabolism, and that there is a likelihood of kisspeptins involvement in the pathophysiology of GDM.

Glucose Dependency and Effect of Kisspeptins on Insulin Secretion

In in vitro studies, kisspeptins enhanced glucose-stimulated insulin secretion (GSIS) in both human and animal pancreatic islets (Table 1) [16, 23, 46, 47]. More specifically, in animal models (pig, rat and mouse), kisspeptin-10 and -13 potentiated insulin secretion by direct activation of islet cells in the presence of 20 mmol/L glucose (Table 1) [47]. Similarly, kisspeptin induces GSIS in human islets and pancreatic cell lines cultured at high glucose concentrations (17 mM but not at 3 mM) [18]. In vivo studies have shown that intravenous (IV) kisspeptin-10 administration in rats and glucose-infused adult male monkeys (both fed and fasting states) resulted in a significant increase in plasma insulin levels (Table 1) [16, 50]. However, in glucose-deprived rhesus monkeys, IV kisspeptin-10 administration had no effect on plasma insulin

Table 1 Summary of studies conducted on the role of kisspeptin in insulin secretion and regulation of appetite

Model	Species	Effect of kisspeptin	Reference
Pancreatic islet cells in vitro	Mouse	↑ GSIS	Schwetz et al. 2014 [46]
		↑ GSIS	Bowe et al. 2012 [47]
		↓ GSIS	Vikman et al., 2009 [48]
		↑ GSIS	Bowe et al., 2009 [16]
		↑ GSIS	Hauge-Evans et al., 2006 [23]
	Rat	↑ GSIS	Bowe et al. 2012 [47]
	Pig	↑ GSIS	Bowe et al. 2012 [47]
	Human	↑ GSIS	Bowe et al. 2012 [47]
	Human	↑ GSIS	Hauge-Evans et al., 2006 [23]
	MIN-6 Cells	↓ GSIS	Hauge-Evans et al., 2006 [23]
Pancreas in vitro	Rat	↓ GSIS ↓ Arginine and exendin-4 induced insulin secretion	Silvestre et al., 2008 [49]
In vivo	Rhesus monkeys	↑ GSIS ↔ Basal insulin levels in both fed and fasting	Wahab et al., 2011 [50]
	Wistar rats	↑ Insulin secretion (IV) ↔ Insulin secretion (ICV)	Bowe et al., 2009 [16]
	Mice	↑ GSIS	Bowe et al., 2019 [17]
	Human	↑ GSIS	Izzi-Engbeaya et al., 2018 [18]
	Mice	↓ Food intake, meal frequency, time spent on meals, total mealtime ↑ Inter-meal interval and satiety	Stengel et al., 2011 [51]

IV intravenous, *ICV* intracerebroventricular

levels in either fed and fasting states, suggesting a glucose-mediated mechanism [50].

Additionally, a glucose-dependent increase in plasma insulin secretion in humans occurred following kisspeptin-54 infusion (Table 1) [18]. These findings suggest that kisspeptins stimulate insulin secretion in a glucose-dependent fashion, simulating an incretin-like effect, and further supporting previous data that hyperglycaemia may be the stimulus for kisspeptin-induced GSIS (Fig. 1) [16].

Differential Effects of Kisspeptins on Pancreatic β cell Responses

In contrast to the GSIS potentiating effect of kisspeptin 10 at higher glucose concentrations (20 mmol/L but not at 2 mmol/L), Vikman et al. found that kisspeptin-13 and kisspeptin-54 (at doses ranging from 10 nM to 1 μM) inhibited insulin secretion in the presence of lower concentrations of glucose (2.8 mmol/L). Glucose at higher concentrations almost abolished the inhibitory effect of kisspeptin-13 on GSIS [48]. Specifically, at 11.1 mmol/L glucose, inhibition of insulin secretion occurred with high doses of kisspeptin-13 and kisspeptin-54. Inhibition of insulin secretion did not appear at 16.7 mmol/L glucose irrespective of dose of kisspeptin-13 (Table 1) [48]. Additionally, kisspeptin-13 selectively inhibited glucose, arginine and exendin-4 induced insulin secretion but not somatostatin and glucagon secretion in the rat pancreas (Table 1) [49]. These data suggest that different kisspeptins, at particular doses and specific glucose concentrations, may have varying physiological effects in the glucose-dependent regulation of pancreatic beta-cell function.

