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The effect of gestational diabetes, and its treatment, on the expression of Occludin, VE-cadherin & Plakoglobin splice variants in the human placental endothelial barrier

Stephany D. Villota Villafuerte

Supervised by: Maria Toledo-Rodriguez
Lopa Leach

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University of Nottingham
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ABSTRACT

The placenta is essential for the development, survival and growth of the embryo, as it forms the critical interface between the fetus and the mother. Gestational diabetes mellitus (GDM) is a type of diabetes typically detected during the second or third trimester of pregnancy. Reduced occupancy of tight and adherens junctional molecules (Occludin and VE-cadherin) and disrupted junctions are a feature of human placental vessels under a diabetic milieu. Differential expression of gene splice variants and resultant protein isoforms may be behind the changes in junctional molecules and was the central hypothesis of this PhD study. The role of angiogenic growth factors and epigenetic control, if any, were also investigated in order to elucidate mechanisms behind gene expression changes. Human placental tissue from normal pregnancies were compared with those complicated by GDM where glucose levels were treated by a change of diet or by medication (metformin).

Placental and umbilical cord tissue was collected from 40 term singleton pregnancies delivered by caesarean section. Gene expression was determined by qPCR; protein expression by immunoblotting, and junctional occupancy by the systematic sampling of immuno-positive vessels.

Diet-controlled GDM placental samples showed differential expression of Occludin gene and protein: *Occludin variant 2* was reduced (1.5-fold), while *Occludin variant 3* was increased (3.3-fold) compared to normal. Occludin isoform-A levels (fully functional isoform) were decreased (1.4-fold), and lower percentage of vessels showed junctional Occludin (12%) compared to normal. Additionally, VE-cadherin gene and protein expression remained unchanged between normal and GDM placental samples. Finally, *Plakoglobin* gene expression was higher in diet-controlled GDM samples (1.6-fold) compared to normal. In metformin-controlled GDM placental samples, Occludin, VE-cadherin and Plakoglobin gene and protein expression were similar to normal placental samples.

We explored if differential expression of angiogenic factors was behind the downregulation of Occludin found in GDM placentae. Assessment of the gene expression of angiogenic/permeability factors (*VEGF-A*, *Ang-1*, *Ang-2* and *MMP-9*) with their receptors (*VEGFR-2* and *Tie-2*) revealed similar expression of these factors between normal and GDM placental samples ($p > 0.05$). Total VEGF-A protein expression was downregulated (1.3-fold) in diet-controlled GDM placental samples compared to normal.

An *ex vivo* model (placental chorionic explants cultured under hyperglycaemic conditions (15 mM D-glucose) for 3-hours ($n= 4$), or 24-hours ($n= 4$)) showed that hyperglycaemia did not cause downregulation of *Occludin* gene expression or junctional localisation in the placental vasculature compared to control ($p > 0.05$).

To determine the mechanism behind the changes in Occludin expression, our study examined whether GDM affected the expression of epigenetic markers in placenta. Quantification of the DNA methylation levels of *Occludin* promoter showed no significant change between normal and GDM placental pregnancies. Additionally, *miR181a-5p* expression was higher (2.1-fold) in diet-controlled GDM placental samples compared to normal. To confirm the effects of *miR-181a-5p* in *Occludin* gene expression, *miR-181a-5p* was overexpressed in HUVEC. Results showed downregulation of *Occludin variant 1* (3.2-fold) and *variant 2* (1.8-fold) gene expression in the presence of *miR181a-5p*.

In conclusion, our study showed that the junctional molecules Occludin, VE-cadherin and Plakoglobin presented several gene splice variants and protein isoforms in human placental samples. Altered gene expression of *Occludin* and *Plakoglobin* were found in diet-controlled GDM placentae. Downregulation of *Occludin* was in part the result of overexpression of *miR-181a-5p*. The stable expression of the target genes in the metformin-controlled GDM group may not be a result of the corrective effects of metformin upon glucose. Our data suggests that, despite good glucose control, the adoption of lifestyle changes alone during a GDM pregnancy (diet-controlled) may not be enough to prevent an alteration in the expression of Occludin.

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LIST OF ABBREVIATIONS

18S	18S ribosomal
AE	Amplification efficiency
AJ	Adherens junction
AMPK	AMP-activated protein kinase
Ang-1/ANGPT1	Angiopoietin 1
Ang-2/ANGPT2	Angiopoietin 2
ARVD	Arrhythmogenic right ventricular dysplasia
ATP	Adenosine triphosphate
B2M	B-2 microglobulin
BBB	Blood-brain barrier
BCRP	Breast cancer resistance protein
BMI	Body mass index
BRB	Blood-retina barrier
BRMEC	Bovine retinal microvessel endothelial cells
BSA	Bovine serum albumin
C-section	Caesarean section
CCD	Coiled-Coil Domain
CCM	Cerebral Cavernous Malformation
cDNA	Complementary DNA
Cme	5-methylcytosine
Ct	Threshold cycle
DMRs	Differentially Methylated Regions
DNMT	DNA methyltransferase
DOHAD	Developmental origins of health and disease
EC	Extracellular cadherin
ECGS	Endothelial cell growth supplement
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FPG	Fasting plasma glucose
FRd	Fibrinogen-Related Domain
GDM	Gestational diabetes mellitus
GFP	Green fluorescent protein
GLUTs	Glucose transporters
GSDS	Gene Structure Display Server
HbA1c	Glycated haemoglobin
HBSS	Hank's balanced salt solution

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPMEC	Human placental microvasculature endothelial cell
HPRT1	Hypoxanthine phosphoribosyl-transferase I
HUAEC	Human umbilical artery endothelial cell
HUVEC	Human umbilical vein endothelial cell
Ig	Immunoglobulin
IP	Immunoprecipitation
IUGR	Intrauterine growth restriction
JAM	Junctional adhesion molecule
JUP	Plakoglobin
KLF4	Kruppel-Like Factor 4
KLF6	Kruppel-Like Factor 6
LADA	Latent autoimmune diabetes of adulthood
LGA	Large for gestational age
M	Stability
M0 media	M199 with 1% penicillin/streptomycin
M20 media	M0, plus 20% fetal calf serum
MAZ	Myc-associated zinc-finger protein
MeDIP	Methylated DNA immunoprecipitation
miRNAs	microRNAs
MIV	Mature Intermediate Villi
MMP-9	Matrix metalloproteinase -9
MODY	Maturity-onset diabetes of the young
mRNA	Messenger RNA
MRP1	Multidrug resistance associated protein
MV	Mesenchymal Villi
NCBI	National Center for Biotechnology Information
NFX1	Nuclear transcription factor, X-box binding 1
NHS	National Health Service
NICE	National Institute for Health and Care Excellence
OCN	Occludin
OCT	Optimal cutting temperature
OCT3	Organic cation transporter
OGTT	Oral glucose tolerance test
P-gp	P-glycoprotein
P0	Passage 0. Cells first isolation and plating
P3	Passage 3. Cells have been split and seeded three times
PBS	Phosphate-buffered saline
PC	Post-conception
PCR	Polymerase chain reaction
PECAM-1	Platelet endothelial cell adhesion molecule
PFA	Paraformaldehyde
PIGF	Placental growth factor

PMSF	Phenylmethanesulfonyl Fluoride
POFUT1	Protein O-fucosyltransferase 1
qPCR	Quantitative polymerase chain reaction
REC	Human retinal endothelial cells
RPE	Human Retinal pigmented epithelial cells
rRNA	Ribosomal RNA
RUNX	Runt-Related Transcription factors
SCD	Super-Clustering Domain
SD	Standard deviation
SDHA	Succinate dehydrogenase complex, subunit A
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
sEng	Soluble endoglin
Ser	Serine
sFlt-1	Soluble fms-like tyrosine kinase also referred to as sVEGFR1
SGA	Small for gestational age
SV	Stem Villi
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TBP	TATA box binding protein
TBST	Tris-buffered saline
TER	Trans-epithelial electrical resistance
Thr	Threonine
Tie-1	Tyrosine kinase with Immunoglobulin (Ig)-like and Epidermal growth factor (EGF)-like -1 receptor
Tie-2	Tyrosine kinase with Immunoglobulin (Ig)-like and Epidermal growth factor (EGF)-like -2 receptor
TJ	Tight junction
TM	Transmembrane
TM20	Transfection medium: antibiotic-free M199 plus 20% FCS
TOP1	DNA Topoisomerase 1
TRITC	Tetramethylrhodamine
tRNA	Transfer RNA
Tyr	Tyrosine
UTR	Untranslated region
UTR	Untranslated Region
V	Variation
VE-cadherin	Vascular endothelial cadherin
VEGF-A	Vascular endothelial growth factor-A

VEGFR-1/Flt-1	Vascular endothelial growth factor receptor 1
VEGFR-2/Flk-1	Vascular endothelial growth factor receptor 2
VEGFR-3/Flt-4	Vascular Endothelial Growth Factor 3
VPF	Vascular permeability factor
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta
ZO (1, 2, 3)	Zona occludens -1

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CHAPTER 1

GENERAL INTRODUCTION

1.1. DIABETES MELLITUS

Diabetes mellitus is a group of metabolic disorders characterised by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both (Alberti & Zimmet, 1998). Diabetes can generate long-term damage, dysfunction and failure of different organs, especially to the eyes, kidneys, nerves, heart and blood vessels. In the UK, there are almost 3.7 million people who have been diagnosed with diabetes as at November 2017 ("Diabetes prevalence 2017," 2017).

Different tests are currently used to determine plasma glucose levels in a patient. As a guideline (Kerner & Brückel, 2014), a person can be diagnosed with diabetes if:

- $\text{HbA}_{1c} \geq 6.5\%$ ($\geq 7.8 \text{ mM}$) or
- Random plasma glucose level $\geq 11.1 \text{ mM}$ or
- Fasting plasma glucose (FPG) levels $\geq 7.0 \text{ mM}$ or
- Oral glucose tolerance test (OGTT) 2-hour glucose in venous plasma $\geq 11.1 \text{ mM}$.

1.1.1. TYPES OF DIABETES

There are different types of diabetes; the most prevalent ones are type 1 (T1DM) and type 2 (T2DM). Additionally, other less common specific types of diabetes are gestational diabetes (GDM), latent autoimmune diabetes of adulthood (diabetes LADA), maturity-onset diabetes of the young (diabetes MODY), among others.

T1DM is an autoimmune disorder caused by an absolute deficiency of insulin secretion due to the destruction of pancreatic β -cells. T1DM commonly occurs in childhood and adolescence, but it can develop at any age. Around 10% of people living with diabetes in the UK have T1DM ("Diabetes the basics," 2017). Individuals with T1DM eventually become dependent on insulin for survival.

T2DM is a metabolic disorder caused by a combination of resistance to insulin action and an inadequate compensatory insulin secretory response. The risk of developing T2DM increases with age, obesity, and lack of physical activity. T2DM is characterised as a silent disease as it can go undiagnosed for many years. Therefore, T2DM patients are at increased risk of developing macrovascular and microvascular complications. In the UK, around 90% of all adults with diabetes have T2DM ("Diabetes," 2016). For some individuals with T2DM, adequate glycaemic control can be achieved with weight reduction, exercise, and oral glucose-lowering agents; in this case, there is no need for insulin. T2DM individuals that present β -cell destruction will require insulin for survival.

1.1.2. DIABETES IN PREGNANCY

According to the UK National Institute for Health and Care Excellence (NICE) records, up to 5% of the women who give birth in England and Wales have either pre-existing diabetes or GDM (NICE, 2015). Of these women with pregnancies complicated with diabetes, approximately 87.5% have GDM, 7.5% have T1DM, and the remaining 5% have T2DM.

During a diabetic pregnancy, maternal glucose concentrations are determined by maternal factors (i.e. type of diabetes, glucose control) and the rate of glucose uptake in the fetus¹ (Desoye & Nolan, 2016). Diabetic

¹ The word fetus (plural fetuses) comes from the Latin *fetus*, meaning offspring, the young in the womb (Skeat, 2013)

mothers are at higher risk of: miscarriage, accelerated retinopathy and nephropathy, hypoglycaemia, pre-eclampsia, among others (Janota *et al.*, 2003; McCance, 2015) These complications are thought to be attributable to poor glycaemic control. For instance, approximately 20% of pregnancies complicated with pre-existing diabetes (T1DM or T2DM) conclude in spontaneous losses. This proportion is higher than in normoglycemic pregnancies (13%) (McGrogan *et al.*, 2014).

Fetal glucose concentrations are determined by both glucose appearance and disappearance. Trans-placental glucose transfer from the mother regulates fetal glucose appearance. While, glucose uptake regulates fetal glucose disappearance into fetal tissues depending on fetal insulin levels, insulin sensitivity and fetal weight (Desoye & Nolan, 2016). A diabetic uterine environment will bring consequences to the fetus. This response is referred to as Pedersen theory, where maternal hyperglycaemia results in fetal hyperglycaemia, and hence results in hypertrophy of fetal pancreatic islet tissue with increased insulin secretion to compensate for the high glucose levels. Thus, fetuses from diabetic pregnancies present higher risks of stillbirth, perinatal mortality, congenital anomalies, small/large for gestational age (SGA/LGA), preterm delivery, shoulder dystocia and birth injury, and neonatal hypoglycaemia among others (McCance, 2015). In the UK, 4% of fetuses from T1DM pregnancies had at least one significant congenital anomaly, being the most common anomaly heart disease (1.7%) (McCance, 2015).

After delivery, there are some guidelines that should be followed in a pregnancy complicated with diabetes to ensure the wellbeing of the mother and the baby (NICE, 2015). Women who were insulin-treated should reduce their insulin doses immediately, and should monitor their blood glucose levels carefully to establish the appropriate dose. Neonates should receive feeding as soon as possible to minimise the risk of hypoglycaemia. Additionally, neonatal blood glucose levels should be tested

two to four hours after birth, and continue for at most a few days until levels are persistently above 2.0 mM.

1.2. GESTATIONAL DIABETES MELLITUS

During a normal pregnancy, a physiological increase in secretion of several hormones (e.g. cortisol, growth hormone, progesterone, and prolactin) will result in a state of insulin resistance starting in the second trimester. In most women, adequate pancreatic β -cell activation will compensate this insulin resistance by increasing the amount of insulin secretion, and will maintain a normoglycaemic pregnancy. However, some women will develop GDM due to insufficient pancreatic β -cell function (World Health Organization, 2014). Hence, GDM is defined as a carbohydrate intolerance resulting in any degree of hyperglycaemia with onset or first recognition during pregnancy. However, this definition does not exclude the possibility that unrecognised glucose intolerance may have antedated or begun concomitantly with pregnancy (American Diabetes Association, 2010). Pre-existent diabetes could be ruled out if glucose tolerance was shown to return to normal at postpartum (A. Ferrara, 2007).

As with T1DM and T2DM, GDM is associated with some pregnancy complications as: fetal overgrowth, polyhydramnios, premature birth, pre-eclampsia, fetal low blood glucose, and stillbirth. Additionally, as a long-term consequence for a GDM mother, 50% of women are expected to develop T2DM within five years of the first registered pregnancy complicated by GDM (C. Kim *et al.*, 2002). Likewise, infants of women with GDM are at increased risk of developing obesity, cardiovascular diseases, impaired glucose tolerance, and diabetes as children or young adults. This increased risk may be independent of genetic factors.

Following the guidelines of the NICE, formal testing for GDM is done during the late second trimester (between 24 and 28 weeks of gestation) by an oral glucose tolerance test (NICE, 2015). However, testing for GDM could be suggested earlier during pregnancy if women present any one of the

following risk factors: BMI above 30 kg/m² (overweight), previous history of glucose intolerance, previous history of pregnancy complications, or being part of high-risk ethnic groups (South Asian, Chinese, African-Caribbean or Middle Eastern). GDM is diagnosed if the woman has either a FPG level ≥ 5.6 mM, or a 2-hour plasma glucose level ≥ 7.8 mM.

Appropriate treatment of GDM can reduce the risk of serious adverse birth complications (e.g. shoulder dystocia). As a baseline, all women diagnosed with diabetes should adjust their diet and adopt a physical exercise routine. Lifestyle changes are sufficient to control hyperglycaemia in 80–90% of GDM patients (McCance, 2015). If a diet change is insufficient to control maternal hyperglycaemia, the use of oral blood glucose-lowering agents (metformin) or insulin therapy will be needed to prevent fetal complications. In this regard, metformin is offered to women with GDM if blood glucose targets are not met using lifestyle changes within 1-2 weeks (NICE, 2015). Metformin is a biguanide compound that works by stopping the liver production of new glucose, and helps to improve insulin sensitivity by making insulin carry glucose into the cells more efficiently. In more severe cases, immediate treatment with insulin, with or without metformin, is offered to women with GDM who have a FPG levels ≥ 7.0 mM at diagnosis.

After delivery, GDM women should discontinue blood glucose-lowering therapy (NICE, 2015). Additionally, women with GDM pregnancy are tested for blood glucose concentrations after delivery to exclude persisting hyperglycaemia. Women diagnosed with GDM and whose blood glucose levels returned to normal after the birth are offer a fasting plasma glucose test 6-13 weeks after the birth to exclude diabetes. Women with fasting plasma glucose level below 6.0 mM have a low probability of having diabetes at present; however, they should continue to follow the lifestyle changes implemented during pregnancy, and will need an annual test to check that their blood glucose levels are normal due to the moderate risk of developing T2DM. Women with fasting plasma glucose levels between 6.0

and 6.9 mM are at higher risk of developing T2DM. Women with fasting plasma glucose level of 7.0 mM or above are likely to have T2DM.

1.3. HUMAN PLACENTA

The placenta forms the critical interface between the fetus and the mother, which is essential for development, survival and growth of the embryo. The central functional unit is the villus, as it oversees the nutrient supply, gas exchange and waste removal between the mother and the fetus. Each villus is surrounded by a multinucleated syncytiotrophoblast layer, which is bathed in maternal blood (Figure 1.1). Underneath the syncytiotrophoblast is the mononucleated cytotrophoblasts, which near-term forms a discontinuous layer.

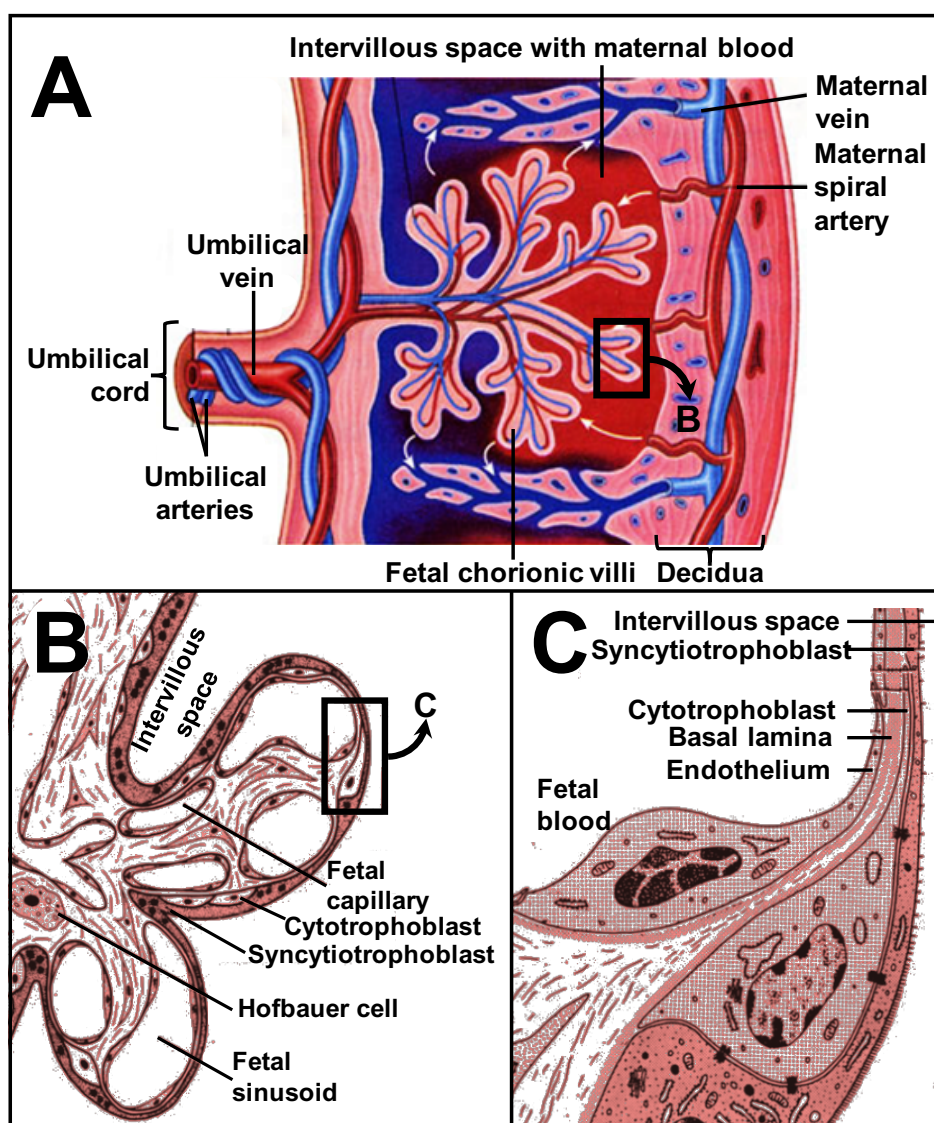


Figure 1.1. Schematic overview of the placental villi morphology. (A) Placenta consists of a fetal and maternal part. In the fetal side, chorionic villi, covered by the syncytiotrophoblast, are bathed in the maternal blood of the intervillous space. The maternal side consists of the decidua layer which presents remodelled spiral arteries that take maternal blood into the intervillous space. (B) Highly simplified light microscopic section of two terminal villi. (C) Representation of an electron microscopic section of the placental barrier, demonstrating its layers. Modified from: (A) (Faas *et al.*, 2014), (B and C) (Benirschke *et al.*, 2006).

There are different types of villi within the placenta: *Stem villi* are characterised by a compact fibrous stroma, with large, centrally located arterioles and venules; *Mesenchymal villi* are characterised by unorganised stromal and vascular structures (precursors of immature intermediate villi); *Immature intermediate villi* are less compact than stem villi, and their cells form intercommunicating channels in which arterioles and venules are embedded; *Mature intermediate villi* are more differentiated, they present capillaries; and *Terminal villi* form the smallest unit that mediates maternal-fetal exchange (Figure 1.2).

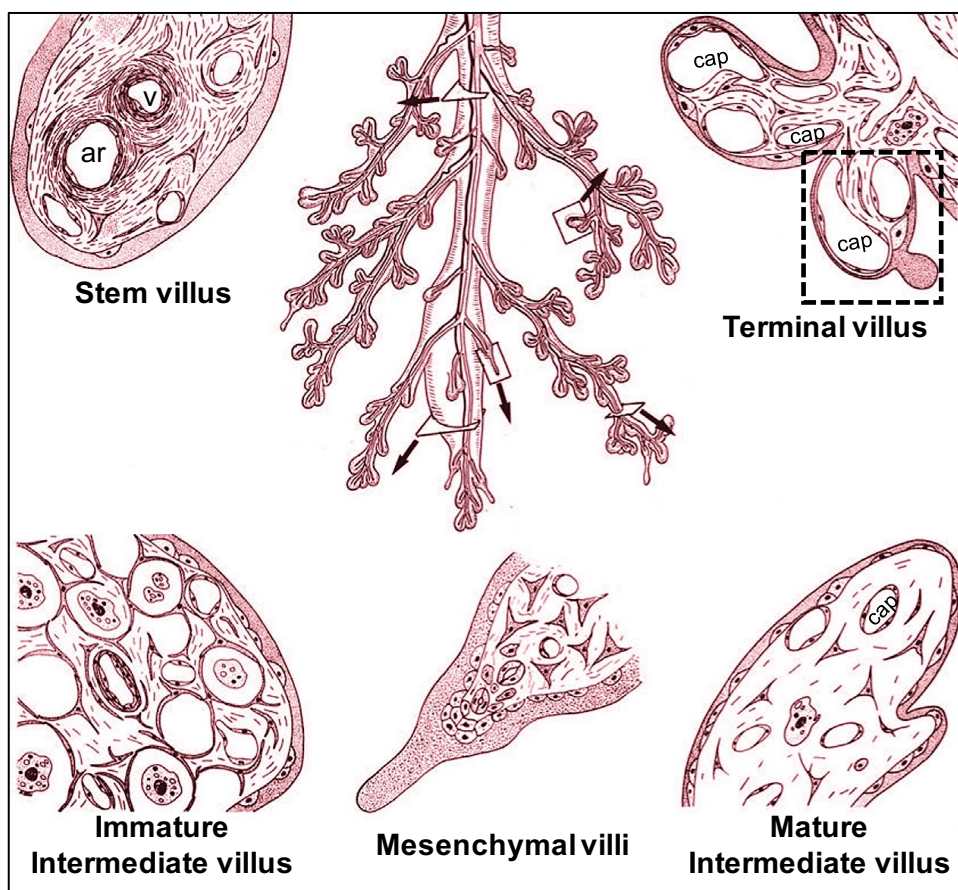


Figure 1.2. Schematic representation of the peripheral part of the mature placental villus tree and villus types. Within the placental there are: Stem villi with centrally located arterioles (ar) and venules (v), Mesenchymal villi, Immature intermediate villi, Mature intermediate villi with capillaries (cap), and an example of Terminal villus (black dotted rectangle) Modified from: (Benirschke *et al.*, 2006).

There are several developmental, environmental or pathological factors that influence the regulation of placental transport. Two models have been postulated about this regulation. The *fetal demand model* suggests that the fetus primarily controls placental function (Constancia *et al.*, 2005). Thus, in the event of maternal under-nutrition, the fetus will compensate for the lack of nutrients by increasing placental nutrient transport. In contrast, the *maternal supply model* suggests that during maternal undernutrition, the placental nutrient flow will be reduced (Jansson *et al.*, 2012). As the placenta presents intrinsic nutrient-sensing signalling pathways, both models are likely to coexist (Gaccioli *et al.*, 2013). For instance, regulation by fetal demand could be predominant when a nutritional challenge is moderate or brief, whereas regulation by maternal supply may dominate if the nutritional challenge is more severe or prolonged.

1.3.1. PLACENTAL ENDOTHELIAL CELLS

Within the stoma of each villus are the fetal blood vessels. These vessels present a layer of endothelial cells lining their internal surface. This endothelium layer maintains homeostasis by controlling blood vessel tone, maintaining blood fluidity, regulating angiogenesis, and controlling the transfer of water, nutrients and leukocytes across the vascular wall. Different endothelial cells can be isolated from placenta and umbilical cord. The first reports go back to 1960s, with the isolation of endothelial cells from umbilical vein (HUVEC) (Maruyama, 1963), umbilical artery (HUAEC) (Joyner & Strand, 1978), and from the placental microvasculature (HPMEC) (Leach *et al.*, 1994) (Figure 1.3). These endothelial cells have been used to study the physiology and pathology of several complications, including diabetes (Altannavch *et al.*, 2004; Azcutia *et al.*, 2010).

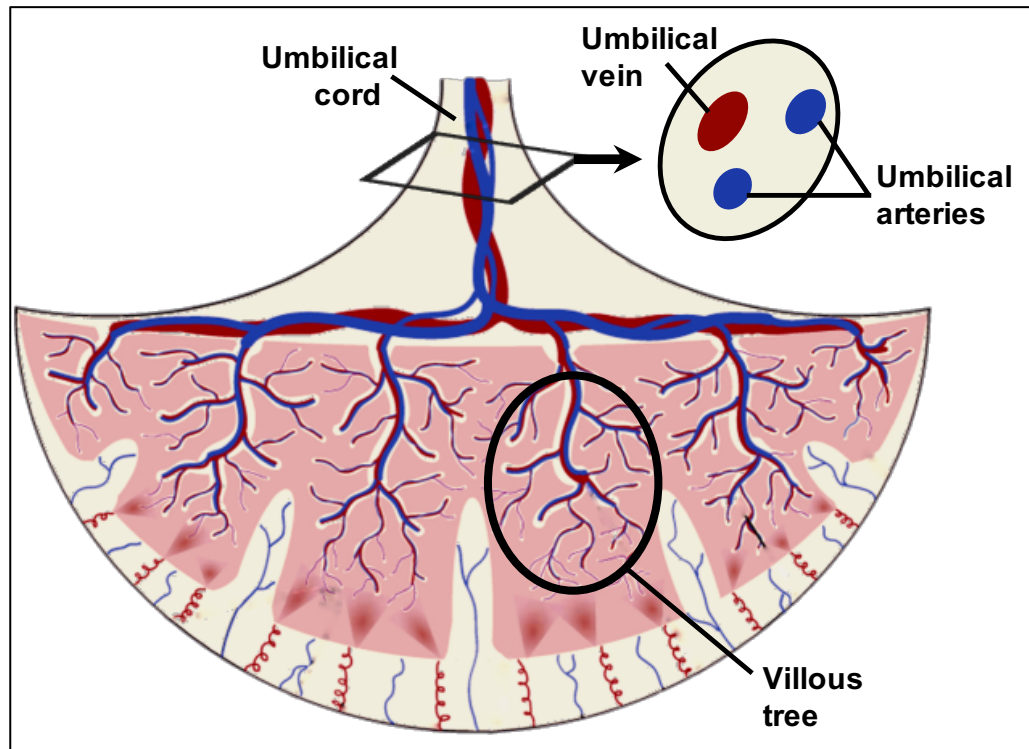


Figure 1.3. Schematic representation of the placental microvessels and the umbilical vein and arteries. Endothelial cells can be isolated from the placental villi trees (HPMEC), umbilical vein (HUVEC), or umbilical artery (HUAEC). Modified from: (Plitman Mayo *et al.*, 2016).

1.3.2. HUMAN PLACENTA AND DIABETES

The placenta and the fetus use glucose as the primary substrate for energy metabolism. From the total amount of glucose taken by the placenta from the maternal circulation, the placenta consumes 30 to 40% by itself (Higgins *et al.*, 2011). This glucose transfer is facilitated by glucose transporters (GLUTs) expressed in the syncytiotrophoblast and, to a reduced extent, by paracellular diffusion.

A diabetic environment will generate structural and morphological consequences for the placenta. For instance, studies have found a change in expression of vascular endothelial growth factor (VEGF), adherens junctional molecules (vascular endothelial cadherin and β -catenin) (Leach *et al.*, 2004), and tight junctional molecules (occludin and zonula occludens-1) (Babawale *et al.*, 2000) in placental samples from pregnancies complicated with diabetes (T1DM and GDM) compared to controls.

Additionally, infarction, hematoma, calcification, and fibrin deposits have been found in the surface of placentae from pregnancies complicated with diabetes (T1DM, T2DM, and GDM) (Salge *et al.*, 2012). The extent and specificity of these abnormalities depend on the gestational period and the severity and type of diabetes. For instance, placentae from pregnancies complicated with T1DM and T2DM have been described as having an impaired vessel wall structure as they present a thinner capillary basement membrane (Janota *et al.*, 2003).

1.4. REGULATION OF GENE EXPRESSION

In molecular terms, a gene is the entire DNA sequence required for synthesis of a functional protein or RNA molecule. Gene expression is the process in which information encoded in a gene is transcribed to RNA. This DNA sequence is transcribed to a precursor pre-mRNA. This pre-mRNA will need to undergo post-transcriptional modifications to become mRNA. Furthermore, gene expression can also be regulated by epigenetic changes, which refer to heritable changes in phenotype or gene expression which do not involve changes in DNA sequence (R. Ma *et al.*, 2015; Nelissen *et al.*, 2010). Epigenetic processes represent a crucial characteristic of cell differentiation and play an essential role in the maintenance of the differentiated state. This regulation can be made at three levels: DNA (DNA methylation, chromatin remodelling), RNA (non-coding RNAs), and protein (histone variants, and post-transcriptional histone modifications).

1.4.1. ALTERNATIVE SPLICING

Constitutive splicing is the process of intron removal and exon ligation of the majority of the exons in the order in which they appear in a gene (Breitbart *et al.*, 1987). Alternative splicing is a deviation from this preferred sequence where certain exons are skipped resulting in various forms of mature mRNA. RNA splicing will increase the complexity of gene expression that results in various forms of mature mRNA (gene splice variants). This is a complex reaction that takes place in the spliceosome

which catalyses this process and holds the various intermediates in the correct orientation relative to one another (Figure 1.4).

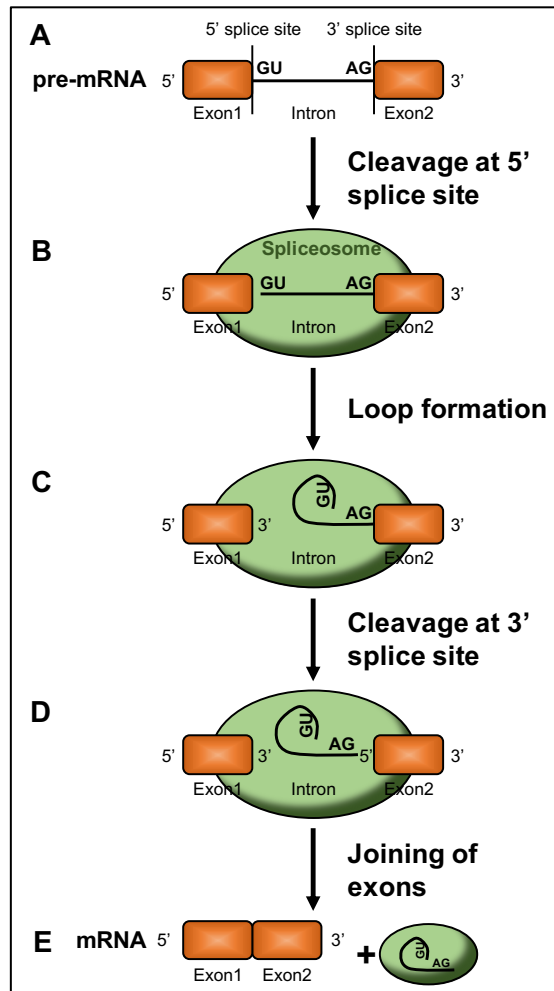


Figure 1.4. RNA splicing. Pre-mRNA containing both exons and introns is transcribed. **(A)** Introns are identified as they begin with the bases GU and end with AG. The spliceosome (green) mediates the catalyses of intron sequences. **(B)** Cleavage of 5' splice site results in a free upstream exon and the intron joined to the Exon2. **(C)** The intron folds back on itself forming a loop. **(D)** Cleavage of 3' splice site results in a free intron and both exons. **(E)** The spliceosome joins both exons to form the spliced product (mRNA). The spliceosome and intron are free from the complex.

Alternative splicing can lead to protein diversity as a single gene can code for multiple proteins due to differential inclusion or exclusion of regions of pre-mRNA. There are different types of gene splicing, including:

- Exon skipping: exon(s) are included or excluded from the final gene transcript leading to different lengths mRNA variants

- Intron retention: an intron is retained in the final transcript
- Alternative 5' or 3' splice site: joining of different 5' and 3' splice sites.

1.4.2. DNA METHYLATION

Among all the different epigenetic changes, DNA methylation is one of the most studied inactivation method. It is a process by which methyl groups are added to the 5th-carbon position of cytosine converting it to 5-methylcytosine (C^{me}) (Robertson, 2005). DNA methylation occurs almost exclusively at regions of high cytosine content. These regions are called CpG islands due to an elevated number of cytosine nucleotide followed by guanine nucleotide along the 5' to 3' sequence. CpG islands are often found in promoter regions and first exon of a gene, but also in regions towards the 3' end. DNA methylation is catalysed by DNA methyltransferases (DNMTs). DNMT1 activity enables maintenance of the DNA methylation pattern during mitosis. DNMT1 recognises hemi-methylated DNA (one DNA strand has C^{me}, and the other strand has C) and restores methylation in both strands. Additionally, DNMT3a and DNMT3b are the *de novo* methyltransferases that will set up DNA methylation patterns early in development. DNA methylation of promoter regions will repress gene transcription by preventing the binding of the transcriptional machinery to the gene.

1.4.3. MICRORNAS

Non-coding RNAs can be classified according to their length as long or small non-coding RNAs. Long non-coding RNAs have been associated with *cis*-acting functions, as they can regulate the expression of one or more genes on the same chromosome. On the other hand, small non-coding RNAs present *trans*-acting functions, as they will regulate the expression of one or more genes on different chromosomes or regulate mature RNAs in the cytoplasm.

microRNAs (miRNAs) are a subtype of small non-coding RNAs, that are generally 21–25 nucleotide long. miRNAs are epigenetic post-

transcriptional regulators in a sequence-specific manner by binding to the target mRNA 3' untranslated region (3' UTR) and repressing its translation and/or degrading it. miRNAs are described as “multivalent”, as one miRNA can target multiple genes.

1.4.4. EPIGENETICS, PLACENTA AND DIABETES

The placenta is susceptible to environmental conditions that could have the capacity to alter placental functions and fetal development. For instance, smoking during pregnancy has been linked to a change in the offspring's DNA methylation levels of genes involved in brain development (Toledo-Rodriguez *et al.*, 2010).

During an uncomplicated pregnancy, some epigenetic modifications are needed for placental formation and maintenance and hence, successful development of the fetus (Sundrani *et al.*, 2013). For instance, the epigenetic profile of the placenta shows a progressive increase of DNA methylation from the first to the third trimester (Nelissen *et al.*, 2010). Likewise, DNA methylation is in part in charge of the cytotrophoblasts differentiation into syncytiotrophoblast (Januar *et al.*, 2015; Nelissen *et al.*, 2010). Additionally, the expression of some miRNAs (*miR-141*, *miR-149*, *miR-299-5p*, and *miR-135b*) was found to be upregulated in maternal plasma during pregnancy, and their concentration decreases after delivery (Chim *et al.*, 2008).

During a pregnancy complicated with diabetes, there is a change in expression of some epigenetic factors. Fetal hyperinsulinemia, due to a diabetic environment, has been linked to epigenetic reprogramming of the fetus genome (Ambra *et al.*, 2014). Additionally, higher levels of genome-wide DNA methylation have been found in placental and cord samples of pregnancies complicated with GDM, when compared to normal pregnancies (Finer *et al.*, 2015).

Furthermore, studies have detected differential expression of several miRNAs obtained from samples (plasma serum, exosomes, and HUVEC) from pregnancies complicated with GDM compared to samples from normal pregnancies. From these different profiles, studies have been able to link specific miRNAs to metabolic pathways and glucose metabolism. For instance, lower expression of *miR-29a* was found in plasma serum samples from GDM women compared to controls; downregulation of *miR-29a* results in increased levels of plasma glucose (C. Zhao *et al.*, 2011). Increased expression of *miR-130b* and *miR-148a* were found in HUVEC from GDM pregnancies compared to controls; these miRNAs reduce the expression of AMPK α -1 and hence lead to lower glucose intake and insulin resistance (Tryggstad *et al.*, 2016). Overexpression of *miR-125a-3p* and *miR-224-5p* were found in exosomes released by placental chorionic villi explants and skeletal muscle tissue from patients with GDM pregnancies compared to controls; these miRNAs are involved in insulin resistance (Nair *et al.*, 2018). Increased expression of *miR-29b-3p*, *miR-122-5p* and *miR-132-3p* were found in plasma serum samples from GDM women compared to controls; these miRNAs are involved glucose level regulation and insulin resistance (Gillet *et al.*, 2019).

1.5. REGULATION OF PROTEIN EXPRESSION

Protein synthesis is called translation because the information language of a base sequence (mRNA) is translated into a different language of amino acid sequences (protein). Translation occurs in the ribosome, where the mRNA sequence will be used as a template to form an amino acid chain (protein). Once a protein is produced, several modifications can change the functionality of the protein by different processes called post-translational modifications (Bürkle, 2001). These modifications are commonly used to regulate cellular activity. Post-translational modifications can be divided into two broad categories: A) *covalent addition*: usually reversible, addition of one or more groups, such as phosphoryl, acetyl, or glycosyl to one or more of the amino acid side chains; B) *hydrolytic cleavage*: usually irreversible, cleavage of one or more peptide

bonds in a protein by proteases. Post-translational modifications occur at distinct amino acid side chains or peptide linkages, and they are most often mediated by enzymatic activity (kinases, phosphatases, transferases, ligases and proteases). Many proteins can also modify themselves using autocatalytic domains (e.g. auto-kinase and auto-proteolytic domains).

1.5.1. PROTEIN PHOSPHORYLATION

Protein phosphorylation is the most common post-translational modification. It is a reversible modification that regulates many cellular processes, including cell cycle, growth, and apoptosis (Krebs & Beavo, 1979). Phosphorylation is catalysed by kinases enzymes that transfer phosphate groups from ATP to specific amino acid residues (serine, threonine or tyrosine), as well as to other amino acids in much smaller amounts. On the other a hand, phosphatases are enzymes responsible for the removal of the phosphate groups from the amino acid residues. There are fewer phosphatases than kinases, and phosphatases are less specific than kinases.

1.6. ENDOTHELIAL JUNCTIONAL ADHESION PROTEINS AND VASCULAR INTEGRITY

Vascular permeability is mediated by the opening and closing of endothelial junctions, and by vesicular transport systems and biochemical transporters. Placental endothelial cells form paracellular clefts that ensure cell-cell adhesion and may control intercellular solute transport. These clefts are maintained by two specialised adhesive junctional regions: adherens junctions (AJs) and tight junctions (TJs) (Figure 1.5). These junctions can transfer intracellular signals that control many endothelial cell functions. Adhesion proteins of both types of junctions bind to cytoskeletal and signalling proteins, by their cytoplasmic tails, and promote anchoring of junctions to actin microfilaments and transfer of intracellular signals to the inside of the cell.

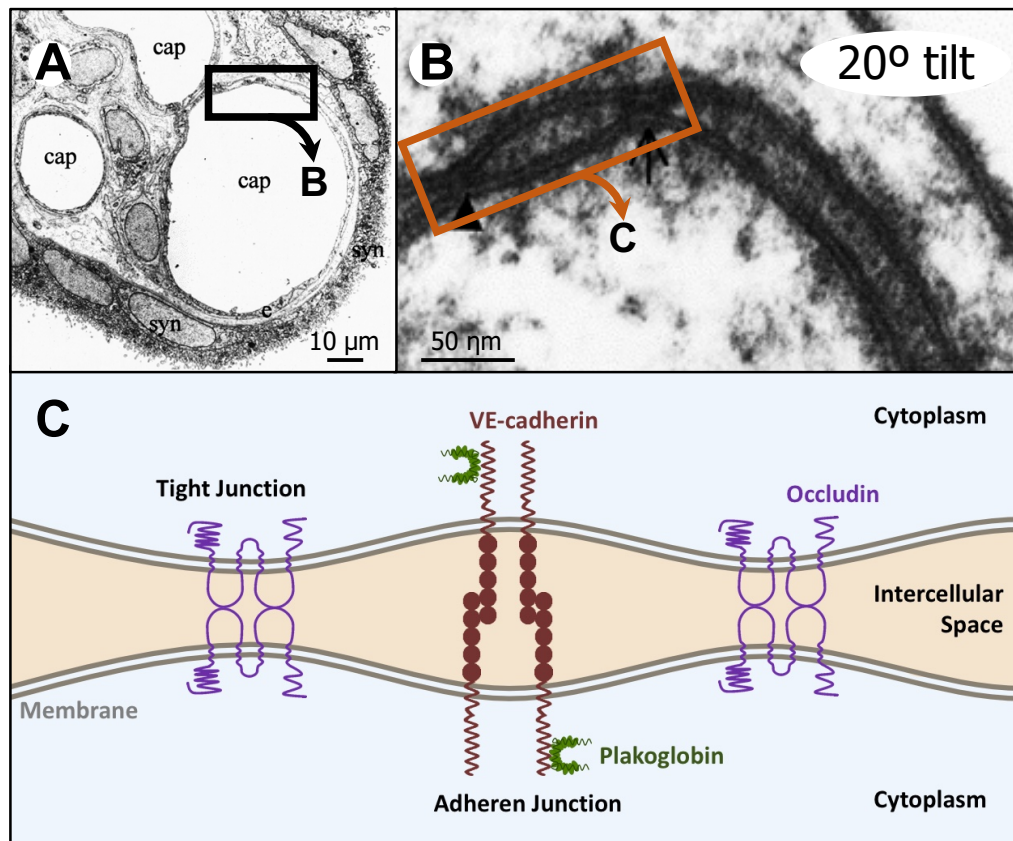


Figure 1.5. Placental vessels endothelial junctions. (A) Electron micrographs of the terminal villus end of a chorionic villus. Close apposition of the endothelium (e) and the syncytiotrophoblast (syn) at the terminal end. (B) Electron micrograph of a paracellular cleft showing two TJ and an AJ (Source: (Leach *et al.*, 2000)). (C) Schematic representation of endothelial junctions. Some members of the TJs (occludin), and AJs (VE-cadherin and plakoglobin) are represented.