Kisspeptins and Insulin Resistance

A number of studies have reported an inverse relationship between plasma kisspeptin levels and markers of insulin resistance such as body mass index (BMI), fasting insulin and Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) in women (Table 2) [19]. In advanced pregnancy, the relevance of the marked rise in plasma kisspeptin levels coinciding with peak insulin resistance remains unclear. A possible explanation for this rise in kisspeptin levels includes suppression of insulin resistance, enhanced insulin secretion by the pancreatic beta cells and increased energy expenditure [35]. Still, these hypotheses require further exploration and validation. In the insulin-resistant states of obesity and polycystic ovarian syndrome, significantly lower plasma kisspeptin levels have been reported [20]. Additionally, obese women considerably manifest lower plasma kisspeptin levels than their non-obese equivalents [19]. Similarly, obese women with polycystic ovarian syndrome (PCOS) have lower plasma kisspeptin-54 levels than their normal-weight counterparts (Table 2) [20]. Although these studies demonstrate a correlation between lower plasma kisspeptin levels and insulin resistance, their observational nature limits the ability for the establishment of a causal link between decreased circulatory kisspeptins and reduced insulin sensitivity.

Role of Kisspeptin in Regulation of Metabolism

Several hormones and neuropeptides such as leptin, adiponectin, ghrelin and glucagon-like peptide 1 (GLP-1) are established role players in appetite regulation and energy balance [53–57]. Studies by Tolson et al. and Stengel et al. showed that kisspeptin expressed in the arcuate nucleus of the hypothalamus influences appetite, weight and energy regulation [51, 57, 58]. Data suggest a negative correlation between plasma kisspeptin and hormones involved in appetite regulation (ghrelin and leptin) and positive correlation with hormones involved in glucose homeostasis (adiponectin and glucagon-like peptide 1) [19]. This association implies that impaired circulatory kisspeptin signalling may alter appetite as well as nutrient utilisation, and potentially lead to obesity and disordered glucose homeostasis. This hypothesis is supported by animal studies which have shown that kisspeptins reduce body weight, suppress appetite and increase bowel movement in mice [51, 57]. Female *KISS1R* knockout mice show less energy utilisation, glucose intolerance, reduced physical activity and increased body weight relative to their male counterparts, independent of differences in sex steroids [57]. An inference can thus be made that dysregulated kisspeptin signalling in females could be an important underlying mechanism governing appetite regulation, hyperglycaemia and obesity.

Kisspeptin and Gestational Diabetes Mellitus

Gestational diabetes mellitus, defined as hyperglycaemia with first detection in pregnancy, is thought to occur when inadequate pancreatic beta-cell insulin secretory response is overwhelmed by peripheral insulin-resistance in pregnancy [7, 8].

Some cross-sectional studies have reported lower circulatory kisspeptin levels in GDM in comparison to non-GDM pregnancies [17, 41]. Cetkovic et al. reported significantly lower plasma kisspeptin levels in the second and third trimesters of pregnant women with GDM ($n = 20$) compared to non-GDM controls ($n = 25$) (Table 2) [41]. Bowe and colleagues, in a study involving a cohort of 91 pregnant women (28.6% had GDM) between 26- and 34-week gestation, reported lower plasma kisspeptin levels in GDM versus non-GDM

Table 2 Summary of cross-sectional studies on correlation between plasma kisspeptin levels and markers of insulin secretion, insulin resistance and appetite regulation

Population	Sample size (*n*)	Findings	Reference
Adult non-diabetic	261	- The highest kisspeptin tertile had significantly lower insulinogenic and disposition indices than other tertiles - Inverse correlation between kisspeptin and OGTT-derived indices of glucose-stimulated insulin secretion - Positive correlation between kisspeptin concentration and age, BP, 2-h glucose	Andreozzi et al., 2017 [52]
Women in first, second and third trimesters:		- ↓↓ Kisspeptin levels in GDM women in the second and third trimesters than controls	Cetkovic et al., 2012 [41]
Type 1 diabetes	16	- ↓↓ Kisspeptin levels in women with type 1 diabetes than controls in all the trimesters.	
Hypertension	22		
Healthy control	25		
Women in second and third trimester:			
GDM	20		
Preeclampsia	28		
Gestational hypertension	18		
Pregnant women between 26 and 34 weeks' gestation	91	- ↓↓ Kisspeptin levels in women with GDM compared to non- GDM - Positive correlation between kisspeptin and basal □ cell secretory function. - Positive correlation between kisspeptin and oral glucose-stimulated insulin levels - No significant correlation between kisspeptin and fasting insulin.	Bowe et al., 2019 [17]
Non-obese women:		- ↓ Kisspeptin levels in the obese than non-obese women.	Kolodziejski et al., 2018 [19]
BMI < 25 kg/m^2	15	- Negative correlation between kisspeptin with BMI, HOMA-IR, insulin, ghrelin and leptin. - Positive correlation between kisspeptin and adiponectin	
Obese women: BMI > 35 kg/m^2	15		
Obese and overweight women with PCOS	28	- ↑↑ Kisspeptin-54 levels in normal-weight women with PCOS and obese controls	Panidis et al., 2006 [20]
Normal-weight women with PCOS	28	- Negative correlation between plasma kisspeptin with BMI, HOMA-IR and fasting insulin	
Obese and overweight women without PCOS (controls)	13		

"↑↑" indicates markedly higher, and "↓↓" indicates significantly lower

pregnancies (Table 2) [17]. The findings by Cetkovic and Bowe et al. suggest possible kisspeptin's involvement in β cell mass increase and function. Kisspeptin-stimulated pancreatic β cell mass adaptation may be serving as a compensatory mechanism for combating maternal peripheral insulin resistance, and when there is kisspeptin-KISS1R signalling failure, glucose intolerance will ensue.

In a study consisting of non-obese and obese women, obese women had lower plasma kisspeptin levels compared to their non-obese counterparts. The same study reported a modest negative correlation between kisspeptin with BMI, HOMA-IR, insulin, ghrelin and leptin (Table 2) [19]. These data seem to suggest that peripherally impaired kisspeptin signalling may contribute to the aetiopathogenesis of GDM through amongst others, unopposed increased peripheral insulin resistance induced by increased obesogenic (leptin and ghrelin) and lactogenic hormones in pregnancy.

Bowe et al. demonstrated that administration of KISS1R antagonist (kisspeptin-234 to pregnant mice) resulted in reduced GSIS and impaired glucose tolerance. This study provides critical evidence and suggests an essential role for kisspeptin-KISS1R signalling in glucose homeostasis during pregnancy [17]. Furthermore, compared to controls, β cell-specific *KISS1R* knockout mice had more impaired glucose tolerance and lower 30 min insulin secretory responses post-glucose challenge in pregnant compared to non-pregnant states [17]. Also, while investigating the metabolic phenotypes of KISS1R KO male and female mice, and WT littermates, Tolson et al. found increased body weight, hyperleptinaemia and impaired glucose intolerance in KISS1R KO female mice compared to WT littermates and KISS1R KO male mice [57], suggesting a sexually dimorphic influence of kisspeptin signalling on obesity and glucose homeostasis. Hence, pregnant women with kisspeptin signalling disruption may develop GDM secondary to unsuppressed appetite, excess weight gain, reduced energy utilisation and disordered glucose balance [17, 57].

Conclusion

Current evidence suggests that kisspeptin and its receptor, KISS1R, expressed in placental syncytiotrophoblasts play various roles in pregnancy. Data from animal and human studies implicate kisspeptins in glucose-stimulated insulin secretion, insulin resistance and appetite regulation, suggesting their involvement in glucose homeostasis and pancreatic β cell mass adaptation—all of which have connotations for the pathobiology of GDM. Mechanistic pathways mediating the biologic effects of kisspeptin on insulin resistance and appetite regulation are not clearly understood. In humans, there is a significant need for both basic and translational research to explore the differential effects of variable concentrations of distinct kisspeptins on pancreatic β cell regulation, insulin secretory response to oral glucose and insulin resistance in pregnancy. The underlying mechanisms of abnormal circulatory and placental kisspeptin expression and their interpretation in the regulation of pancreatic β cell function in GDM require further investigation.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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Appendix 10