AJs initiate cell-to-cell contacts and promote their maturation and maintenance, playing a pivotal role in tissue integrity. AJs are characterised by two apposing membranes separated by ~ 20 nm (Schulze & Firth, 1993). AJs are essential for the initiation and maintenance of intercellular adhesion. Endothelial AJs are formed by: VE-cadherin, claudin-5, and platelet endothelial cell adhesion molecule (PECAM-1). In turn, VE-cadherin binds to multiple intracellular partners inside the cells such as β -catenin, plakoglobin, and others. AJs organise before TJs, which cannot form in the absence of AJs.

TJs regulate the passage of ions and hydrophilic solutes through the paracellular route. This type of junction can also act as a membrane barrier

to limit the movement of lipids and proteins between the apical and the basolateral surface. TJs present ~4 nm separation between membranes of adjoining endothelial cells (Leach & Firth, 1992). These junctions are formed by three transmembrane adhesive proteins: the family of claudins, occludin, and junctional adhesion molecule (JAM). Additionally, there are several TJ-associated intracellular proteins like zona occludens (ZO)-1, ZO-2, ZO-3, cingulin, among others.

Our study will focus on the expression of two AJ proteins: VE-cadherin and Plakoglobin; and one TJ protein: Occludin. Here are some details about the description, role, and published information about their gene splice variants.

1.6.1. OCCLUDIN

Occludin protein was first discovered as a member of TJs in chicken (Furuse *et al.*, 1993). Later on, *Occludin* gene sequences were identified in mammals (Ando-Akatsuka *et al.*, 1996). Occludin is associated with increased TJ barrier function, as it plays a role in both barrier and boundary functions. However, claudins are the backbone of TJ assembly, while Occludin regulates permeability by incorporating itself into the claudin-based strands (Furuse *et al.*, 1998). Although Occludin is not essential for TJ formation, its expression is essential for barrier maintenance. Occludin-knockout mice presented a complex phenotype, such as growth retardation, mineral deposits in the brain, male sterility, and gastritis, suggesting barrier impairment in multiple organs (Saitou *et al.*, 2000). Additionally, Occludin-knockout cells lack apoptosis communication to surrounding cells.

Occludin downregulation has been observed in various disease state associated with blood-brain (BBB) or blood-retina barriers (BRB) disruption, such as multiple sclerosis, stroke, hypoxia and diabetes. For instance, in diabetic retinopathy, the inner BRB presents vascular leakage leading to macular oedema, and potentially to vision loss. Occludin and claudin-5 are the major proteins in the retinal endothelial TJs. A previous

study showed a decrease in Occludin content in the retinas of diabetic rats (Felinski & Antonetti, 2005). This loss of Occludin from TJs in diabetic endothelial TJs is thought to be induced by VEGF. A study using bovine retinal endothelial cells showed a 50% decrease in Occludin contents when cells were exposed to VEGF for 6 hours (Antonetti *et al.*, 1998). Moreover, the primary structures responsible for the BBB's barrier property are the TJs. A change in BBB permeability and a decrease in Occludin, and ZO-1 expression have been found in multiple sclerosis, stroke, cerebral ischemia, among other diseases (Förster, 2008).

Human *Occludin* gene (*OCLN*) includes nine exons. Published literature suggests the presence of several *OCLN* splice variants depending on the tissue of study (Figure 1.6) (Feldman *et al.*, 2005; Ghassemifar *et al.*, 2002; J. M. Gu *et al.*, 2008; Kohaar *et al.*, 2010; Mankertz *et al.*, 2000; Muresan *et al.*, 2000). Additionally, an NCBI and Ensembl database search supports the existence of three splice variants and one pseudogene (Pruitt *et al.*, 2014; Yates *et al.*, 2016) (Ensembl release 94 – October 2018). To our knowledge, there is no information about the expression of *OCLN* splice variants in human placental samples from pregnancies complicated with GDM.

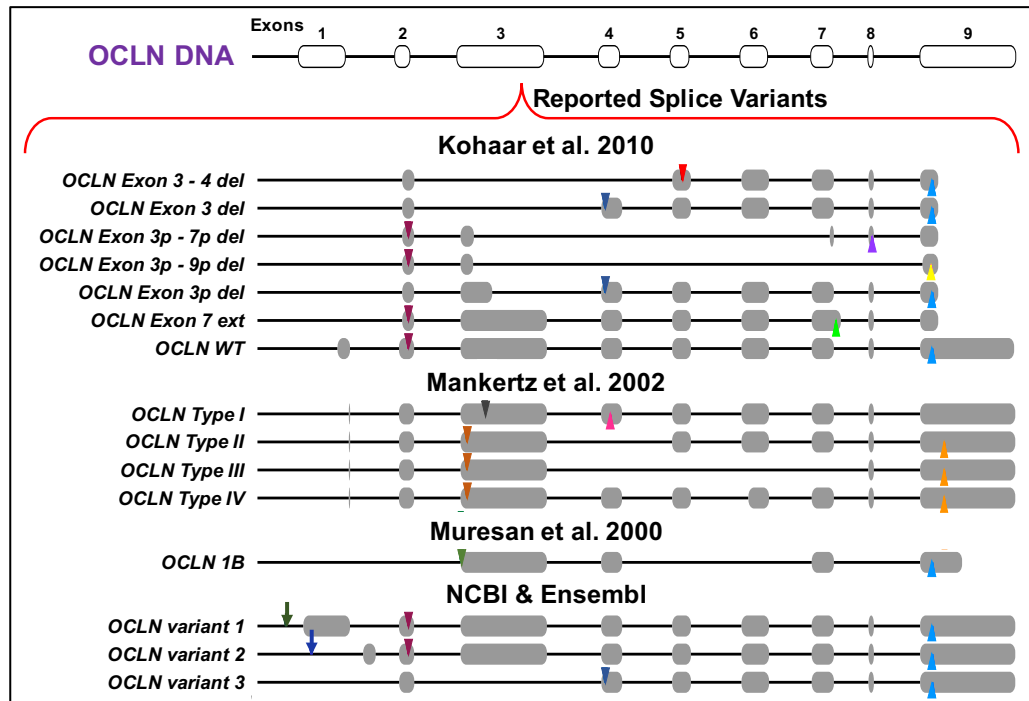


Figure 1.6. Schematic representation of *Occludin* (*OCLN*) gene splice variants reported in literature and databases. Several splice variants have been reported in human liver tissue (Kohaar *et al.*, 2010), human epithelial colonic cells (Mankertz *et al.*, 2002), human colon carcinoma cells (Muresan *et al.*, 2000), and in NCBI and Ensembl databases (Pruitt *et al.*, 2014; Yates *et al.*, 2016). *OCLN* gene consists of nine exons; these splice variants are formed by a modification of these exons due to alternative splicing. Diagram shows: two alternative transcriptional start sites (green and blue arrows); six translational start sites (magenta, dark green, dark orange, dark grey, dark blue, and red wedges facing down); and six stop sites (pink, light green, purple, light blue, yellow, and light orange wedges facing up). Exon sequences obtained from NCBI and Ensembl databases (Pruitt *et al.*, 2014; Yates *et al.*, 2016). The intron/exon structure of each variant was displayed using the Gene Structure Display Server (GSDS 2.0) web-based bioinformatics tool (<http://gsds.cbi.pku.edu.cn>) (B. Hu *et al.*, 2015).

1.6.2. VASCULAR ENDOTHELIAL CADHERIN (VE-CADHERIN)

VE-cadherin was first identified by using rat brain and retina cDNA to clone eight novel cadherin family members, one of which (clone 5) was found in endothelial cells (S. Suzuki *et al.*, 1991). This same cadherin was also identified by a monoclonal antibody that labelled the protein localising to intercellular boundaries of cultured endothelial cells (Lampugnani *et al.*, 1992). As cadherin-5 presents common features to other cadherin family members (five cadherin-like repeats in the extracellular domain, a single-pass transmembrane region, and a well-conserved cytoplasmic tail), and

because of its selective expression in endothelial cells, it was renamed as vascular endothelial cadherin (VE-cadherin) (Breviario *et al.*, 1995). VE-cadherin maintains endothelial integrity and controls vascular permeability. VE-cadherin-deficient mice embryos died at mid-gestation (E9.5 days) due to vascular insufficiency, caused by increased endothelial apoptosis (Carmeliet *et al.*, 1999). Additionally, VE-cadherin-deficient endothelial cells lose contact-inhibition, presenting a higher cell density phenotype (Lampugnani *et al.*, 2003).

Several medical conditions can be related to a change in VE-cadherin expression. For instance, cerebral cavernous malformation (CCM), a pathology that presents vascular malformations localised at the venous side of the central nervous system vessels, is associated with reduced *VE-cadherin* gene expression (Boulday *et al.*, 2011). Furthermore, diseases involving inflammation, particularly tumours inducing angiogenesis, could be associated with cell-cell adhesion defects. In human endothelial tumours like angiosarcoma or haemangiomas, lower VE-cadherin protein expression or junctional localisation is found, leading to alterations to vascular structures (Martín-Padura *et al.*, 1995).

Human *VE-cadherin* gene includes 12 exons. The first exon is entirely untranslated, and exon 12 contains untranslated regions. An NCBI and Ensembl database search supports the presence of four splice variants, and predicts the existence of seven splice variants (Pruitt *et al.*, 2014; Yates *et al.*, 2016) (Ensembl release 94 – October 2018) (Figure 1.7). *VE-cadherin* predicted splice variants found in the NCBI database were derived from a genomic sequence (NC_000016.10) annotated using gene prediction method: Gnomon. Gnomon incorporates descriptions of the basic transcriptional, translational and splicing signals, as well as length distributions and compositional features of exons, introns and intergenic regions (Souvorov *et al.*, 2010). Therefore, Gnomon decides if the sequences are completely or partially supported. *VE-cadherin* predicted splice variants found in the Ensembl database were derived from a gene source database:

GENCODE Basic. GENCODE annotates protein-coding genes, pseudogenes, long non-coding RNAs and small non-coding RNAs from publicly available data and analysis, along with the research literature to identify and characterize gene loci to the highest standard (Frankish *et al.*, 2019). Hence, GENCODE produces foundational reference genome annotations for the human genome. Each *VE-cadherin* variant translates into a different protein. To our knowledge, the expression of these predicted splice variants has not been reported so far in the literature.

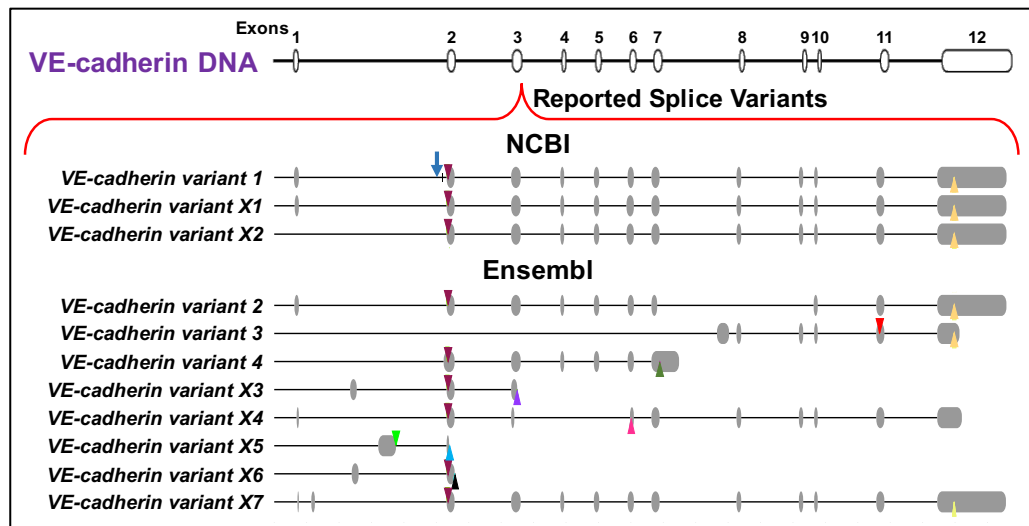


Figure 1.7. Schematic representation of *VE-cadherin* gene splice variants reported in databases. Several splice variants have been reported in NCBI and Ensembl databases (Pruitt *et al.*, 2014; Yates *et al.*, 2016). *VE-cadherin* gene consists of 12 exons; these splice variants are formed by a modification of these exons due to alternative splicing. *VE-cadherin variants X* are predicted sequences. Diagram shows: one transcriptional start site (blue arrow); three translational start sites (green, magenta, and red wedges facing down); and six stop sites (light blue, black, purple, pink, green, and orange wedges facing up). Exon sequences obtained from NCBI and Ensembl databases (Pruitt *et al.*, 2014; Yates *et al.*, 2016). The intron/exon structure of each variant was displayed using the Gene Structure Display Server (GSDS 2.0) web-based bioinformatics tool (<http://gsds.cbi.pku.edu.cn>) (B. Hu *et al.*, 2015).

1.6.3. PLAKOGLOBIN

Plakoglobin was first identified as an 83 kDa protein part of the desmosomal plaque from bovine epidermis by Franke and collaborators in 1983 (Franke *et al.*, 1983). Later on, the same group used a monoclonal antibody and a cDNA clone to determine that this desmosomal protein was

also part of AJs and thus, was given the name plakoglobin (also known as γ -catenin) (Cowin *et al.*, 1986). One of the most documented roles of Plakoglobin is cell-cell adhesion, as the protein is found in both AJs and desmosomes, by linking cadherins to the actin cytoskeleton. This protein is also involved in cell signalling, typically associated with tumour and metastasis suppressor activities (Simcha *et al.*, 1996). Furthermore, studies in Plakoglobin-null mice embryos showed that this protein is an essential component of myocardial desmosomes, as it plays a crucial role in protein sorting, desmosomal plaque assembly and intermediate filament anchorage. Plakoglobin-null mice embryos died during early development (E10 – E16) due to severe heart defects (Bierkamp *et al.*, 1996; Ruiz *et al.*, 1996)

A mutation in *Plakoglobin* gene is associated with Naxos disease, which is characterised by arrhythmogenic right ventricular dysplasia (ARVD), thickening of the skin of the hands and sole, and woolly hair (Pötter *et al.*, 2001). This inherited condition presents a recessive form of transmission. Additionally, changes in Plakoglobin gene and protein expression are found in different complications. For instance, decreased levels of *Plakoglobin* gene expression are found in several types of cancer: thyroid, breast, ovarian, cervical, among others (Pötter *et al.*, 2001; Williams *et al.*, 2000). Likewise, *Plakoglobin* overexpression can decrease the tumorigenicity and proliferation of transformed cells (Pötter *et al.*, 2001). Additionally, a study has shown a downregulation of Plakoglobin protein levels in the pancreas of rats with induced T1DM (Jiang *et al.*, 2011).

Human *Plakoglobin* gene (*JUP*) is a reverse strand transcript (transcription reads from right-to-left) that includes 14 exons. The first exon is entirely untranslated, and exon 14 contains untranslated regions. An NCBI and Ensembl database search supports the presence of seven splice variants (Pruitt *et al.*, 2014; Yates *et al.*, 2016) (Ensembl release 94 – October 2018) (Figure 1.8). *Plakoglobin* splice variants found in NCBI database are the result of the Reference Sequence (RefSeq) project and

were derived from genomic sequence to make the sequence consistent with the reference genome assembly. *Plakoglobin* predicted splice variants found in Ensembl database were derived from a gene source database: GENCODE Basic. *Plakoglobin* translation sequence starts in exon two and finishes at the beginning of exon 14; hence, all *Plakoglobin* splice variants translate into the same protein. To our knowledge, the expression of these splice variants has not been reported so far in the literature.

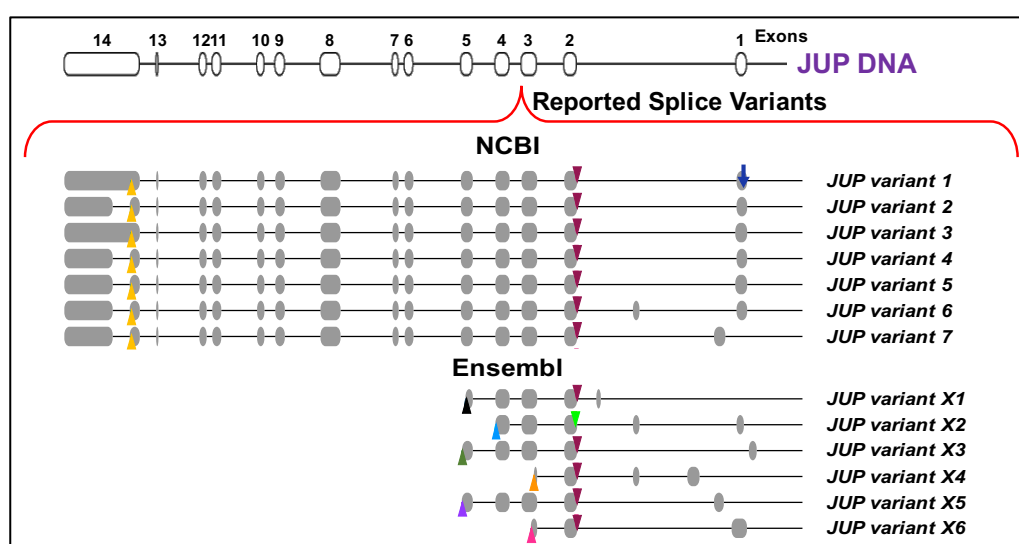


Figure 1.8. Schematic representation of *Plakoglobin* (*JUP*) gene splice variants reported in databases. Several splice variants have been reported in NCBI and Ensembl databases (Pruitt *et al.*, 2014; Yates *et al.*, 2016). *Plakoglobin* gene consists of 14 exons; these splice variants are formed by a modification of these exons due to alternative splicing. *Plakoglobin* variants X are predicted sequences. Diagram shows: one transcriptional start site (blue arrow); two translational start sites (green, and magenta wedges facing down); and seven stop sites (yellow, black, light blue, green, orange, purple and pink wedges facing up). Exon sequences obtained from NCBI and Ensembl databases (Pruitt *et al.*, 2014; Yates *et al.*, 2016). The intron/exon structure of each variant was displayed using the Gene Structure Display Server (GSDS 2.0) web-based bioinformatics tool (<http://gsds.cbi.pku.edu.cn>) (B. Hu *et al.*, 2015).

1.6.4. JUNCTIONAL MOLECULES, PLACENTA AND DIABETES

Studies have determined the localisation of Occludin, VE-cadherin and Plakoglobin in placental vasculature from first-trimester and term uncomplicated pregnancies. First-trimester placental vessels are characterised by an absence of Occludin and Plakoglobin; while VE-

cadherin is localised in large and small vascular profiles within the chorionic villi (Leach *et al.*, 2002). On the other hand, term-placental vessels are characterised by the presence of these three junctional molecules. Occludin is present in arteries, veins and arterioles from term-placental villus trees (Leach *et al.*, 2000). Plakoglobin can be found in clefts of stromal villus vessels, with weak or negative immunoreactivity in intermediate and terminal villi (Leach *et al.*, 2000). Similar to the first-trimester profile, VE-cadherin is localised in all chorionic villi (Leach *et al.*, 2000).

In pregnancies complicated with diabetes, the junctional localisation of Occludin and VE-cadherin is affected. For instance, reduced junctional Occludin localisation was found in placental vessels from GDM pregnancies compared to normal samples (Babawale *et al.*, 2000). Likewise, reduced junctional VE-cadherin localisation was found in placental vessels from diabetic pregnancies (T1DM, T2DM and GDM) compared to normal placental samples (Babawale *et al.*, 2000; Baumüller *et al.*, 2015; Leach *et al.*, 2004). To our knowledge, the localisation of Plakoglobin in placental samples from pregnancies complicated with diabetes has not been reported so far in the literature.

The reduction of junctional VE-cadherin in placental vasculature produces alterations to the paracellular endothelial barrier. A study used a perfusion system, and determined an increase in dextran tracer (76-M_r) leakage in placental vessels from T1DM pregnancies compared to normal pregnancies (Leach *et al.*, 2004). This placental vasculature leakage was associated with a reduced junctional VE-cadherin localisation with no overall loss of VE-cadherin protein (Leach *et al.*, 2004). Furthermore, perfusion of placental vasculature from non-diabetic pregnancies with 15 mM glucose for 3 hours (maternal circuit) showed increased dextran tracer (76-M_r) leakage and reduced junctional VE-cadherin localisation in vessels compared to controls (5 mM glucose perfusion) (Sciota, 2012). Hence, hyperglycaemia may contribute to paracellular leakage and increase transplacental glucose transport. Furthermore, a pilot study from our group

used perfusion of term-placentae to show that placental vessels from GDM pregnancies were significantly leaky (20% vascular profiles) to dextran tracer (76-M_r) compared to normal placentae (10% vascular profiles) (Reeve *et al.*, 2006). However, the extent to which a GDM environment affects the gene expression of junctional molecules has not been determined. Therefore, this study will focus on the gene and protein expression of Occludin, VE-cadherin, and Plakoglobin splice variants in placental vasculature from pregnancies complicated with GDM compared to samples from normal pregnancies.

1.7. PLACENTAL VASCULATURE – ANGIOGENESIS

Placental vasculature has to proliferate to supply the increasing metabolic needs of the growing fetus. This is why the placental vasculature is plastic and constantly changing throughout gestation to meet these demands. The development of the placental vasculature network is tightly controlled by two processes throughout gestation: vasculogenesis and angiogenesis. *Vasculogenesis* is the process by which new blood vessels are formed by the differentiation and migration of endothelial progenitor cells to form interconnecting capillaries. On the other hand, *Angiogenesis* is the sprouting of new capillaries from pre-existing vessels.

Several growth factors and their receptors control placental vasculogenesis and angiogenesis (Figure 1.9). Vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) are the most predominant growth factors (Cvitic *et al.*, 2014). At early stages of placentation (the start of vasculogenesis), mesenchymal villi present FGF, VEGF, and its receptors: VEGFR1 and VEGFR2 (Demir *et al.*, 2007; Wang, 2017). The first sign of placental vasculogenesis can be detected around day 21 post-conception (PC), during the development of first villus vessel as a result of FGF inducing the differentiation of hemangiogenic stem cells into hemangioblasts and angioblasts (Figure 1.9) (Demir *et al.*, 2007; Wang, 2017). Later on, VEGF will mediate the assembly of primordial vessels. Around day 32 PC, villous endothelial tubes start to contact each other and the fetal allantoic vessels

in the probable umbilical cord, establishing a primitive fetoplacental circulation system (Charnock-Jones *et al.*, 2004; Wang, 2017). Finally, the transition from vasculogenesis to angiogenesis is mediated by VEGFR1, VEGFR2, and VEGF. During angiogenesis, placental vascular remodelling is coordinated by VEGF, placental growth factor (PIGF), and Angiopoietin (Ang) -1 and -2. The following angiogenic processes can be grouped in three overlapping periods: 1) Week 7 to 24 PC: Formation of capillary networks by branching angiogenesis mediated by a higher expression of VEGF-A and VEGFR-2. 2) Week 15 to 32 PC: Regression of peripheral webs and formation of central stem vessels. PIGF expression increases steeply as non-branching angiogenesis gradually replaces branching angiogenesis. 3) Week 25 PC to term: placental villi present an increased expression of VEGFR-1, PIGF, Ang-1 and Ang-2. During this period, terminal villi are formed, and their structure is determined by a balance between branching (controlled by VEGF-A) and non-branching angiogenesis (controlled by PIGF). Well-balanced secretory levels of VEGF and PIGF are responsible for a balance of branching and non-branching angiogenesis; therefore, evenly formed grape-like terminal villi (Benirschke *et al.*, 2006). Moreover, Ang-1 and -2 are also present in the terminal villi (Leach *et al.*, 2002) (Figure 1.9).