Diabetes/Obesity/Dyslipidemia

ICE2020-1040

SYNCYTIOTROPHOBLAST EXTRACELLULAR VESICLES KISSPEPTIN EXPRESSION IN GESTATIONAL DIABETES MELLITUS

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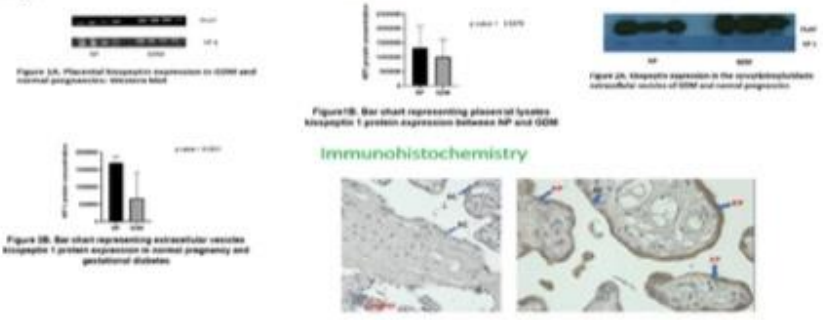
Introduction: Gestational diabetes mellitus (GDM) is reported to complicate up to 17% of pregnancies globally and 1 in 4 pregnancies in South Africa. More recent studies have challenged the theory which has suggested that insulin resistance in the later stages of normal pregnancies is due to placental hormones such as progesterone, oestrogen, human placental lactogen, and cortisol. Kisspeptin is largely produced by placental syncytiotrophoblasts with a consequent dramatic rise in circulatory levels in advanced pregnancy. Studies have shown that kisspeptin induces glucose-stimulated insulin secretion and has an inverse relationship with markers of insulin resistance. The role kisspeptin in the syncytiotrophoblast extracellular vesicles of pregnancies complicated by GDM is unknown.
Objectives: To determine the syncytiotrophoblast extracellular vesicles kisspeptin protein expression in GDM complicated pregnancies.
Methods: An exploratory experimental study was performed.
Eight archival placental lysates (GDM- 4, non GDM- 4) and 6 syncytiotrophoblast extracellular vesicles (STEVs) (GDM- 3, non GDM- 3) from University of Oxford Nuffield Department of Women's and Reproductive Health (Manu Lab) were used for the experiment.
STEVs were isolated by placental perfusion method using freshly delivered placentae of both GDM and normal pregnancies and stored at -80 degrees Celsius (**all samples received ethical approval**).
Western blotting, immunoprecipitation and immunohistochemistry were performed using anti kisspeptin 1 monoclonal antibody from Santa Cruz (Kiss-1(24Q): sc-101246), USA.
Results: The kisspeptin protein expression in the placental lysates was lower in GDM complicated pregnancies compared to normal pregnancies (p value= 0.5379).
Kisspeptin protein expression was lower in the STEVs of GDM complicated pregnancies than normal pregnancies (p value= 0.0831).
Co-expression of placental alkaline phosphatase (PLAP) and kisspeptin in the PLAP positive pulled out STEVs was found.

Image:



Conclusion: Kisspeptin protein expression is lower in both the placenta and STEVs of GDM complicated pregnancies compared to normal pregnancies. Findings suggest that in GDM pregnancies, lower STEVs kisspeptin levels are insufficient enough to stimulate insulin secretion by the pancreatic cells to counteract insulin resistance thereby leading to GDM.

Disclosure of Interest: None Declared

Appendix 11

bred fully wild type mice to establish the genetic and environmental impact of two-fold increased expression of Phlda2 on the offspring.
Results: Offspring exhibited increased anxiety-like behaviours, deficits in cognition and atypical social behaviours alongside changes in the transcriptional signatures of key brain regions with males relatively more impacted than females. Many of the behavioural and molecular alterations were shared by Phlda2 transgenic and non-transgenic offspring in comparison to controls.
Conclusion: This work establishes, for the first time, that placental endocrine insufficiency results in atypical behaviour in both mothers and their offspring. Importantly, we have evidence that the same phenomenon may underpin the co-occurrence of perinatal mood disorders and atypical behaviour of children in humans.

S3.3.
PATERNAL PROGRAMMING: SEX-DEPENDENT EFFECTS IN THE PLACENTA OF FETUSES FROM MALE DIABETIC RATS

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Objectives: We propose that paternal exposure to diabetes can lead to the transmission of metabolic disorders in the offspring. Our aim was to assess sex-dependent lipid alterations in the placenta of fetuses from diabetic males.
Methods: Control and type 2 diabetic male rats (diabetes obtained by intrauterine programming in the offspring of streptozotocin-induced diabetic rats, glycemia: 140-190 mg/dL) were mated with control female rats. On day 21 of gestation, the placenta of male and female fetuses was obtained for the evaluation of lipid levels (by TLC) and mRNA of genes involved in lipid metabolism (by RT-qPCR).
Results: Fetal weight was increased in both males and females of diabetic group ($p<0.05$) but placental weight showed no differences between groups. Triglyceride (46%), cholesterol (43%) and free fatty acid (47%) levels were increased in the male's placenta ($p<0.05$) but not in the female's placenta from diabetic group.
The *PPARalpha* mRNA ($p<0.001$) was only increased in the male's placenta from diabetic group (168%, $p<0.001$). No differences were found in the *PPARgamma* mRNA in both male's and female's placentas from diabetic group.
The mRNA levels of *Fasn*, *Acc1* and *Scd-1* (genes involved in lipid synthesis) show no differences between groups, and the mRNA levels of *Aco* (42%), *Cd36* (55%), *Fatp1* (96%), *Lipg* (121%) and *Lpl* (79%) (genes involved in lipid oxidation and transport) were increased only in male's placenta from diabetic group when compared to controls ($p<0.05$).
Conclusion: Paternal diabetes has sex-dependent effects on the regulation of lipid metabolism in the placenta, where only males are affected with an increase of lipid accumulation but also with an increased expression of genes involved in lipid oxidation and transport pathways.

S3.4.
PATERNAL OBESITY IS ASSOCIATED WITH ENDOPLASMIC RETICULUM STRESS-INDUCED PLACENTAL HYPOXIA AND ALTERED VASCULARIZATION WITHOUT CHANGES IN MATERNAL GLUCOSE TOLERANCE IN PREGNANCY

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Objectives: We determined whether paternal obesity alters placental vascular development and whether endoplasmic reticulum (ER) stress is involved. We also investigated whether paternal obesity impacts placental endocrine structures and function, impairing maternal metabolic adaptations to pregnancy.
Methods: C57BL/6J male mice were fed a control (CON, 17% kcal fat) or a high fat (PHF, 60% kcal fat) diet for 10 weeks before mating with control-fed C57BL/6J female mice to generate pregnancies. At mid-gestation (embryonic day (E) 14.5) and late gestation (E18.5), dams underwent a glucose tolerance test to assess glucose tolerance. Markers of hypoxia (CA IX), angiogenesis (VEGF-A), vessel maturity (CD31:α-SMA), ER stress (GRP78, phospho-PERK, phospho-IRE1α, *Atf6*), and endocrine function (*Igf2*, *Irs1*, *Csh1*, Csh2, parietal trophoblast giant cells (P-TGCs)) were semi-quantified using immunostaining, RT-qPCR, and/or Western blotting in CON and PHF placentae at either or both E14.5 and E18.5. Significance was assessed by Student's t-test, two-way ANOVA, and two-way repeated measures ANOVA or mixed-effects model with Bonferroni's *post-hoc*.
Results: Paternal obesity induced placental hypoxia and altered angiogenesis at E14.5 and E18.5, and was associated with activation of PERK and IREa branches of ER stress. *Igf2* ($P_{Diet} = 0.0002$) and *Irs1* ($P_{Diet} = 0.0483$) transcript levels were increased in PHF placentae at E14.5. Maternal glucose tolerance was similar between dams mated with CON and PHF males at both time points. Placental *Csh1* and *Csh2* transcript levels were unchanged by paternal obesity at E14.5 and E18.5, as were P-TGC numbers at E14.5.
Conclusion: Paternal obesity results in ER stress-induced placental hypoxia and altered angiogenesis. Impacts on offspring appear to be related to placental function, rather than maternal metabolic adaptations, since paternal obesity-induced changes in placental vasculature were not associated with changes in P-TGC numbers, placental lactogen transcript levels, or maternal glucose tolerance.