As placental transport of nutrients and waste is dependent on vascular development; vasculogenesis and angiogenesis play an essential role in pregnancy outcome and fetal development. There is evidence of alterations in these parameters in pregnancies complicated by intrauterine growth restriction (IUGR), pre-eclampsia or diabetes. For instance, an increase in circulating glucose concentrations in diabetic mothers will promote atherosclerosis that may impair uteroplacental blood flow and lead to hypoxia (Pietro *et al.*, 2010). This hypoxic condition, during the last term of pregnancy, will lead to increased levels of VEGF and branching angiogenesis. Additionally, IUGR placentae display alterations in vasculogenesis and branching angiogenesis. These complications lead to a decreased surface area, volume, and the number of terminal villi in IUGR placentae when compared to normal pregnancies (Mayhew *et al.*, 2003). In

pre-eclampsia, increased levels of anti-angiogenic factors (sFlt-1 and sEng) that antagonise the effects of pro-angiogenic factors (VEGF, PlGF) will affect the maintenance of the placental vasculature. Unfortunately, pre-eclampsia not only affects the blood supply to the placenta (and hence the fetus) but also affects the mother's kidneys and higher blood pressure.

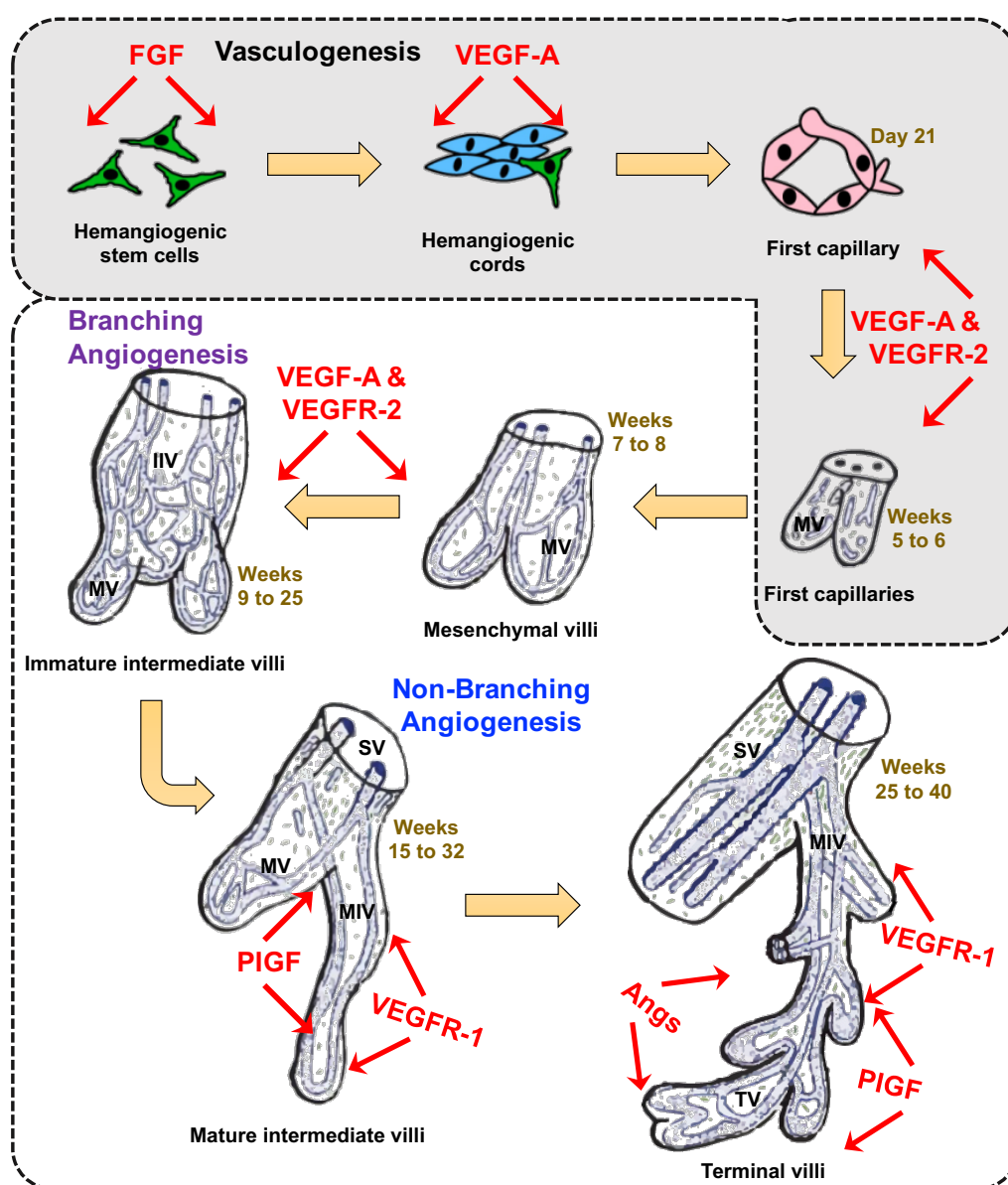


Figure 1.9. Placental villus development: vasculogenesis and angiogenesis processes. Vasculogenesis (grey panel) Stage I: hemangiogenic stem cells (green) differentiate into hemangioblasts and angioblasts (blue); FGF expression controls this step. Stage II: development of first villus vessel (pink), around day 21 to week 6 PC. VEGF-A and VEGFR-2 will define the end of vasculogenesis and the beginning of angiogenesis; both will mediate the next stages. Angiogenesis (white panel) Stage III: the first vessels fuse to form a simple capillary bed, mesenchymal villi (MV) (weeks 7 to 8 PC). Stage IV: development of immature intermediate villi (IIV)

from MV (weeks 9 to 25 PC); capillary bed expands by branching angiogenesis. Stage V: most of the IIV are transformed into stem villi (SV). Additionally, periphery MV are transformed into mature intermediate villi (MIV) by non-branching angiogenesis (weeks 15 to 32 PC). This stage is controlled by increased levels of PIGF and VEGFR-1 and decreased levels of VEGF-A. Stage VI: MIV present longitudinal capillary growth that is bulging against the surface, causing the development of terminal villi (TV) (weeks 25 to 40 PC). Well-balanced levels of VEGF-A and PIGF will generate a balance of branching and non-branching angiogenesis; thus, evenly formed grape-like TV. Additionally, Ang-1 and -2, and VEGFR-1 regulate this stage with VEGF-A and PIGF. Modified from: (Benirschke *et al.*, 2006)

Our study will focus on the expression of three angiogenic factors: VEGF-A, Ang-1, and Ang-2; and their respective receptors: VEGFR-2 and Tie-2. These molecules are also considered permeability factors due to their interactions with junctional molecules. Furthermore, our study also focused on the expression of a matrix metalloproteinase (MMP-9), as it contributes to the maintenance and remodelling of the extracellular matrix during angiogenesis. MMP-9 also presents proteolytic activity towards junctional molecules.

1.7.1. VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) – A

VEGF is a family of growth factors and their receptors, which are essential regulators of blood vessel formation. This family includes four different VEGF subtypes termed: VEGF-A, -B, -C, and -D; plus, placental growth factor (PlGF). Their biological function is mediated by a family of protein tyrosine kinase receptors: VEGFR-1/Flt-1, VEGFR-2/Flk-1, and VEGFR-3/Flt-4.

VEGF-A was first described as a vascular permeability factor (VPF) found in a tumour taken from guinea pigs (Senger *et al.*, 1983). Later in 1989, two groups independently reported the purification and sequencing of an endothelial cell-specific mitogen, which they respectively called: VEGF (N. Ferrara & Henzel, 1989) and vasculotropin (Plouët *et al.*, 1989). Subsequent molecular cloning of VEGF and VPF revealed that both activities

were expressed by the same molecule (Keck *et al.*, 1989). During the 90s, several properties specific to VEGF-A were determined:

- Secreted by endothelial cells and exerts a direct and restrictive effect on them via interaction with cellular receptors VEGFR-1 and VEGFR-2.
- It induces a pleiotropic response (endothelial cells proliferate, migrate, and assemble into tubes) and increases permeability.
- Hypoxia highly regulates its expression.
- It is a potent growth factor.

Additionally, inactivation of the *VEGF-A* gene in mice resulted in embryonic lethality (E11 days) (N. Ferrara, 1999). These embryos presented significant defects in the vasculature of different tissues and organs, including the placenta, heart, brain, and nervous system. The human *VEGF-A* gene transcribes into several splice variants due to alternative splicing. Therefore, *VEGF-A* mRNA splicing generates two families of proteins: VEGF-A_{xxx} (pro-angiogenic) and VEGF-A_{xxx}b (anti-angiogenic), xxx is the number of amino acid of the mature protein (Perrin *et al.*, 2005).

VEGF-A contributes to the pathogenesis of a diversity of diseases in which the integrity of blood vessels is compromised, including brain tumours, heart complications and diabetic retinopathy (Harhaj *et al.*, 2006). For instance, VEGF-A expression is induced under pathological conditions associated with ischemia, such as ischemic heart disease (Poltorak *et al.*, 2000). Increased levels of VEGF-A have also been found in the retinas of animal models of diabetes and patients with diabetes (Antonetti *et al.*, 1998; Harhaj *et al.*, 2006). Previous studies in human placentae from pregnancies complicated with diabetes have found differing results regarding VEGF-A expression. For instance, the expression levels of VEGF-A and both receptors (VEGFR-1 and -2) were increased in syncytiotrophoblast and terminal villi of T1DM placental samples when compared to samples from normal pregnancies (Dubova *et al.*, 2012). On the contrary, maternal and cord plasma levels of VEGF-A were similar between GDM and normal

pregnancies, though VEGF-A levels tended to be lower in GDM samples (Gauster *et al.*, 2012).

1.7.2. VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR (VEGFR) -2

VEGFR-2 is a type III receptor tyrosine kinase, which is the principal VEGF receptor on endothelial cells. VEGFR-2 was first described in a human endothelial cell cDNA library (Terman *et al.*, 1991). VEGFR-2 is essential for endothelial cell biology during development and in the adult, in physiology and pathology. The VEGFR-2 expression is highest in the early stages of pregnancy and declines steeply as pregnancy advances. It is mostly expressed in the fetoplacental endothelium and mediates mitogenic cellular responses (Clark *et al.*, 1996). In the adult, VEGFR-2 is mostly expressed on vascular endothelial cells, although expression is also detectable on neuronal cells, megakaryocytes, and haematopoietic stem cells (Katoh *et al.*, 1995). VEGFR-2 is the principal mediator of several physiological and pathological effects of VEGF-A on endothelial cells, such as differentiation, proliferation, migration, survival, and permeability. Studies in VEGFR-2 knockout mice have shown the importance of this receptor for recruitment and early differentiation of haemangiogenic cells into fetoplacental capillaries (Shalaby *et al.*, 1995, 1997). Knockout mice die at E8.5 days with a phenotype similar to that of VEGF-A-deficient mice.

VEGFR-2 is implicated in both tumour angiogenesis, and diabetic retinopathy. Studies in rat brains transplanted with glioma cells showed that the expression of VEGFR-2 is upregulated in the tumour vasculature endothelial cells when compared with normal brain vasculature (Plate *et al.*, 1993). Additionally, studies in diabetic retinopathy have shown higher protein levels of VEGFR-2 in retinal and choroidal vessels of diabetic animals, when compared to standard controls (Sun *et al.*, 2014). As well as higher VEGFR-2 levels in macular vessels from diabetic patients, when compared to nondiabetic controls (Sun *et al.*, 2014). Furthermore, mixed results have been shown for VEGFR-2 expression levels in human GDM placental samples. For instance, lower gene and protein expression of

VEGFR-2 was found in placental tissue from diet-controlled GDM pregnancies when compared to normal pregnancies (Meng *et al.*, 2016). On the contrary, lower gene and higher protein levels of VEGFR-2 were found in placental samples of GDM pregnancies when compared to normal pregnancies (Troncoso *et al.*, 2017).

1.7.3. TIE-2 RECEPTOR

The Tie-receptor family is endothelial cell-specific. This family received its name due to their Tyrosine kinase with Immunoglobulin (Ig)-like and Epidermal growth factor (EGF)-like homology domains. The Tie-receptor family is involved in the regulation of two antagonistic processes: vascular homeostasis maintenance and vessel maturation, as well as vascular destabilisation and remodelling. During the early 1990s, different groups were searching for novel tyrosine kinase proteins expressed by endothelial cells; two receptors were initially isolated: Tie-1 and Tie-2 (Maisonpierre *et al.*, 1993; Partanen *et al.*, 1992; Sato *et al.*, 1993; Ziegler *et al.*, 1993). Tie-2 has at least four known ligands: Angiopoietin (Ang)-1, Ang-2, Ang-3, and Ang-4. To our knowledge, there have not been found ligands for the Tie-1 receptor. Following Tie-2 activation, intracellular signalling pathways are activated, resulting in cell migration, cell survival, inflammation and permeability. Tie-2 knockout mice die by E10.5 due to severe vascular remodelling defects, insufficient vessel stabilisation, and perturbed vascular maturation (Dumont, Gradwohl, *et al.*, 1994; Suri *et al.*, 1996).

Tie-2 is mostly expressed by endothelial cells during development. However, its expression persists in quiescent adult endothelial cells, although at reduced levels in some organs (e.g. heart and brain) (Partanen & Dumont, 1999). On a pathology level, Tie-2 expression is upregulated during tumour angiogenesis. Higher Tie-2 expression was found in rat tissue showing angiogenesis (tumours and healing skin wounds) when compared to quiescent tissue (Wong *et al.*, 1997). Likewise, higher levels of Tie-2 were found in human carcinomas (breast and lung cancer) (Peters *et*

al., 1998; Takahama *et al.*, 1999). Additionally, studies in human placental samples from pregnancies complicated with pre-conception diabetes, preeclampsia, and IUGR have shown that VEGF and Tie-2 expression are not altered between groups (Vuorela & Halmesmäki, 2006).

1.7.4. ANGIOPOIETINS 1 AND 2 (ANG-1 AND ANG-2)

A second family of growth factors specific for the vascular endothelium was identified as a potential source of ligands for the Tie-2 receptor, named Angiopoietins (Ang). There are three Angiopoietins known in humans: Ang-1, Ang-2, and Ang-4 (Ang-3 is the mouse orthologue of human Ang-4). The best-characterised Angiopoietins are Ang-1 and Ang-2, two secreted glycoproteins that bind with similar affinity to Tie-2. Ang-1 was identified as a ligand for the Tie-2 receptor by a secretion-trap cloning approach (Davis *et al.*, 1996). Later on, Ang-2 was identified by homology screening, and it was shown to be a naturally occurring antagonist of Ang-1 and Tie-2 (Maisonpierre *et al.*, 1997). Ang-1 is expressed in perivascular cells in the quiescent vasculature, whereas Ang-2 expression occurs in areas of endothelial activation and angiogenesis (Davis *et al.*, 1996; Maisonpierre *et al.*, 1997). Ang-1 is required for correct organisation and maturation of newly formed vessels and promotes quiescence and structural integrity of adult vasculature. Ang-1 knockout mice die by E12.5 days with heart defects. These mice display a vasculature with decreased complexity, dilated vessels, diminished branching, reduced numbers of large vessels and fewer endothelial cells (Suri *et al.*, 1996). Ang-2 can activate Tie-2 receptor or suppress the effects of Ang-1; allowing vessel growth or regression depending on the presence or absence of VEGF-A (Maisonpierre *et al.*, 1997). Ang-2 knockout mice are born healthy, but develop chylous ascites within a few days after birth. Depending on the genetic background, Ang-2 deficient mice die within the first 14 days after birth or develop to adulthood with persistent vascular defects and an impaired response to inflammatory challenges (Fagiani & Christofori, 2013). On the other hand, Ang-2 overexpression disrupts blood vessel formation. Transgenic mice die at E9-9.5 with heart abnormalities similar to those observed in embryos

lacking Ang-1 or Tie-2 (Maisonpierre *et al.*, 1997). Since Ang-2 antagonises the stabilising effects on vessels of Ang-1, the ratio of these factors appears to be of importance for vessel formation.

Ang-1 is an essential player in the early development of the heart. Its role increases as the rest of the vasculature mature (Davis & Yancopoulos, 1999). In human placenta, Ang-1 is found in the cyto-syncytiotrophoblast bilayer of the first-trimester placenta, and in paravascular tissues of stem villi at term (Leach *et al.*, 2002). Under pathological conditions, Ang-1 might be involved in inflammatory diseases. In rheumatoid arthritis, higher levels of angiogenic factors (VEGF-A) and of Ang-1 were found in synovial fibroblasts (Gravallese *et al.*, 2003). Studies using placental tissue or plasma samples from patients with diabetes have shown no change in Ang-1 expression levels (Janota *et al.*, 2003; H. S. Lim *et al.*, 2005; Loukovaara *et al.*, 2004). Additionally, Ang-1 role in tumour angiogenesis is controversial. Studies have shown that overexpression of *Ang-1* leads to reduced tumour growth in several tumour models (Hawighorst *et al.*, 2002; Hayes *et al.*, 2000; Stoeltzing *et al.*, 2003); while studies in rat gliomas and plasma cell tumours show that overexpression of *Ang-1* promotes tumour growth (Machein *et al.*, 2004; Nakayama *et al.*, 2004).

Hypoxia upregulates Ang-2 levels. Under physiological conditions, Ang-2 is expressed in regions of vascular remodelling. For instance, during pregnancy, higher levels of Ang-2 are found in term placental stem and terminal villi (Leach *et al.*, 2002). Ang-2 expression is also upregulated under pathological conditions, such as in the endothelium of tumours, and in the tumour cells themselves. Hence, Ang-2 seems to be the earliest marker of blood vessels that are perturbed by invading tumour cells. Additionally, Ang-2 role in diabetes is controversial. Studies using plasma from T1DM pregnant women showed lower levels of Ang-2 in T1DM when compared to normal pregnancies (Loukovaara *et al.*, 2004). On the contrary, studies using placental tissue from T1DM pregnant women found similar gene levels of *Ang-1*, *Ang-2*, *Tie-2*, *VEGF-A*, *VEGFR-1*, *VEGFR-2*, when

compared to normal pregnancies (Janota *et al.*, 2003). Furthermore, studies in T2DM using plasma samples showed higher levels of Ang-2 in diabetic patients, when compared to controls (H. S. Lim *et al.*, 2005).

1.7.5. MATRIX METALLOPROTEINASE (MMP) -9

Matrix metalloproteinases (MMPs) are a family of structurally related but genetically distinct enzymes that degrade extracellular matrix and basement membrane components. There are more than 20 human MMPs described that, based on the substrate specificity and molecular structure, can be classified into collagenases, gelatinases, stromelysins, membrane-type MMPs, and other MMPs. MMP-9 is a member of the gelatinases, also known as gelatinase B, that was initially identified as a secretion product of macrophages and neutrophils (Hibbs *et al.*, 1987; Mainardi *et al.*, 1984). MMP-9 plays an essential role in the maintenance and remodelling of the extracellular matrix that occurs in morphogenesis, tissue repair, angiogenesis, uterine involution and tumour invasion. Due to MMPs involvement in the preparation of the endometrium for pregnancy, implantation into the uterus and or embryo development, it is surprising that MMP-9 knockout mice are viable and able to reproduce. Several studies have implemented animal models with knockout MMP-9 to address different questions (J. Hu *et al.*, 2007). For instance, MMP-9-deficient mice showed a phenotype of growth retardation with bones that were shorter than those of control mice; revealing that MMP-9 has an essential role in the maintenance of bone structure (Vu *et al.*, 1998).

On a pathology level, MMP-9 can be expressed by tumour cells or stromal cells surrounding the tumour. Upregulation of MMP-9 was found in breast cancer (Merdad *et al.*, 2014). Additionally, MMP-9 may play a key role in metastatic tumour cell behaviour. For instance, MMP-9 levels were higher on normal lung endothelial cells from patients carrying distant tumours than patients without tumours (Hiratsuka *et al.*, 2002). This study also showed that expression of VEGFR-1 by distant primary tumours would induce MMP-9 expression in pre-metastatic lung endothelial cells, hence

promoting lung metastasis. Furthermore, a study with diabetic-induced rats showed higher expression of MMP-9 in their retinas. Normal retinal endothelial cells exposed to high glucose have shown elevated levels of MMP-9, proteolytic degradation of Occludin, and disruption of TJs (Giebel *et al.*, 2005). Moreover, MMP-9 knockout diabetic-induced mice were used to determine the development of diabetic retinopathy (Kowluru *et al.*, 2011). This study showed that regulation of MMP-9 expression prevents retinal capillary cells from undergoing apoptosis. Thus, suggesting a new target to prevent the development of diabetic retinopathy. On the other hand, lower levels of MMP-9 can also generate consequences. In normal pregnancies, MMP-9 is found in trophoblast villus cells and mesenchyme of the placenta (Matjila *et al.*, 2013). However, pregnancies complicated with preeclampsia present a downregulation of MMP-9 expression (Qiao *et al.*, 2005). Reduced expression of MMP-9 will limit the ability of the fetus to invade the maternal tissue via degradation of extracellular matrix, generating complications for the mother (preeclampsia) and fetus (IUGR).