S3.5.
PLACENTAL ENDOCRINE FUNCTION IS ALTERED IN OBESE WOMEN IN ASSOCIATION WITH CHANGES IN PLACENTAL STRUCTURE AND GDM DIAGNOSIS

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Objectives: Maternal obesity and gestational diabetes mellitus (GDM) are associated with immediate and long-term health risks for the mother and child. The placenta produces hormones, including steroids and cytokines, that influence maternal glucose control. This work examined the expression of placental hormones in a cohort of South African pregnant women classified into four groups depending on GDM diagnosis and body mass index (non-obese: <30 kg/m2 and obese: ≥30 kg/m2).
Methods: The study involved four groups of women: Non-GDM, Non-obese (n=14), Non-GDM, Obese (n=19), GDM, Non-obese (n=15), GDM-Obese(n=23). At delivery, placental tissue was fixed and processed for immunohistochemistry and histological assessment or snap-frozen for RT-qPCR and Western Blot analysis. Maternal and cord serum concentrations of placenta-derived factors were measured by ELISA. Data were compared by two-way ANOVA with Bonferroni pairwise as required (significant when $p<0.05$).
Results: The expression of genes encoding steroidogenic enzymes (HSD3B1, CYP11A1, CYP19A1), placental lactogen-related members (GHV, CSH1-2), growth factors/other endocrine signals (IGF2, VEGF, KISS1) and cytokines (TNFα, IL-1B, IL-6) were not significantly different between the four groups. Maternal obesity reduced placental leptin and KISS1R (GPR54) gene expression, an effect significant in women without GDM. Overall, placental, and maternal circulating TNFα levels were decreased in women with GDM and cord TNFα concentration lower in obese women. Despite these observations, TNFα staining of terminal villi syncytiotrophoblast was increased in obese women. While placenta and maternal and cord IL-6 levels did not vary with groups, IL-6 staining of terminal villi

stroma and vessels was reduced in obese women, an effect significant in those with GDM. Stereology revealed that placenta from obese women showed fewer terminal villi with fewer vessels and stroma, but the specific effect depended on GDM diagnosis.
Conclusion: Placental endocrine function is altered in obese women and may be related to structural alterations and the development of GDM.

S3.6.
PLACENTAL PROTEOMICS REVEALS SEXUALLY DIMORPHIC ADAPTIVE CHANGES TO MATERNAL OBESITY AND GESTATIONAL DIABETES

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Objectives: Pregnancies with obesity and gestational diabetes (GDM) have significant adverse outcomes for both mother and fetus with the male fetus at increased risk. We previously demonstrated sexual dimorphism in placental lipid profiles in these conditions and hypothesized that proteomic analysis may identify dimorphism of cellular pathways.
Methods: Villous tissue was collected at term C section (no labor) from lean, obese or type A2GDM women with either male or female fetus (n=4/group). Peptides were analyzed via liquid chromatography tandem mass spectrometry (Q Exactive Orbitrap Mass Spectrometer). Differentially expressed ($p<0.05$) proteins between lean, obese or A2GDM groups were identified to determine significantly affected GO pathways for male, female or combined. Heatmaps were constructed using pathway enrichment scores >2 for each comparison. DAVID analysis recognized 2980 proteins to create functional annotation clusters of pathways.
Results: Significantly regulated proteins were identified comparing specimens from lean vs obese (53 up, 143 down), A2GDM (57 up, 220 down) or A2GDM vs obese (34 up, 44 down). In female placentas, pathways corresponding to extracellular region and extracellular space were significantly enriched in A2GDM and obese vs lean whereas those involved in protein transporters, centrioles and glucose metabolic process were downregulated comparing A2GDM vs lean or obese. In contrast, in male placentas, pathways involved in cytoskeleton, extracellular space, heme binding, inflammatory response, antioxidant activity, complement activation and bicarbonate transport were upregulated, whereas multiple pathways related to translation, mRNA and rRNA processing, ribosomal subunit biogenesis, RNA splicing, nucleosome and focal adhesion were downregulated comparing obese or A2GDM with lean .
Conclusion: We show a profound sexual dimorphism in the placental cellular response to maternal obesity and GDM which may represent fetal sex-specific adaptive responses. Dramatic alterations seen in male placentas may maintain or enhance fetal growth in this environment but with risk of demise while the female placenta may adapt to ensure survival.