1.7.6. ANGIOGENIC FACTORS AND VASCULAR PERMEABILITY

VEGF-A, Angiopoietins and MMP9 not only contribute to the endothelial angiogenic pathway. These factors also play an important role in the stability of the endothelial paracellular barrier (Figure 1.10). For instance, high levels of VEGF-A increase vascular permeability by downregulation of Occludin protein content (Antonetti *et al.*, 1998), and by phosphorylation of VE-cadherin, β -catenin and Plakoglobin (Wallez & Huber, 2008). Upon binding, VEGF-A promotes autophosphorylations of VEGFR-2. Afterwards, the Src family of tyrosine kinases are activated. Src directly phosphorylates VE-cadherin (Wallez *et al.*, 2007), Plakoglobin (Miravet *et al.*, 2003), p120 (Castaño *et al.*, 2007) and Occludin (Elias *et al.*, 2009; Murakami *et al.*, 2009) affecting the paracellular barrier stability. Additionally, Src phosphorylates multiple substrates, including focal adhesion kinase (FAK) and p21-activated kinase (PAK). FAK will bind to VE-cadherin cytoplasmic tail and phosphorylate β -catenin, this will facilitate VE-cadherin- β -catenin dissociation and AJ breakdown (X. L. Chen *et al.*,

2012). PAK will phosphorylate VE-cadherin cytoplasmic tail resulting in the recruitment of β -arrestin2 to the phosphorylated site; β -arrestin2 will promote VE-cadherin internalization into a vesicle and disassembly of AJs (Gavard & Gutkind, 2006). Furthermore, VEGF-A promotes Occludin degradation through an ubiquitin-proteasome system (Murakami *et al.*, 2009). VEGF-A also activates TR3/Nur77, which will affect the vascular barrier stability by increasing the expression of nitric-oxide synthase, downregulating VE-cadherin and Occludin protein expression, and reducing the stability of VE-cadherin mRNA (D. Zhao *et al.*, 2011). Ang-2 will bind to Tie-2, and the activation of the receptor will depend on Ang-2 multimerization. As a tetramer, Ang-2 will bind to Tie-2 and induce its phosphorylation. This activation will only generate angiogenesis. As a dimer, Ang-2 will bind to Tie-2 but does not induce phosphorylation or angiogenesis. However, Ang-2 dimer seems to be involved in the loss of junctional VE-cadherin and increase endothelial permeability (Hakanpaa *et al.*, 2015). Additionally, MMP-9 proteolytic activity downregulates Occludin (Caron *et al.*, 2005; Giebel *et al.*, 2005) and VE-cadherin (Navaratna *et al.*, 2007) protein expression and increases endothelial barrier permeability.

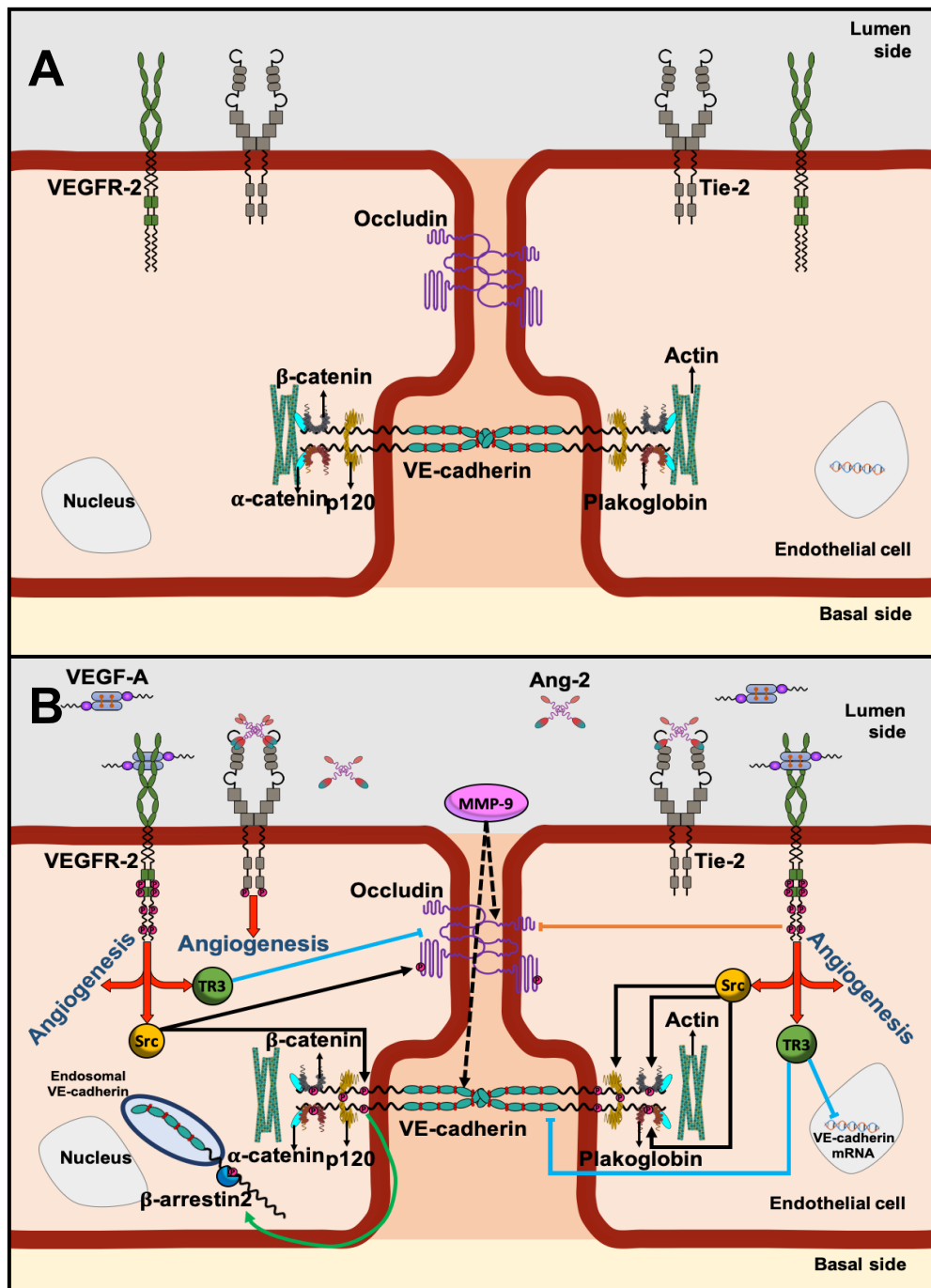


Figure 1.10. Schematic representation of a model of molecular mechanisms by which VEGF-A and MMP-9 promote vascular permeability. (A) Endothelial cell with junctional molecules: Occludin (purple), VE-cadherin (green), p120-catenin (yellow), β-catenin (grey), α-catenin (light blue), and Plakoglobin (brown) maintaining the paracellular barrier. **(B)** Autophosphorylation of VEGFR-2 (green) by binding of VEGF-A (blue) leads to angiogenesis, and activation of Src family and TR3. Src will phosphorylate (pink circles) Occludin, VE-cadherin, β-catenin, p120-catenin and Plakoglobin (black arrows). VE-cadherin phosphorylation will result in its internalisation (endosomal VE-cadherin) by β-arrestin2 (blue) (green arrow). TR3 will inhibit Occludin and VE-cadherin protein expression, as well as VE-cadherin mRNA (light blue inhibition). VEGFR-2 phosphorylation will degrade Occludin protein by ubiquitination (orange inhibition). Ang-2 (green and orange)

dimer will not activate Tie-2 receptor (grey). Ang-2 tetramer will induce autophosphorylation of Tie-2 and activate angiogenesis. MMP-9 proteolytic activity downregulate Occludin and VE-cadherin proteins will (black dashed arrows). As a result of these events, these endothelial cells will undergo angiogenesis and the endothelial barrier permeability will increase.

Nonetheless, considering VEGF-A multiple splice variants, VEGF-A can exert different levels of permeability and angiogenesis properties. For instance, VEGF-A₁₆₅ induces higher endothelial permeability than VEGF-A₁₂₁ (Fearnley *et al.*, 2015). Furthermore, VEGF-A_{165b} is categorised as anti-angiogenic factor and a protector of the paracellular barrier. A study using human placentae showed that perfusion of the placental microvascular bed with VEGF-A_{165a} increases vascular permeability and decreases junctional VE-cadherin vascular profiles (Pang *et al.*, 2017). However, sequential addition of VEGF-A_{165b} to VEGF-A_{165a} perfusions resulted in a recovery of junctional VE-cadherin at the paracellular barrier (Pang *et al.*, 2017). Similarly, human primary retinal pigmented epithelial (RPE) cells cultured with VEGF-A_{165a} showed reduced junctional Occludin protein expression and localisation, as well as increased paracellular permeability (TEER) (Ved *et al.*, 2017). Nonetheless, co-culture of RPE cells with VEGF-A_{165a} and VEGF-A_{165b} resulted in a recovery of junctional Occludin expression and localisation, and reduction of RPE permeability (Ved *et al.*, 2017). These responses could be due to the fact that VEGF-A_{165b} binds to VEGFR-2 with the same affinity as VEGF-A_{165a}, but it does not activate the receptor or stimulate the downstream signalling pathways (Woolard *et al.*, 2004) (Figure 1.11). As VEGF-A, Ang-1 promotes endothelial survival, proliferation, and migration by activation of Tie-2 receptor. However, whereas VEGF-A causes vascular permeability, Ang-1 is also considered an anti-permeability factor, as it promotes the stabilisation and the maturation of growing blood vessels (Fukuhara *et al.*, 2010; Ngok *et al.*, 2012; Senger *et al.*, 1983; Thurston *et al.*, 2000) (Figure 1.11). Ang-1 can induce *Occludin* gene expression (Hori *et al.*, 2004), and inhibit VEGF effects over Occludin (Siddiqui *et al.*, 2015) and VE-cadherin phosphorylation and internalisation in endothelial cells (Gavard *et al.*, 2008). Therefore, beyond angiogenesis, angiogenic growth factors also control vascular permeability. Hence, this

study will determine the gene expression of *VEGF-A*, *Ang-1*, *Ang-2*, *VEGR-2*, *Tie-2*, and *MMP-9* in placental vasculature from normal pregnancies and pregnancies complicated with GDM.

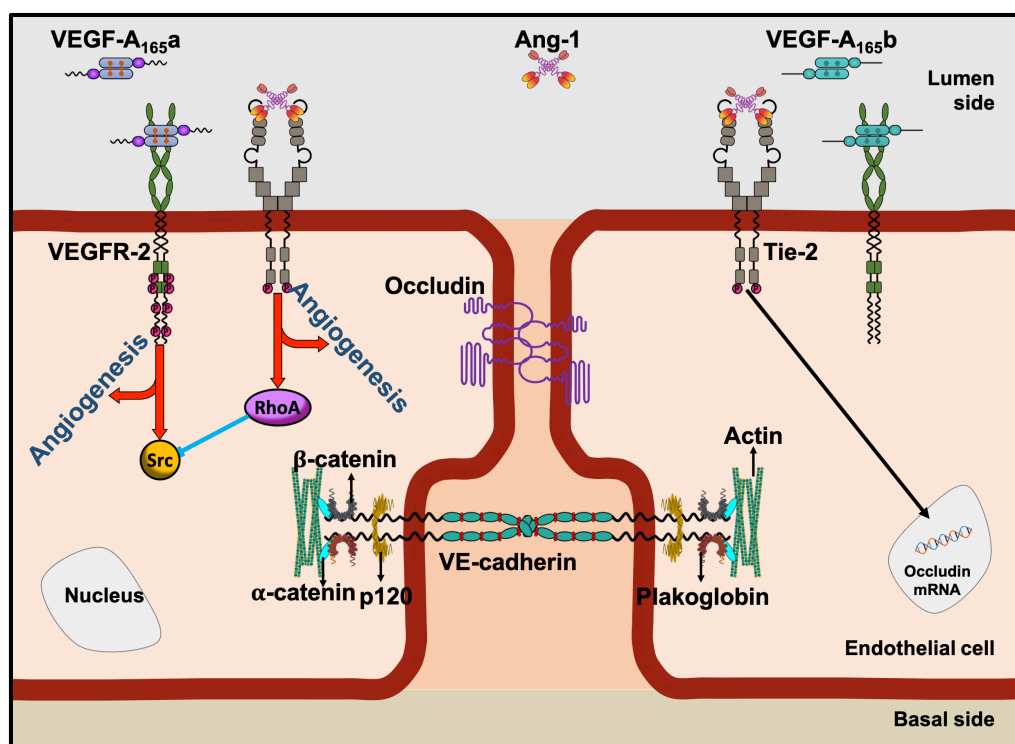


Figure 1.11. Schematic representation of a model of molecular mechanisms by which Angiopoietin-1 and VEGF-A_{165b} promote vascular integrity. VEGF-A_{165a} (blue) binds to VEGFR-2 (green) and autophosphorylation leads to angiogenesis and activation of the Src kinase family (yellow). Ang-1 (orange and yellow) binds to Tie-2 (grey) receptor and autophosphorylation leads to angiogenesis and activations of RhoA (purple). RhoA inhibits Src, preventing the phosphorylation of junctional molecules (Gavard *et al.*, 2008). Tie-2 activation promotes Occludin gene expression (black arrow). As a result of these events, these endothelial cells undergo angiogenesis, but maintain paracellular barrier integrity. When, VEGF-A_{165b} (turquoise) binds to VEGFR-2 (green), the receptor does not phosphorylate; hence, VEGF-A_{165b} acts as an anti-angiogenic and anti-permeability factor.

1.8. CENTRAL HYPOTHESIS

Exposure to the diabetic milieu during pregnancy (gestational diabetes) leads to changes in the expression of the splice variants of junctional adhesion molecules in placental tissue, despite treatments used to control glucose levels

1.9. AIMS

To test this hypothesis, the aims of this study were:

- To measure the gene and protein expression of Occludin (TJ), Plakoglobin, and VE-cadherin (AJ) splice variants in human placental tissue of normal pregnancies, diet-controlled and metformin-controlled GDM pregnancies (Chapter Three).
- To compare the gene expression of the angiogenic/permeability factors (VEGF-A, Ang-1, Ang-2, MMP-9) and their receptors (VEGFR-2, Tie-2) in human placental tissue from normal and GDM pregnancies (Chapter Four).
- To test the effect of a hyperglycaemia insult (15 mM D-glucose) on Occludin gene expression and junctional localisation in a placental explant *ex vivo* model (Chapter Five).
- To evaluate the expression of regulatory factors that target Occludin expression (DNA promoter methylation, microRNAs, and phosphorylation) in human placental tissue of normal and GDM pregnancies (Chapter Six).
- To test the effect of *miR-181a-5p* overexpression in *Occludin* gene expression in HUVEC transfection *in vitro* model (Chapter Six).

CHAPTER 2

GENERAL METHODOLOGY

2.1. STUDY DESIGN

Power analysis was performed to determine the minimum number of samples required to detect a significant difference between placental samples from normal pregnancies and pregnancies complicated with GDM. These calculations were based on the results obtained by Babawale and collaborators using human placental samples from normal and GDM pregnancies (Babawale *et al.*, 2000) (Table 2.1). Sample sizes of 11 (VE-cadherin), seven (Occludin) or two (Phospho-Tyrosine) would be required to achieve a power of 0.80 with an alpha of 0.05 using two-tailed t-tests. A minimum sample size of seven per condition should be enough to determine a change in expression between normal and GDM placental samples, considering an average of the power analysis. This analysis was performed using a Java Applet (Lenth, 2006).

Table 2.1. Information of the relative incidences of immuno-location of molecules in placental vessels from normal and GDM pregnancies used for power calculations for this study. The percentage of vessel profiles are expressed as mean (SEM) (Babawale *et al.*, 2000). Standard deviation was calculated from SEM values ($SD = SEM \times \sqrt{N}$; where $N= 5$ per group). True difference of mean was calculated as the absolute difference between mean values.

Antibody	Vessel profiles		Standard Deviation		True Diff Means
	Normal	GDM	Normal	GDM	
VE-cadherin	99.3 (0.43)	79.6 (8.72)	0.96	19.5	19.7
Occludin	97.7 (0.57)	96.0 (0.27)	1.27	0.60	1.70
Phospho-Tyrosine	19.2 (0.56)	56.4 (1.23)	1.25	2.75	37.2

2.2. STUDY POPULATION

Pregnant women, with singleton pregnancies, were recruited at the Queen's Medical Centre University Hospital (Nottingham, UK). This study was in accordance with the Declaration of Helsinki and had the approval of the Queen's Medical Centre and University Hospital NHS Trust Ethics Committee (Ref: OG010101).

A total of 40 women with term pregnancies (>37 weeks), delivering by elective caesarean section, were asked for written informed consent for placental tissue collection. Twenty-two samples were used for gene and protein expression from normal ($n=9$), diet-controlled GDM ($n=7$), and metformin-controlled GDM ($n=6$) pregnancies. Additionally, samples from normal pregnancies were used for cell isolation ($n=10$), and placental chorionic villus explants ($n=8$). We ensured that pregnant women in all groups had similar characteristics and were non-smokers. The clinical details of the patients are shown in Table 2.2.

Table 2.2. Characteristics of the study population used for gene and protein expression.

Characteristic	Normal ($n=9$)	GDM - Diet ($n=7$)	GDM - Metformin ($n=6$)
Maternal age, years, mean (SD)	34.67 (6.04)	30.57 (6.78)	31.83 (4.71)
Maternal BMI, kg/m ² , mean (SD)	25.71 (4.27)	29.20 (7.54)	32.38 (6.89)
Gestational age at delivery, weeks, mean (SD)	38.69 (1.18)	39.17 (0.26)	39.40 (0.37)
HbA _{1c} , %, mean (SD)		5.32 (0.30)	5.60 (0.34)
HbA _{1c} , mmol/mol, mean (SD)		34.60 (3.51)	36.60 (2.97)
Neonatal gender	7M 2F	3M 4F	3M 3F
Placental weight, g, mean (SD)	717.6 (103.3)	674.4 (119.4)	644.2 (98.4)
Baby weight, kg, mean (SD)	3.41 (0.29)	3.54 (0.69)	3.62 (0.71)
Placenta efficiency, fetal/placental weight ratio, mean (SD)	5.14 (0.58)	5.28 (0.63)	5.64 (0.86)

BMI, body mass index; glycated haemoglobin (HbA_{1c}); data reported as mean $\pm$ the standard deviation (SD).

2.3. PLACENTAL TISSUE COLLECTION

Placentae were collected immediately after delivery. Once placenta arrived at the laboratory, it was weighed, and tissue sampling was done following the recommendations of Burton and collaborators (Burton *et al.*, 2014). Placing the placenta with the basal plate (maternal side) uppermost (Figure 2.1), four quadrants of chorionic villi, midway between the umbilical cord insertion and the placental rim, were sampled. Care was taken to avoid maternal and fetal surfaces (basal and chorionic plate).

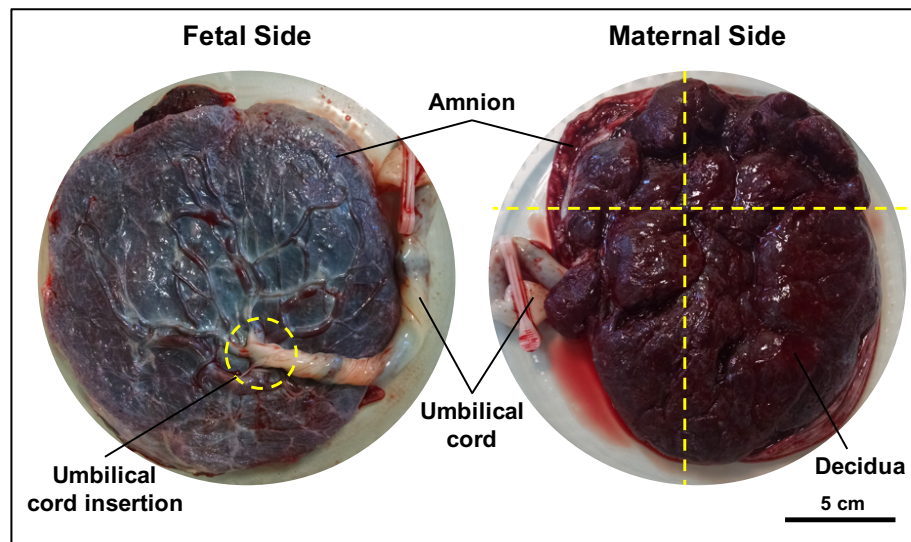


Figure 2.1. Human placenta collected immediately after delivery. Samples of chorionic villi obtained from four quadrants (delimited by yellow lines), midway between the umbilical cord insertion (yellow circle) and the placental rim. Scale bar = 5 cm.

From each quadrant, three biopsies were taken and processed depending on future experiments (Figure 2.2):

- RNA and DNA extraction: tissue was washed with cold-sterile phosphate-buffered saline (PBS) to remove as much blood as possible. Once blood was removed, samples were weighed, and an appropriate amount of RNeasy lysis buffer was added (1 ml per 100 mg of tissue) (Wolfe *et al.*, 2014). Samples were then stored at 4°C for 24 –

72 hours. Afterwards, RNAlater was removed, and samples were stored at -20°C.

- Protein extraction: tissue was snap-frozen and stored at -80°C.
- Immunofluorescence: tissue was fixed in 1% paraformaldehyde (PFA) at 4°C for 24 hours. Tissue was rinsed in PBS, covered on optimal cutting temperature (OCT) compound and snap frozen with liquid nitrogen cooled isopentane to minimise freeze damage. Tissue was stored at -80°C.

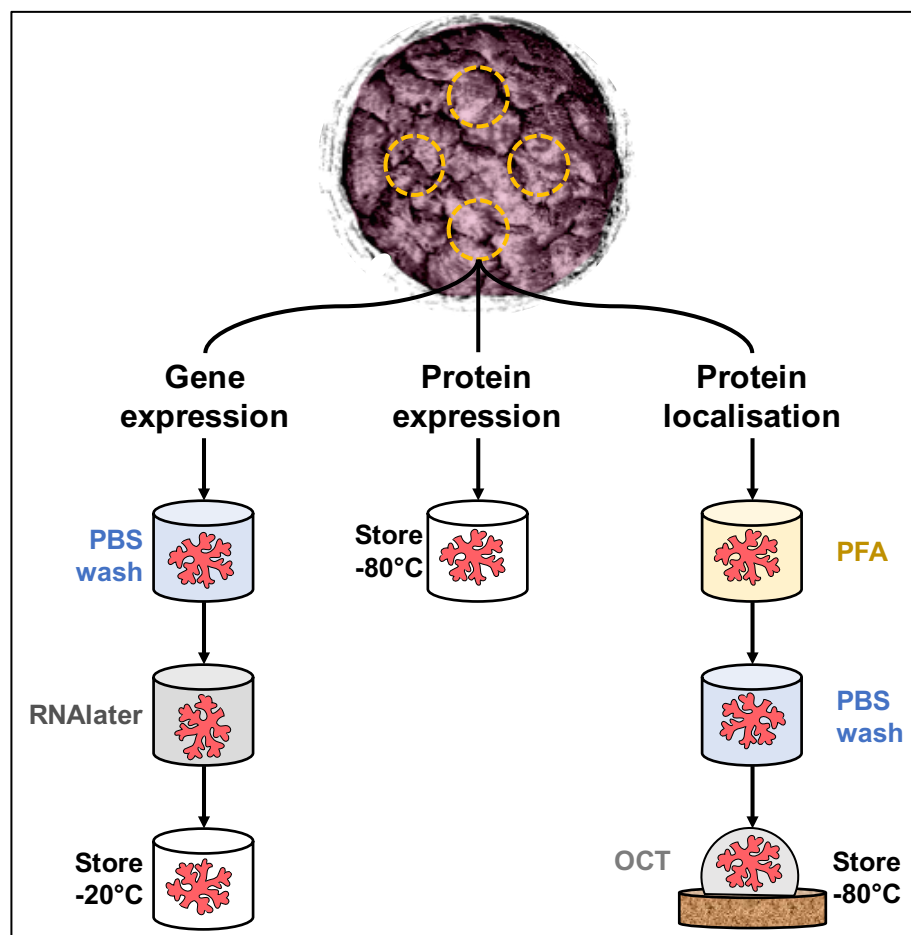


Figure 2.2. Human placenta sampling. Four quadrants of chorionic villi were sampled; from each quadrant three different samples were processed depending of future experiments. Gene expression: tissue was washed in PBS, stored in RNAlater 4°C, and stored at -20°C. Protein expression: tissue was snap frozen and stored at -80°C. Protein localisation: tissue was fixed in 1% PFA, rinsed in PBS, covered with OCT, snap frozen and stored at -80°C.