S4.1.
CELLULAR SENESCENCE IN EXTRAVILLOUS TROPHOBLASTS

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Cellular senescence defines a state of irreversible growth arrest in combination with phenotypic changes including high transcriptional, secretory and metabolic activity. A long-standing paradigm assigned senescence with adverse cellular phenotypes such as aging and was shown to be triggered by replicative exhaustion, DNA damage or oncogenes. This view has been challenged by describing cellular senescence as part of the developmental program in mice. During placental development senescence has been associated with syncytiotrophoblast formation and suggested to coincide with the expression of the cell-cycle inhibitors. Available studies in the context of adverse pregnancy outcome give a somewhat unclear picture by relating either induced or attenuated signs of cellular senescence to placental pathologies. Our own data demonstrate a robust induction of senescence-associated signatures in extravillous trophoblasts (EVTs). These included senescence-associated beta-gal (SAβG) activity in situ, induction of a senescence-associated secretory phenotype, a metabolic switch in fatty acid synthesis, signs for DNA damage, and secretion of pro-inflammatory cytokines. Prior to the development of cellular senescence, HLA-G+ distal cell column trophoblasts (dCCTs) undergo endoreduplication. As this event is vastly limited to a tetraploid status we assume that senescence induces an endoreplicative block to prevent exacerbated polyploidization and as a consequence genomic instability. This barrier to potential malignancy is likely controlled by p57 and cyclin E, which are both induced in decidual EVTs and absent from actively, endocycling dCCTs. Indeed, knockdown of cyclin E and p57 significantly reduced SAβG activity in cultivated EVTs. Well in line, hyperplastic complete hydatidiform moles showed strongly exacerbated polyploidization in EVTs, reduced signs for cellular senescence and downregulated levels of p57 and cyclin E. Altogether, we suggest that cellular senescence in EVTs fulfills a functional role by provoking specific phenotypic changes and likely controls the extent of polyploidization. This study was supported by the Austrian Science Fund P 33485).

S4.2.
OXIDATIVE STRESS-INDUCED SENESCENCE IN MATURE, POST-MATURE AND PATHOLOGICAL PLACENTAS

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Objectives: Placental dysfunction is the main cause of many placental pathologies. Premature placental ageing might be involved in this process. Cell senescence, which can be activated by oxidative stress and DNA damage, has also been implicated in cell fusion. Placental oxidative stress is a key intermediary event in the pathophysiology of pre-eclampsia. We examined senescence markers across normal gestation, and in pathological and post-mature pregnancies. Inducers of oxidative stress were used to mimic senescence changes in term explants.
Methods: Placental samples were collected with ethical approval and informed consent: first and second trimester samples from surgical terminations; term and pre-term controls, and early-onset pre-eclampsia samples from caesarean deliveries. Paraffin and EM blocks of post-mature placentas were from an archival collection. Term explants were subjected to hypoxia-reoxygenation (HR) or hydrogen peroxide (H_2O_2).
Results: p21 was increased significantly in term homogenates compared to first and second trimester samples, and was significantly higher in both preterm controls and PE+IUGR compared to term controls. Immunostaining revealed nuclear localisation of p21 and phosphorylated histone, γH2AX, in the syncytiotrophoblast, with abundant foci in pathological and post-mature placentas. Abnormal nuclear appearances were observed in post-mature placentas. Sudan-Black-B staining demonstrated abundant expression of lipofuscin, an aggregate of oxidised proteins, lipids and metals, in post-mature and pathological placentas. In addition, an increased percentage of nuclei were positive for 8-hydroxy-2'-deoxyguanosine, a marker of oxidised DNA, in pathological placentas compared to age-matched controls. These changes could be mimicked *in vitro* by challenge with HR or H_2O_2.
Conclusion: Evidence of senescence markers increases with gestational age in normal placentas, and is exaggerated in post-mature and pathological placentas. Oxidative stress triggers these changes in placental explants, and may be the precipitating insult *in vivo*. The consequent pro-inflammatory senescence-associated secretory phenotype may contribute to the pathophysiology of early-onset pre-eclampsia.

S4.3.
MORPHOLOGICAL CHANGES IN PLACENTAS FROM PROLONGED PREGNANCY

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