2.4. HUMAN PLACENTAL ENDOTHELIAL CELLS ISOLATION

Three different types of primary endothelial cells were isolated from human placental tissue. Cells were obtained from the umbilical cord vessels: artery and vein; and from the placental microvasculature. Placentae were collected immediately after C-section delivery. Once the placenta arrived at the laboratory, it was weighed, and tissue was processed according to the target primary cell.

2.4.1. HUMAN UMBILICAL VEIN ENDOTHELIAL CELL (HUVEC)

The umbilical cord was clamped at both ends and stored in saline solution (0.9% NaCl, 1% penicillin/streptomycin) at 4°C until isolation (within 24 hours). The isolation of human umbilical vein endothelial cells (HUVECs) was performed as described by Hamilton and Leach (Hamilton & Leach, 2011). Briefly, the cord vein was cannulated (Figure 2.3B) and flushed with pre/warmed saline to remove blood cells and proteins (Figure 2.3C). Once the saline solution flowed through the vein back and forth, the cord was filled to full turgor to ensure no leak or puncture marks were present and to determine how much collagenase was needed. The cord was filled with fresh and pre-warmed collagenase solution (0.1% collagenase type II) in M0 media (M199 with 1% penicillin/streptomycin) and incubated at 37°C for 15 minutes in a water bath containing saline (Figure 2.3D). Afterwards, collagenase solution was agitated back and forth through the vein to remove and collect as many endothelial cells as possible. Pre-warmed M20 media (M0, plus 20% fetal calf serum (FCS)) was added into the vein to stop the collagenase activity. This cell suspension was collected and centrifuged at 1000 rpm for 5 minutes (Figure 2.3E). The re-suspended cell pellet was seeded in a 1% (w/v) gelatine coated T25 cm² flask with growing media (M20 supplemented with 75 µg/ml endothelial cell growth supplement (ECGS), and 50 µg/ml heparin) and cultured at 37°C humidified incubator with 5% CO₂ in air (Figure 2.3F). This initial cultured HUVEC in a small flask is known as passage 0 (P0). The growing media was refreshed every 24 or 48 hours until confluency. HUVECs were used until passage 3 (P3).

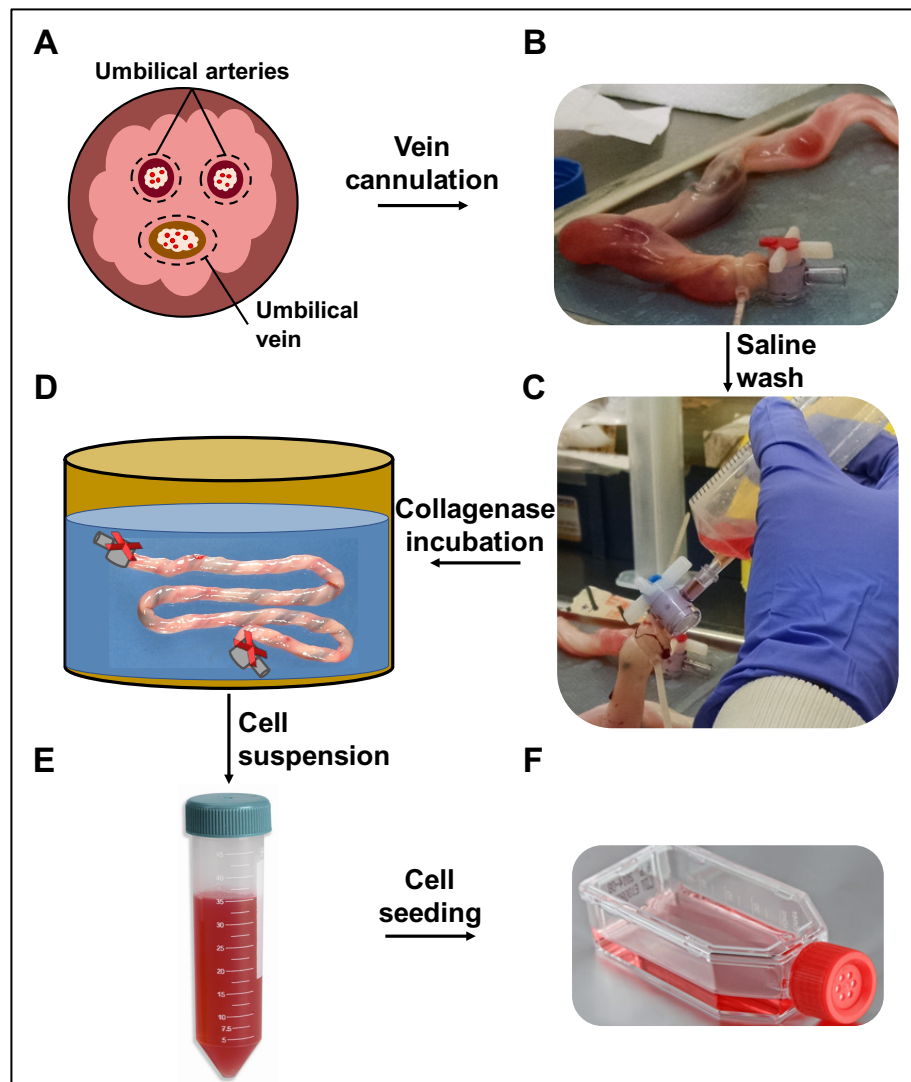


Figure 2.3. Isolation of human umbilical vein endothelial cells (HUVEC). (A) Diagram of the vessels in the umbilical cord, there is one umbilical vein (light brown) that carries fresh oxygenated and nutrient-rich blood towards the fetus. (B) Cord vein was cannulated. (C) Vein was washed with pre-warmed sterile saline to remove blood cells and proteins. (D) Vein was filled with collagenase solution and incubated in a water bath at 37°C for 15 minutes. (E) Cell suspension was collected in a tube and centrifuged to obtain a cell pellet. (F) Cells were re-suspended in growing media and seeded in a T25 cm² flask, and cultured at 37°C humidified incubator with 5% CO₂ in air.

2.4.2. HUMAN UMBILICAL ARTERY ENDOTHELIAL CELL (HUAEC)

The umbilical cord was clamped at both ends and stored in saline solution (0.9% NaCl, 1% penicillin/streptomycin) at 4°C until isolation (within one hour). The isolation of human umbilical artery endothelial cells (HUAECs) was prepared following the protocol described by Mano and

collaborators (Mano *et al.*, 1983) with minor modifications. One arterial vessel was separated from the umbilical cord by tearing the cord tissue longitudinally (Figure 2.4B). The intact artery was cannulated with a nasogastric (5 mm) tube, and heparin (25 µg/ml heparin in M0) was used as a vasodilator to flush the vessel (Figure 2.4C). The artery was filled with fresh and pre-warmed collagenase solution (0.1% collagenase type II in M0) (Figure 2.4D) and incubated at 37°C for 10 minutes (Figure 2.4E). Afterwards, collagenase solution was collected, and pre-warmed M20 media was added to stop the collagenase activity. The cell suspension was centrifuged at 1000 rpm for 5 minutes (Figure 2.4F). The re-suspended cell pellet was seeded in a 1% (w/v) gelatine coated T25 cm² flask with M20 media and cultured at 37°C humidified incubator with 5% CO₂ (Figure 2.4G). The M20 media was refreshed every 48 hours until confluency.

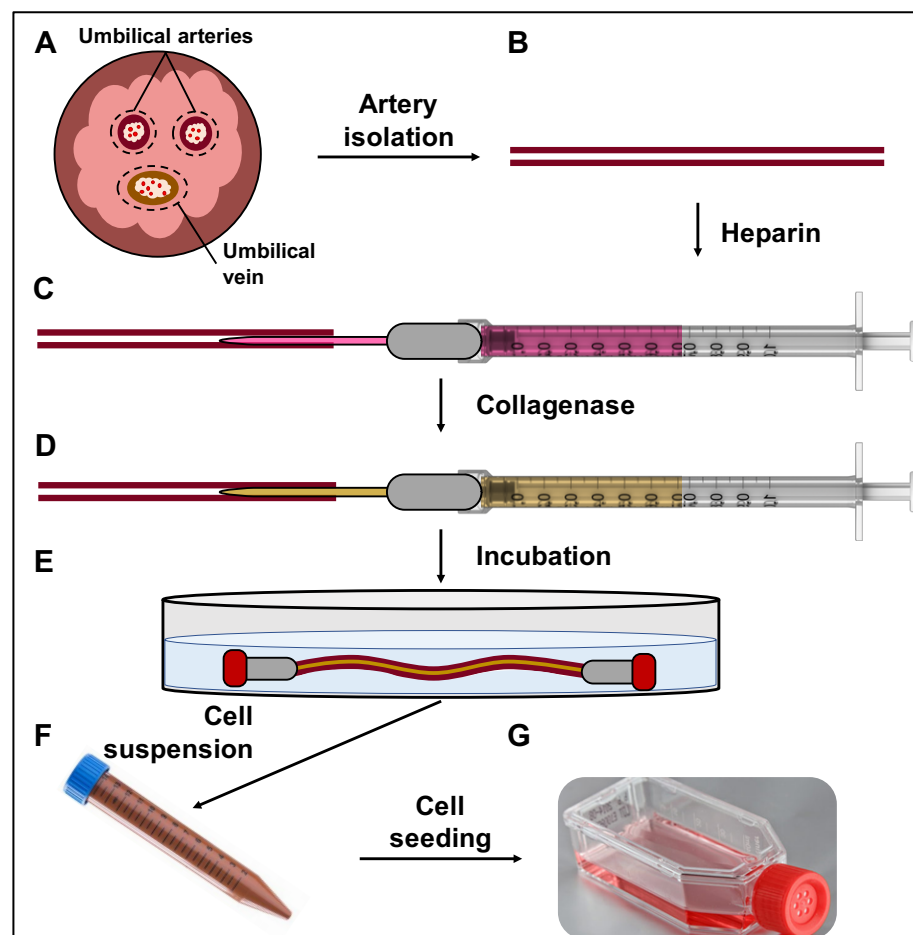


Figure 2.4. Isolation of human umbilical artery endothelial cells (HUAEC). (A) Diagram of the vessels in the umbilical cord, there are two umbilical arteries (dark red) that carry deoxygenated and nutrient-depleted fetal blood from the fetus to

the placenta and maternal circulation. **(B)** One of the arteries was isolated from the cord. **(C)** Artery was cannulated and heparin solution was used as vasodilator. **(D)** Artery was filled with collagenase solution. **(E)** Artery was incubated in a water bath at 37°C for 10 minutes. **(F)** Cell suspension was collected in a tube and centrifuged to obtain a cell pellet. **(G)** Cells were re-suspended in M20 media and seeded in T25 cm² flask, and cultured at 37°C humidified incubator with 5% CO₂ in air.

2.4.3. HUMAN PLACENTAL MICROVASCULAR ENDOTHELIAL CELL (HPMEC)

Human placental microvascular endothelial cells (HPMECs) were isolated following published protocols (Dye *et al.*, 2001; Troja *et al.*, 2014) with minor modifications. The placenta was placed on ice during excision (Figure 2.5A). Small fragments from placental cotyledons were cut on ice into 1 – 2 cm³ pieces until a 50 ml tube was filled up to 15 – 20 ml (Figure 2.5B). Tissue was washed thoroughly three times with cold-sterile PBS to remove as much blood as possible (Figure 2.5C). Tissue was transferred into a petri dish containing Hank's balanced salt solution (HBSS) and dissected on ice into very small fragments (Figure 2.5D). The minced tissue was centrifuged at 400 *xg* for 5 minutes at 4°C. The supernatant was discarded, and minced tissue was incubated in digestion buffer (0.28% collagenase type II, 0.25% dispase type II, 0.01% DNase-I in HBSS with HEPES) at 37°C with agitation for 20 to 60 minutes (Figure 2.5E). Solution was checked every 20 minutes to make sure some visible pieces of tissue were remaining. A cold solution of 5% BSA in HBSS was added, and the cell suspension was shaken by inversion at least 20 times (Figure 2.5F). The suspension was centrifuged at 600 *xg* for 7 minutes at 4°C. The BSA wash and centrifugation steps were repeated four more times. The remaining pellet was resuspended in M20, and cells were seeded in a 1% (w/v) gelatine coated T25 cm² flask, and cultured at 37°C humidified incubator with 5% CO₂ in air (Figure 2.5G). The media was refreshed every 48 hours. After two weeks, cells proliferation was limited and pulling out of endothelial cells with magnetic beads was not possible. Cells died after 30 – 40 days after isolation without reaching confluency.

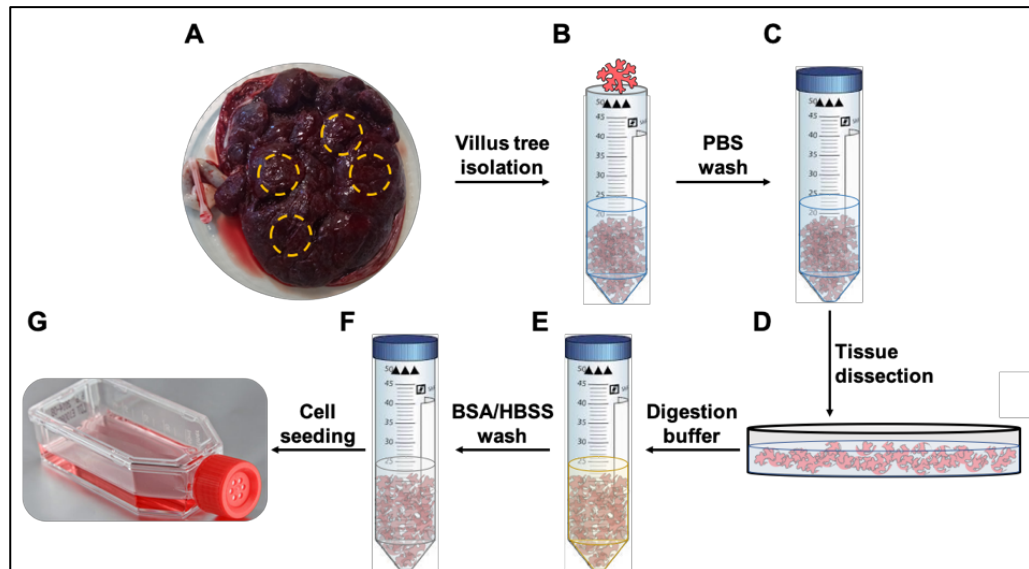


Figure 2.5. Isolation of human placental microvascular endothelial cells (HPMEC). (A) Villi trees were isolated from different cotyledons (orange circles); maternal tissue was carefully removed. (B) Tissue was collected in a tube until it was filled up to 15 – 20 ml. (C) Tissue was washed three times with cold-sterile PBS to remove blood. (D) Tissue was dissected into small fragments on a petri dish containing HBSS. (E) Minced tissue was incubated in digestion buffer at 37°C with agitation for 20 to 60 minutes. (F) Cell suspension was washed with cold solution of 5% BSA in HBSS, suspension was shaken by inversion at least 20 times and centrifuged to collect cell pellet (five washes in total). (G) Cells were re-suspended in M20 media and seeded in T25 cm² flask, and cultured at 37°C humidified incubator with 5% CO₂ in air.

2.5. PLACENTAL CHORIONIC VILLUS EXPLANTS – EX VIVO MODEL OF HYPERGLYCAEMIA

Placentae from normal pregnancies ($N= 8$) were collected immediately after delivery. Once the placenta arrived at the laboratory, it was weighed, and tissue sampling was done following the recommendations of Sciota (Sciota, 2012). The placenta was placed on ice during excision; explants were processed within 30 minutes of delivery. The explants (0.5 x 0.5 cm) were sampled from different quadrants, taking care not to include maternal and fetal tissue and cutting from the central part of the cotyledon (Figure 2.6 A and B). The explants were washed three times in ice-cooled sterile PBS to remove blood (Figure 2.6C). Once all the explants were cut ($n= 24$ per placenta), tissue was placed on nets in a 24-well plate. Explants were incubated in 1.5 ml of explant media (M199, 1%

penicillin/streptomycin, 15 mM dextran, 0.5% BSA, 26 mM sodium bicarbonate, 5000 IU/ml heparin) at 37°C humidified incubator with 5% CO₂ in air for 24 hours (Figure 2.6D). Afterwards, the media was removed, and 1.5 ml of treatment media was added (Figure 2.6E). Explants from each placenta were exposed to three conditions ($n = 8$ per condition, per placenta): *Normoglycaemic Control*: explant media; *Hyperglycaemic Control*: explant media, plus 10 mM L-glucose (15 mM glucose final concentration); *Hyperglycaemia*: explant media, plus 10 mM D-glucose (15 mM glucose final concentration). Explants were incubated at 37°C humidified incubator with 5% CO₂ in air for 3 or 24 hours. Finally, explants ($n = 4$ per condition, per placenta) were processed for gene expression and immunofluorescence as described on future sections (Figure 2.6F).

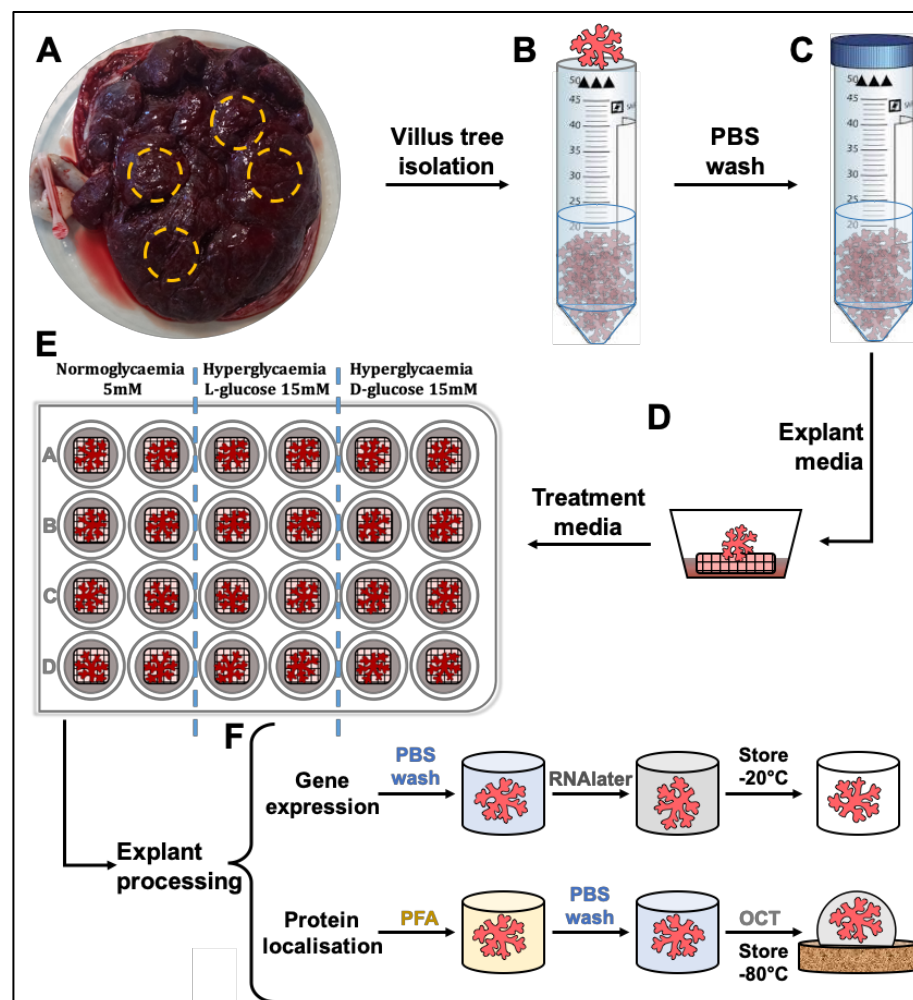


Figure 2.6. Chorionic villi explant experiment set up. (A) Villi trees were isolated from different cotyledons (orange circles); maternal and fetal tissue was

carefully removed. **(B)** Explants were collected in a tube with PBS and kept on ice until all the explants were excised. **(C)** Explants were washed three times with cold-sterile PBS to remove blood. **(D)** Tissue was placed on nets in a 24-well plate and incubated in explant media at 37°C humidified incubator with 5% CO₂ in air for 24 hours. **(E)** Media was changed to treatment media at a final glucose concentration of 5 or 15 mM (condition dependent). Explants were incubated in these media at 37°C humidified incubator with 5% CO₂ in air for 3 or 24 hours. **(F)** Explants were processed for gene expression or protein localisation.

I am particularly grateful to the undergraduate students who helped perform the immunofluorescence experiment on placental chorionic villus explants under my supervision: S. Dhaliwal, S. Klanwarin, D. MacDonald, R. Newman, N. Ockenden, and H. Shallcross.

2.6. RNA ISOLATION

Total RNA was extracted from placental tissue using TRI Reagent™ (T9424; Sigma) and a TissueLyser LT (Qiagen) homogeniser. Samples were weighed and transferred to 2.0 ml microcentrifuge tubes containing one stainless steel bead. TRI Reagent™ was added to each sample (1 ml to each), and the tissue was homogenised for 3 minutes at 50 Hz. After a complete tissue disruption, the solution was centrifuged at 12,000 ×g for 5 minutes and the supernatant was transferred to a new tube and stored at -80°C until RNA isolation (within a month).

Homogenized solutions stored at -80°C were thawed and 200 µl of chloroform was added. Samples were agitated vigorously for 15 seconds and incubated at room temperature for 15 minutes, followed by a centrifugation step at 12,000 ×g for 15 minutes at 4°C. Centrifugation separated the mixture into 3 phases, leaving a colourless upper aqueous phase containing total RNA. This phase was transferred to a new 1.5 ml tube and 500 µl of isopropanol was added. The solution was mixed by inversion and incubated at room temperature for 10 minutes before being centrifuged at 12,000 ×g for 10 minutes at 4°C. The supernatant was discarded, and 1 ml of 75% ethanol was added to wash the pellet. The pellet was mixed by vortex and centrifuged at 12,000 ×g for 5 minutes at 4°C. The

supernatant was carefully discarded, and the pellet was air-dried. Pellet was re-suspended in 50 µl water free of RNAses and incubated at 54°C for 5 minutes. Samples were stored at 4°C until quantification, and at -80°C for more extended storage.

RNA samples were then analysed for purity, integrity and concentration using a NanoDrop 2000c Spectrophotometer (Thermo Scientific). Some RNA samples had residual chemical contamination (260/230 ratio below 2.0) (Scientific, 2016). These samples were treated with an additional ethanol precipitation step by adding 10 µl of sodium acetate (3 M, pH 5.2), 300 µl of 100% ethanol and overnight incubation at -20°C. Afterwards, samples were centrifuged at 12,000 ×g for 30 minutes at 4°C. The supernatant was discarded; the pellet was washed by inversion with 400 µl of cold 75% ethanol and centrifuged at 12,000 ×g for 15 minutes at 4°C. The supernatant was carefully discarded, and the pellet was air-dried. Pellet was re-suspended in water free of RNAses and incubated at 60°C for 5 minutes. Samples were stored at 4°C until quantification, and at -80°C for more extended storage.

2.7. TOTAL RNA - REVERSE TRANSCRIPTION

Total RNA was reverse transcribed using SuperScript® III Reverse Transcriptase (18080; Invitrogen) and random primers (Promega) in a 25 µl final volume reaction. Each reaction required (values in parentheses are final concentrations): RNA (5 µg), 1.25 µl dNTPs (0.5 mM) and 3.75µl N15 oligonucleotides (0.52 µg). Samples were incubated at 65°C for 5 minutes and set on ice for at least one minute. Afterwards, 5 µl first strand buffer (1X), 1.25 µl DTT (5 µM), 0.5 µl RnaseIn (20 U), and 1µl Superscript III (200 U) were added to each sample. The reaction was incubated at 25°C for 5 minutes, followed by 50°C for one hour and finally at 75°C for 15 minutes. cDNA was stored at -20°C.

2.8. PRIMER DESIGN AND POLYMERASE CHAIN REACTION (PCR)

STANDARDISATION

For each gene of interest, reference sequences of all splice variants were downloaded from NCBI and Ensembl databases (Pruitt *et al.*, 2014; Yates *et al.*, 2016). Primers were designed to target specific splice variants, or all variant, depending on the purpose of the experiment and the gene of interest. Primer3web version 4.0.0 (Koressaar & Remm, 2007; Untergasser *et al.*, 2012) was used to design primer sequences. Each primer pair was optimised to contain 18–22 bases, 40–60% GC content, 50–60°C melting temperature, and yield 75–200 bp polymerase chain reaction (PCR) products. Additionally, AmplifX 1.5.4 (Jullien, 2008) was used as an *in silico* PCR software to determine possible primer dimers between designed primer pairs. Primers specificity was validated using the NCBI BLAST tool (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

PCRs were needed to determine each primer pair annealing temperature and if the reaction required betaine to improve specificity. Reactions were performed in 25 µl final volume with (values in parentheses are final concentrations): 0.5 µl of placenta cDNA (1:4 dilution), 0.25 µl Taq DNA polymerase (1.25 U) (D6677-1.5 KU; Sigma), 2.5 µl buffer (1X), 3.5 µl MgCl₂ (3.5 mM), 2.0 µl dNTPs (200 µM), and 0.8 µl of each primer (0.3 µM). If it was required, betaine (5.0 µl; 1 mM) was added to the reaction. The thermal profile was as follows: 94°C for 2 minutes, 35 cycles of 94°C for 20 seconds, annealing temperature for 30 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 10 minutes. The PCR products were separated on a 2% agarose gel at 90 V for 20 minutes, stained with SYBR® Safe DNA Gel Stain (S33102; Invitrogen), and visualised under UV light. After the molecular weight of the PCR product was confirmed, the PCR product was sent to sequence to validate the primer specificity (Appendix A).

2.9. QUANTITATIVE POLYMERASE CHAIN REACTION (qPCR)

Quantitative polymerase chain reactions (qPCRs) were used to determine gene expression. qPCRs were performed in triplicate with SYBR® Green JumpStart™ Taq ReadyMix™ (S4438; Sigma) master mix, and detection was performed using the Rotor-Gene 6000 cyclor (Corbett Research). Reactions used 0.8 µl cDNA (dilution depends of primer), and 7.5 µl JumpStart (1X) in a 15 µl final volume reaction. If required, 3.0 µl betaine (1 mM) were added to the reaction. Primers concentration and cDNA dilutions were optimised for each primer (detailed information available in each chapter). The thermal profile included 95°C for 10 minutes, 40 cycles of denaturation at 95°C for 15 seconds, annealing temperature (specific of each primer) for 30 seconds, and elongation at 72°C for 40 seconds. The resultant mean threshold cycle (Ct) values were used for reference gene normalisation and gene expression analyses.

2.10. REFERENCE GENES SELECTION (GENORM)

We performed a literature search for genes whose expression in human placental tissue is not affected by GDM, nor treatment to manage GDM. There were nine possible reference genes: *β-2 microglobulin (B2M)*, *Hypoxanthine phosphoribosyl-transferase I (HPRT1)*, *Nuclear transcription factor, X-box binding 1 (NFX1)*, *Protein O-fucosyltransferase 1 (POFUT1)*, *Succinate dehydrogenase complex, subunit A (SDHA)*, *TATA box binding protein (TBP)*, *DNA Topoisomerase 1 (TOP1)*, *Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (YWHAZ)*, and *18S ribosomal (18S)* (Bakhashab *et al.*, 2014; G. Chen *et al.*, 2013; Meller *et al.*, 2005). Primers were designed for each gene (Table 2.3), and normal and GDM samples were amplified following the protocol described above.

Table 2.3. Primer information of potential reference genes. Detailed information about primers designed for: *β-2 microglobulin (B2M)*, *Hypoxanthine phosphoribosyl-transferase I (HPRT1)*, *Nuclear transcription factor, X-box binding 1 (NFX1)*, *Protein O-fucosyltransferase 1 (POFUT1)*, *Succinate dehydrogenase complex, subunit A (SDHA)*, *TATA box binding protein (TBP)*, *DNA Topoisomerase 1*

(*TOP1*), Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (*YWHAZ*), and 18S ribosomal (*18S*). Forward (F) and reverse (R) sequences, primer final concentration ([Final]), cDNA dilution (cDNA), annealing temperature (°C) and if betaine was needed for the specificity of the reaction (*).

Gene	Sequence	[Final]	cDNA	°C
<i>B2M</i>	F: 5'-TGTA CTACACTGAATTCACCCC-3' R: 5'-CTTACATGTCTCGATCCCACTT-3'	700 ηM	1:64	60
<i>HPRT1</i>	F: 5'-TCGTGATTAGTGATGATGAACCA-3' R: 5'-CCATCTCCTTCATCACATCTCG-3'	700 ηM	1:8	60*
<i>NFX1</i>	F: 5'-GTGGAGTTGTCAGAGCTGTTAC-3' R: 5'-TTGCCACAGAAACAAGTGTAGG-3'	500 ηM	1:64	60*
<i>POFUT1</i>	F: 5'-GCATCACAAGCCTCCTTTCA-3' R: 5'-GTCTTCTTATCTGGGCTTCGC-3'	500 ηM	1:16	60*
<i>SDHA</i>	F: 5'-GTGCCGTGGTGTTCATCGC-3' R: 5'-CTGGTGTGGGCAGACGTG-3'	300 ηM	1:256	60*
<i>TBP</i>	F: 5'-CCTAAAGACCATTGCACTTCGT-3' R: 5'-TTCGTGGCTCTCTTATCCTCAT-3'	400 ηM	1:64	62
<i>TOP1</i>	F: 5'-TGGATGGTCAGGAATATGTGGT-3' R: 5'-GGGCTGCTTGTTCTCCATAAAT-3'	600 ηM	1:12	60*
<i>YWHAZ</i>	F: 5'-TGGCTCGAGAATACAGAGAGAA-3' R: 5'-TGGGGATCAAGAACTTTTCCAA-3'	200 ηM	1:256	58
<i>18S</i>	F: 5'-TCGTGATTAGTGATGATGAACCA-3' R: 5'-CCATCTCCTTCATCACATCTCG-3'	500 ηM	1:16384	60*

The geNorm algorithm was used, through the R-based SLqPCR package (Version 1.36.0) (Kohl, 2007), to determine which set of reference genes were more stable. geNorm calculates the gene expression stability “*M*” value for each candidate reference gene and ranks from low to high stability (Lanoix *et al.*, 2012). Genes with an *M* value lower than 1.5 are considered the most stable (Arenas-Hernandez & Vega-Sanchez, 2013). Additionally, the optimal number of reference genes required for normalisation can be calculated by determining the pairwise variation “*V*” between two sequential normalisations containing an increasing number of genes. A cut-off value of 0.15 was established below which an additional

reference gene is not required (Lanoix *et al.*, 2012; Vandesompele *et al.*, 2002).

Placental samples from normal ($n= 8$) and GDM ($n= 7$) pregnancies were used to test the variation of expression of the nine potential reference genes. Ct values were used to run the geNorm algorithm. Results showed that *TBP* and *YWHAZ* present the most stable expression in placental tissue between normal and GDM pregnancies (Figure 2.7A). Additionally, three genes were the optimal number needed as reference ($V = 0.99$) (Figure 2.7B).

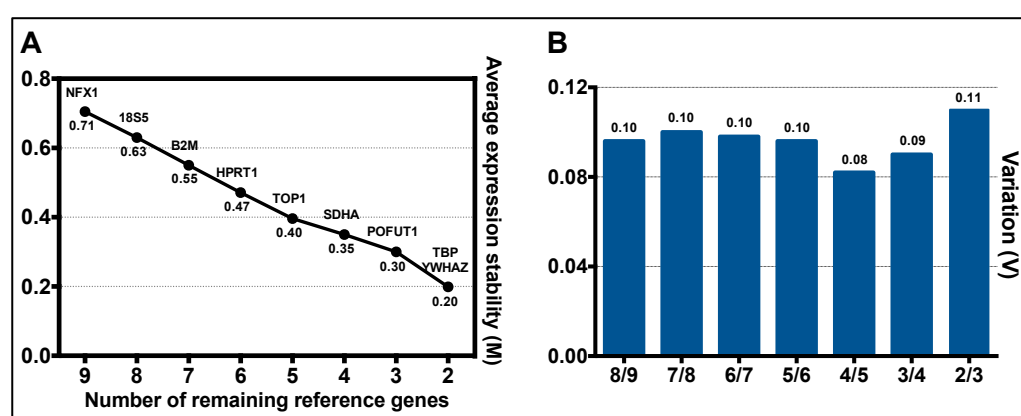


Figure 2.7. Gene stability of nine potential reference genes for human placenta of normal and GDM pregnancies. Placental samples from normal ($n= 8$) and GDM ($n= 7$) pregnancies were used to test gene stability. **(A)** Stability ranking of the nine tested genes. *TBP* and *YWHAZ* are the most stable genes from these group, as they present lower average expression stability values (M). **(B)** Optimal number of reference genes was three; Variation (V) values below 0.15.

The expression of *POFUT1*, *TBP* and *YWHAZ* were used as a normalisation factor and the expression of the candidate reference genes was compared between normal and GDM placental samples (Figure 2.8). *POFUT1* and *TOP1* present significantly higher expression in placental samples from GDM pregnancies ($p < 0.05$). Hence, the use of *POFUT1* as a reference gene was rejected.

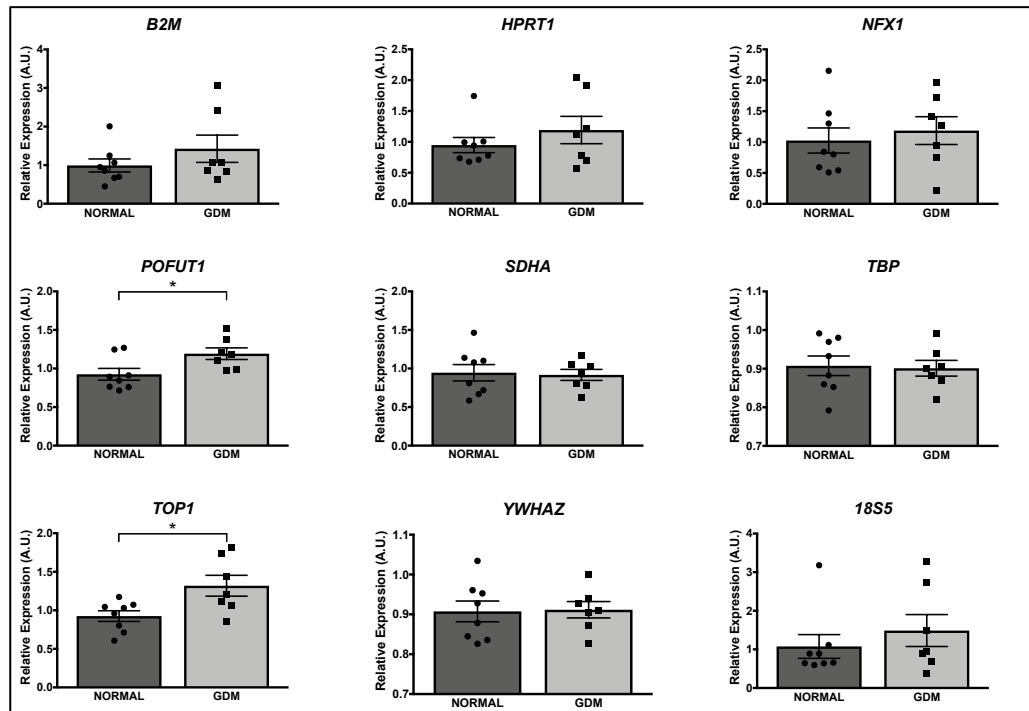


Figure 2.8. Gene expression of nine potential reference genes using *POFUT1*, *TBP* and *YWHAZ* as reference genes. Placental samples from normal (n= 8) and GDM (n= 7) pregnancies were used to test gene stability. The relative expression of each gene was compared between normal and GDM placental samples. Mean $\pm$ SEM; * $p < 0.05$.

A new run of the geNorm algorithm using the remaining eight reference genes showed that *TBP* and *YWHAZ* were still the most stable genes in placental tissue between normal and GDM pregnancies (Figure 2.9A). Additionally, three genes were the optimal number needed as reference ($V = 0.10$) (Figure 2.9B).

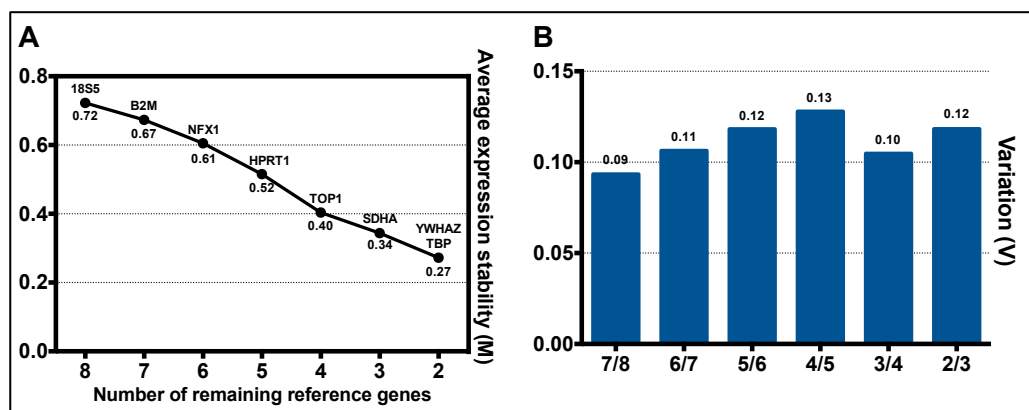


Figure 2.9. Gene stability of eight potential reference genes for human placenta of normal and GDM pregnancies. Placental samples from normal (n=

8) and GDM ($n= 7$) pregnancies were used to test gene stability. **(A)** Stability ranking of the eight tested genes. *TBP* and *YWHAZ* are the most stable genes from these group. **(B)** Optimal number of reference genes was three.

The expression of *SDHA*, *TBP* and *YWHAZ* was used as a normalisation factor and the expression of the reference genes candidates was compared between normal and GDM placental samples (Figure 2.10). *POFUT1* and *TOP1* present significantly higher expression in placental samples from GDM pregnancies ($p < 0.05$). The three reference genes present a similar expression between normal and GDM placental samples. Therefore, *SDHA*, *TBP* and *YWHAZ* will be used as reference genes for all gene expression assays in placental samples.

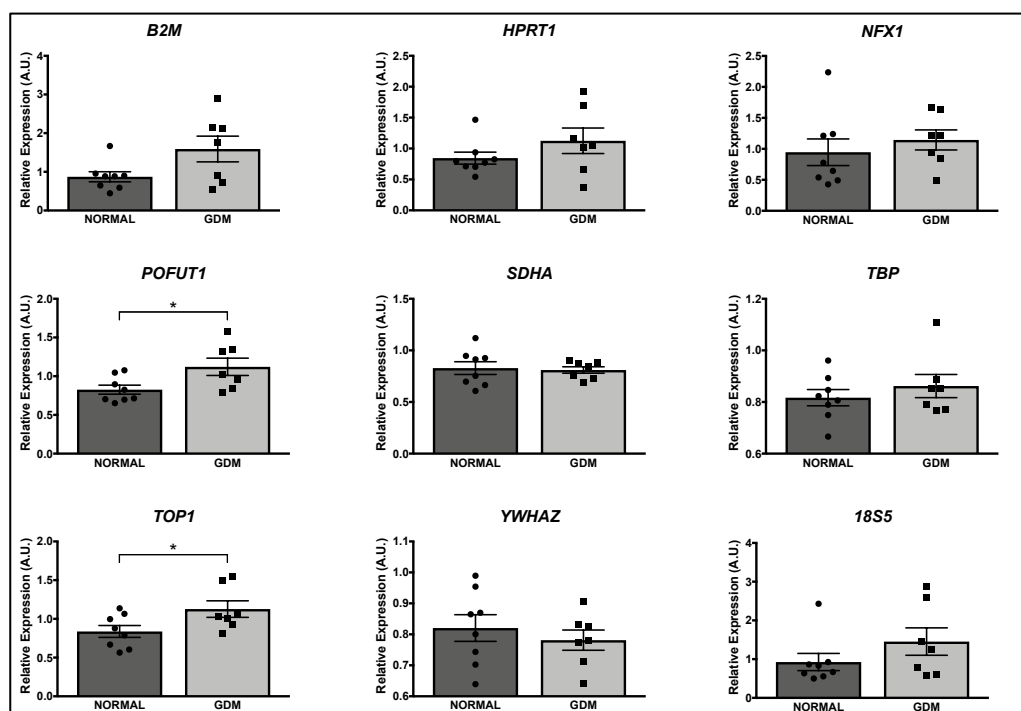


Figure 2.10. Gene expression of nine potential reference genes using *SDHA*, *TBP* and *YWHAZ* as reference genes. Placental samples from normal ($n= 8$) and GDM ($n= 7$) pregnancies were used to test gene stability. The relative expression of each gene was compared between normal and GDM placental samples. Mean $\pm$ SEM; * $p < 0.05$.

2.11. NORMALISATION OF GENE EXPRESSION

Placental samples were amplified for each gene of interest by qPCR. Ct values were normalised to a geometric average of the three reference

genes, confirmed by geNorm results (*YWHAZ*, *TBP*, *SDHA*) (Meller *et al.*, 2005). Relative expression values were calculated using the relative quantification ($2^{-\Delta\Delta Ct}$) method (Pfaffl, 2001).

2.12. DNA ISOLATION

Total DNA was extracted from placental tissues using TNES buffer (10 mM Tris, pH 7.5; 400 mM NaCl; 100 mM EDTA; 0.6% SDS) and a TissueLyser LT (Qiagen) homogenizer. Samples were weighed (40–50 mg) and transferred to 2.0 ml microcentrifuge tubes containing one stainless steel bead. TNES buffer (600 μ l) was added to each sample and the tissue was homogenised for 10 minutes at 50 Hz. After a complete tissue disruption, 35 μ l proteinase K (20 mg/ml) was added, and samples were incubated overnight at 55°C. Once tissue was completely lysed, samples were vortexed for 15 seconds and 200 μ l 5 M NaCl were added. Samples were mixed by shaking for 15 seconds and centrifuged at 12,000 $\times$ g for 5 minutes at room temperature. The supernatant was collected and one volume of 95% cold ethanol plus 1 μ l of glycogen were added. The solution was mixed by inversion and centrifuged at 12,000 $\times$ g for 15 minutes at 4°C. The supernatant was discarded and 500 μ l of 75% ethanol was added to wash the pellet. Pellet was vortexed and centrifuged at 12,000 $\times$ g for 10 minutes at room temperature. Air-dried DNA pellet was re-suspended in 50 μ l of TE buffer and incubated at 55°C for 10 minutes. Samples were stored at 4°C until quantification, and at -80°C for longer storage. DNA quantity and quality were assessed using the NanoDrop spectrophotometer ND-2000 (Thermo Fisher Scientific).

2.13. METHYLATED DNA IMMUNOPRECIPITATION (MEDIP)

Methylated DNA immunoprecipitation (MeDIP) was performed as described by (Borgel *et al.*, 2012) with some modifications. Briefly, DNA (1.2 μ g DNA in 60 μ l TE buffer) was fragmented by sonication to 500–1,000 bp size using a Bioruptor Plus sonication device (Diagenode, Inc.). An aliquot of 200 ng of each sonicated DNA sample (10 μ l) was set aside to serve as

input reference DNA for qPCR analyses. Remaining sonicated DNA was denatured at 95°C for 10 min, samples were immediately cooled on ice for 10 minutes. Samples were then incubated in 460 µl 1X immunoprecipitation buffer (10 mM Na-Phosphate pH 7.0; 140 mM NaCl; 0.05% Triton X-100) (IP buffer) containing rabbit anti 5-methylcytosine antibody (61255, Active Motif, 1 mg/ml) for 2 hours at 4°C in overhead rotation. Following the manufacture's guidelines, Dynabeads M-280 sheep anti-rabbit IgG magnetic beads (11204D; Life Technologies) were washed twice with 1 ml 0.1% PBS/BSA. Beads were vortexed for 5 minutes and collected on the magnetic rack. Beads were resuspended in one volume of 1X IP buffer and added to each sample (10 µl). Samples were incubated for an additional 2 hours at 4°C with overhead rotation. Magnetic beads were collected and washed three times with 700 µl of 1X IP buffer by incubating for 10 minutes at room temperature on shaker. Beads were collected on the magnetic rack and 250 µl proteinase K digestion buffer (50 mM Tris pH 8.0, 10 mM EDTA, 0.5% SDS) plus 7 µl proteinase K (20 mg/ml) were added. Beads were incubated for 3 hours at 50°C in a dry incubator with overhead rotation. Afterwards, one volume (250 µl) of phenol:chloroform buffer was added, and samples were vortexed until strong emulsification was formed. Samples were centrifuged at 13,000 ×g for 10 minutes at room temperature. The supernatant was transferred to a new tube, and 250 µl of chloroform were added. Samples were vortexed until strong emulsification was formed, and centrifuged at 13,000 ×g for 10 minutes at room temperature. The supernatant was transferred to a new tube and 10 µl 5M NaCl, 1 µl glycogen plus 750 µl 100% ethanol were added. Samples were vortexed and left to precipitate at -20°C for 30 minutes. Samples were centrifuged at 20,000 ×g for 30 minutes at 4°C. Pellet was washed with 500 µl 70% cold ethanol and vortexed. Samples were centrifuged at 20,000 ×g for 15 minutes at 4°C. Pellet was air-dried, and methylated DNA was re-suspended in 20 µl of TE buffer. Samples were incubated overnight at 4°C with overhead rotation, followed by an incubation at 55°C for 10 minutes. Samples were then stored at -80°C.

2.14. QUANTIFICATION OF METHYLATION LEVELS IN *Occludin* PROMOTER

qPCR reactions were performed for either MeDIP or input genomic DNA with primers flanking the *Occludin variant 2* promoter region (forward 5'-GAAGCAGAAAAGTGTCTGTGA-3'; reverse 5'-CTTGGGGTGTCTGATGGA GAG-3'). Reactions were run in triplicate in a final volume of 15 µl with (values in parentheses are final concentration): 7.5 µl SYBR® Green JumpStart™ Taq ReadyMix™ (1X) (S4438; Sigma), 0.75µl primers (500 nM), 3 µl betaine (1mM), plus 2 µl of MeDIP (1:2 dilution) or input (1:10 dilution) genomic DNA. Detection was performed using the Rotor-Gene 6000 cycler (Corbett Research). The thermal profile included 95°C for 15 minutes followed by 50 cycles of 95°C for 30 seconds, annealing at 58°C for 45 seconds, and elongation at 72°C for 40 seconds. qPCR efficiency and Ct values of MeDIP and input fractions were used to calculate the percentage of recovery of target sequences after MeDIP (Taiwo *et al.*, 2012). First, the input Ct of each samples was adjusted using the formula:

$$Adjusted\ Input\ CT = Input\ Ct - \log[2AE (10)]$$

Where AE was the amplification efficiency and 10 represents the dilution factor of input to MeDIP. Then the percentage of recovery was calculated using the equation:

$$Recovery\ (\%) = 2AE^{(Adjusted\ Input\ Ct - MeDIP\ Ct)} \times 100$$

2.15. PROTEIN ISOLATION

Total protein was isolated from placental tissue using a Tissue Master 50 (OMNI International) homogenizer and protein lysis buffer [50 mM Tris pH 8.0, 150 mM NaCl, 0.5% deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulphate (SDS)] plus 2 mM phenylmethylsulphonyl fluoride (PMSF) and commercial protease (11836153001; Roche) and phosphatase inhibitors cocktails (P5726; Sigma). Samples were weighed, and a volume of lysis buffer was added to each sample (500 µl buffer per 100 mg tissue).

Tissue was homogenised on ice for one minute. The solution was centrifuged at 13 000 xg for 30 min at 4°C, the supernatant was collected. Five μ l of each sample was stored at 4°C for quantification, samples stocks were stored at -80°C.

Protein concentration was measured using the DC Protein Assay (5000116; Bio-Rad). Six bovine serum albumin (BSA) standards were prepared in 1:10 lysis buffer: 1.50 mg/ml, 1.25 mg/ml, 1.00 mg/ml, 0.75 mg/ml, 0.50 mg/ml, and 0.20 mg/ml. A control was added to subtract the background from the lysis buffer. Samples were diluted 1:10 in water. Standards and samples were loaded in duplicate to a 96-well microplate. Reagent A' (1:49 Reagent S: Reagent A) and Reagent B were added to each well. The microplate was incubated at room temperature for 15 minutes on agitation. The absorbance was measured at 750 nm. Samples were prepared for Western blot analysis to a final concentration of 3.33 μ g/ μ l in lysis buffer and Laemmli buffer.

2.16. WESTERN BLOT

Protein samples were heated at 95°C for 5 minutes and centrifuged at 13 000 xg for 1 minute. Due to the number of samples used for this study, it was necessary to run two gels for each protein of interest. To control for variation between runs, one sample was run in each gel (control sample). Depending on the protein of interest, an appropriate amount of total protein per sample was separated on a 4–20% SDS-PAGE (Bio-Rad) by electrophoresis at 175V for 45 minutes. The proteins were transferred to a nitrocellulose membrane on ice at 100V for 45 minutes. A quick-reversible staining with 1:10 Ponceau S (Sigma) confirmed the presence of proteins in the membrane. Afterwards, the membrane was washed until clear with 1X Tris-buffered saline (TBST)/0.1% Tween 20 solution. Blocking buffer (5% milk/TBST/0.1% Tween) was added, and the membrane was incubated for one hour at room temperature in agitation. Membranes were then incubated overnight at 4°C with primary antibodies (detailed information available in Chapters 3 and 4) diluted in blocking buffer. Membrane was

washed six times in TBST/0.1% Tween (three times of 5 minutes, followed by three times of 10 minutes), followed by an hour incubation at room temperature with secondary antibodies: goat anti-rabbit (926-32211, LI-COR, Inc.), and goat anti-mouse (925-68070, LI-COR, Inc) diluted in blocking buffer to 0.1 µg/ml. Membranes were scanned with an Odyssey Infrared Imaging System 3.0 (LI-COR, Inc.).

Quantification of immuno-positive bands was performed using ImageJ Software Version 2.0 (National Institutes of Health), following the instructions described previously with some modifications (Davarinejad, 2015). In summary, measurements for each band and gel background were obtained with ImageJ (Figure 2.11 A). The net-values were calculated (Band – Background) for each sample and protein. To control for variation between gels, a mean average was obtained from the net-values of the control sample from each run (Figure 2.11 B). A variation factor was calculated for each gel (Average control sample – Net control sample gel X) (Figure 2.11 C). Each sample was normalized with the variation factor for the gel where it run (Net sample + Variation gel X) (Figure 2.11 D). These calculations were done for the loading control protein (β -actin) and the target proteins. Once all values were normalized, the relative quantification of the target protein can be obtained as a ratio of the loading control (Sample normalized target protein / Sample normalized loading control) (Figure 2.11 E). These last results were used for statistical analyses.

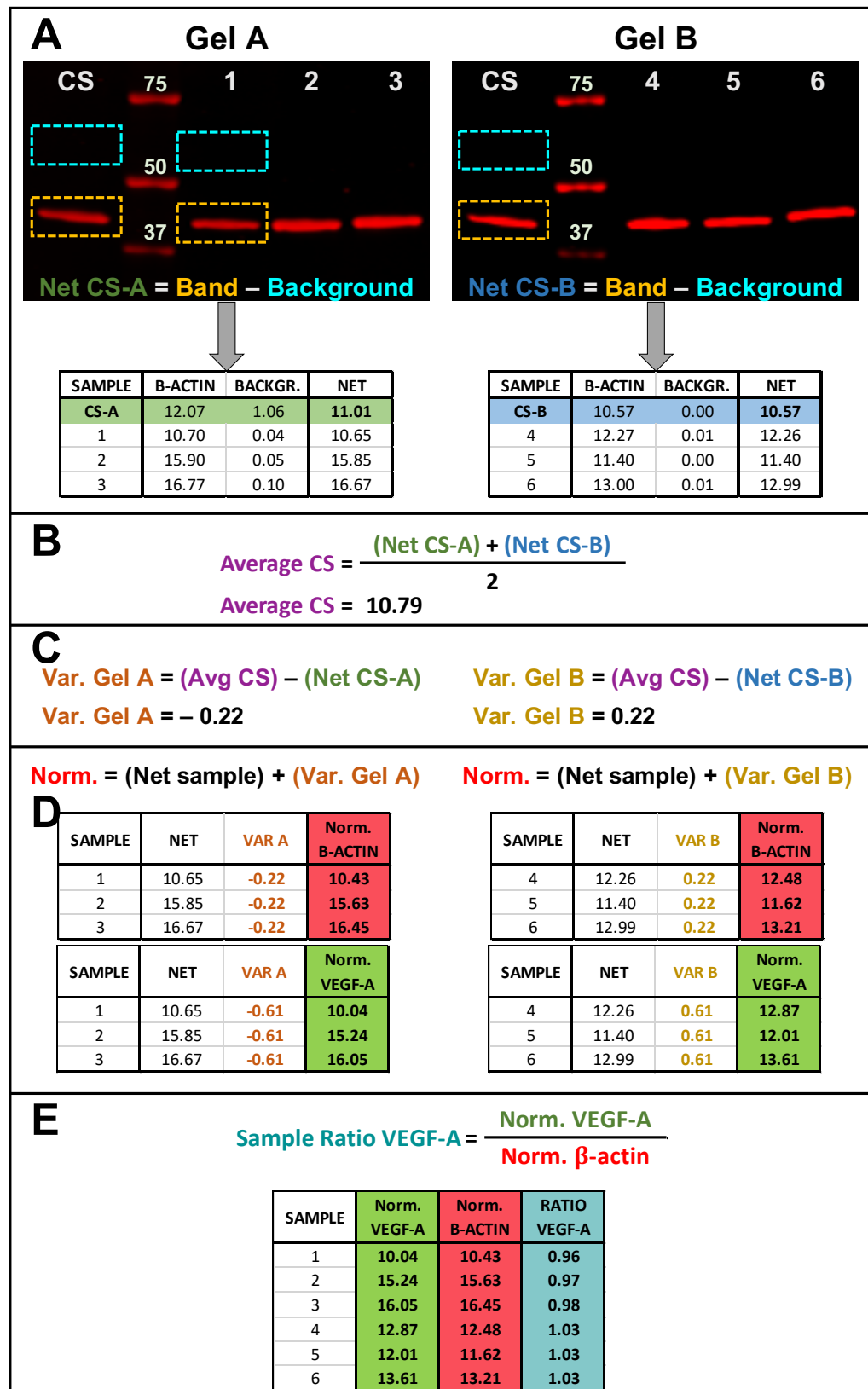


Figure 2.11. Example of calculations used to obtain the relative expression of target proteins with Western blotting. (A) Images of two gels used to quantify β -actin (red) in human placental protein samples; one sample was run in both gels as a control (CS). ImageJ measures the intensity of the band (orange square) and the background (blue square). Net-values were obtained by subtracting the

background from each band. **(B)** Mean average was calculated using the net-values of the control sample. **(C)** The difference between the average and the net-value of the control sample gives a variation factor for each gel. **(D)** This variation factor is added to each sample as a normalization. These calculations are done for the target protein (e.g. VEGF-A) and the loading control (β -actin). **(E)** A ratio between the target protein and the loading control gives the relative expression of the protein.

2.17. PROTEIN IMMUNOPRECIPITATION

Protein immunoprecipitation (IP) assays aimed to quantify Occludin's phosphorylation levels in placental samples. Different primary antibodies (Table 2.4) and magnetic beads (Table 2.5) were used during the optimisation.

Table 2.4. Antibodies used for the optimisation of the protein immunoprecipitation assay. Detailed information about each antibody, catalogue number, manufacturer, and antibody final concentration in $\mu\text{g/ml}$ ([Final]).

Antibody	Catalogue Nº	Company	[Final]
Occludin rabbit polyclonal	71-1500	Thermo Fisher	2, 3, 4
Occludin rabbit monoclonal	ab167161	Abcam	4
Occludin mouse monoclonal	sc-133256	Santa Cruz Biotechnology	2
Occludin rabbit monoclonal	ab216327	Abcam	4
VE-cadherin rabbit monoclonal	2500	Cell Signalling	2
Phospho-Tyrosine mouse monoclonal	9411	Cell Signalling	15
Phospho-Serine mouse monoclonal	9606	Cell Signalling	10

Table 2.5. Magnetic beads used for the optimisation of the protein immunoprecipitation assay. Detailed information about each bead, catalogue number, and manufacturer.

Beads	Catalogue Nº	Company
Dynabeads M-280 sheep anti-rabbit IgG	11204D	Life Technologies
Protein A	LSKMAGA02	PureProteome
Protein G	LSKMAGG02	PureProteome
Protein G Mag Sepharose	28-9513-79	GE Healthcare

Protein IP, from placental samples, using Occludin or phosphorylated antibodies was unsuccessful. However, VE-cadherin precipitation was successful and helped confirm the efficacy of the IP protocol. To reduce the non-specific protein binding, a two-cycle IP was performed as described by Morgan (Morgan, 2016) with some modifications. Pre-clear: Total placental protein (1 mg/ml) was incubated with 15 µl pre-washed Protein G Sepharose beads (GE Healthcare) in IP buffer (50 mM Tris, 150 mM NaCl pH 7.5) at 4°C for 1 hour in an overhead rotation to remove any nonspecific protein binding to the protein G (final volume of 200 µl). Antibody Binding: pre-cleared protein supernatant was transferred to a clean tube and incubated with VE-cadherin antibody (Table 2.3) at 4°C overnight in an overhead rotation. An antibody rabbit IgG was used as control (4 µg/ml). IP: 25µl of pre-washed Protein G Sepharose beads were added to each sample and incubated 4°C for 1 hour in an overhead rotation. Beads were collected with the magnetic rack. Subsequently, beads were washed five times with IP buffer. Elution: 50 µl of 0.2 M glycine pH 2.5 were added to the beads, samples were incubated at room temperature for 2 minutes. Supernatant was collected in a new tube, and 2.5 µl of 1M Tris pH 8.5 were added to neutralise the reaction. Samples were prepared for Western blot analysis by taking 12.5 µl IP sample and adding 2.5 µl of 6X Laemmli buffer. Samples were stored at -20°C.

2.18. MICRORNAs – REVERSE TRANSCRIPTION

Total RNA (5 ng) was reverse transcribed using miRCURY LNA™ Universal RT microRNA PCR Kit (203301; Exiqon) in a final volume of 10 µl. Two µl of 5X Reaction buffer, 5 µl nuclease free water, 1 µl of the 10X enzyme mix, and 2 µl of the sample were used for the reaction. One sample was randomly used as a control, 4.5 µl of nuclease free water and 0.5 µl of synthetic RNA spike were added to the previous reaction. Samples were incubated at 42°C for one hour and finally at 95°C for 5 minutes. microRNA-cDNA was stored at -20°C.

2.19. MICRORNAs – QUANTIFICATION

MicroRNAs (miRNAs) that target *Occludin* expression were selected based on literature and a bioinformatics search using three databases: miRBase (<http://www.mirbase.org>), miRTarBase (<http://mirtarbase.mbc.nctu.edu.tw>), and DIANA (<http://diana.imis.athena-innovation.gr/DianaTools/index.php>).

Five miRNAs were selected: *miR-18a-5p*, *miR-21-3p*, *miR-122-5p*, *miR181a-5p*, and *miR-340-5p*; Exiqon primers were used for amplification (Table 2.6). qPCRs were conducted in triplicate using ExiLent SYBR® Green master mix (203421; Exiqon) in a final volume of 10 µl. Reaction consisted of: 5 µl of 2X qPCR master mix, 1 µl of primer, and 4 µl microRNA-cDNA (1:80 dilution). Detection was performed using the Rotor-Gene 6000 cyclers (Corbett Research). The thermal profile included 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds, and 60°C for 1 minute. *U6 sn-RNA* gene was used as an internal control.

Table 2.6. microRNA primers sequences. Information about *miR-18a-5p*, *miR-21-3p*, *miR-122-5p*, *miR181a-5p*, and *miR-340-5p* Exiqon primers.

microRNA	Sequence	Catalogue Nº
<i>hsa-miR-18a-5p</i>	5'- UAAGGUGCAUCUAGUGCAGAUAG	YP00204207
<i>hsa-miR-21-3p</i>	5'- CAACACCAGUCGAUGGGCUGU	YP00204302
<i>hsa-miR-122-5p</i>	5'- UGGAGUGUGACAAUGGUGUUUG	YP00205664
<i>hsa-miR-181a-5p</i>	5'- AACAUUCAACGCUGUCGGUGAGU	YP00206081
<i>hsa-miR-340-5p</i>	5'- UUAUAAAGCAAUGAGACUGAUU	YP00206068
<i>U6 snRNA (hsa, mmu)</i>	N/A	YP00203907

N/A: Not available

2.20. HUVEC TRANSFECTION

HUVEC ($N = 4$), from passages 2 or 3, were transfected with 0.75 μ l DharmaFECT-1 Transfection Reagent (0.75%, T-2001-01, Dharmacon Research Inc.), according to the manufacturer's instructions. HUVEC were seeded into 12-well plates at $\sim 2.0 \times 10^5$ cells/ml per well in TM20 (antibiotic-free M199 plus 20% FCS) and cultured overnight at 37°C humidified incubator with 5% CO₂ in air. Media was removed, and transfection was performed using 3 μ l miRIDIAN miR-181a-5p Mimic (15 η M, C-300552-05-0005, Dharmacon Research Inc.) or 3 μ l miRIDIAN microRNA Mimic Negative Control (15 η M, CN-001000-01-05, Dharmacon Research Inc.) (Figure 2.12). Mimics were mixed with 0.75 μ l DharmaFECT-1 Transfection Reagent and TM20 in a final volume of 1 ml. An untreated control (TM20) and a control of transfection without mimic (0.75 μ l DharmaFECT-1 and TM20) were prepared. After 24 hours, the transfection medium was replaced with fresh TM20 for a further 24-hour incubation. All transfections were performed in triplicate.

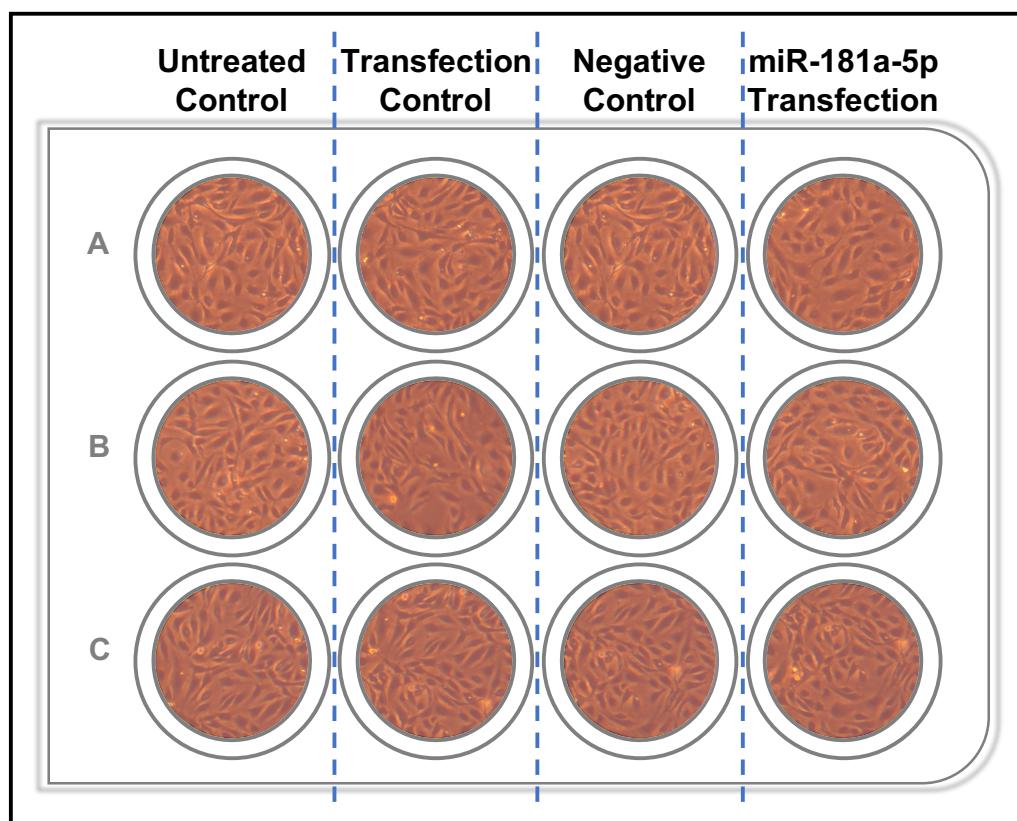


Figure 2.12. HUVEC transfection set up experiment. HUVEC were seeded at 2.0×10^5 cells/ml per well in TM20 and incubated overnight at 37°C humidified incubator with 5% CO₂ in air. Medium was removed and 1 ml of each condition was added in triplicates: untreated control (TM20), transfection control (TM20 + DharmaFECT1), negative control (negative control mimic + TM20 + DharmaFECT1), and miR-181a-5p (miR-181a-5p mimic + TM20 + DharmaFECT1). After 24 hours, transfection medium was replaced with fresh TM20 for a further 24-hour incubation.

After 48 hours, medium was removed and cells were washed with sterile cold PBS. RNA extraction was performed as previously described (Section 2.6) with some modifications. Plate was placed on ice, and 750 µl TRI Reagent (Sigma) were used for extraction. Homogenised solutions were snap-frozen and kept on dry-ice until all the wells were processed. One µl of glycogen was added to the isopropanol step to aid RNA precipitation. RNA was re-suspended in 25 µl water free of RNases. Transfection efficacy of miR-181a-5p mimic, as well as *Occludin* and *KLF6* gene expression were evaluated by qPCR (Section 2.11).

2.21. OCCLUDIN IMMUNOFLUORESCENCE IN PLACENTAL SAMPLES

Placental frozen tissue (covered with OCT) was cryo-sectioned at 10 μm thickness, and transferred onto 1% gelatinised slides. Frozen sections were stored at -80°C until immunofluorescence.

Occludin immunostaining was performed as described by Babawale and collaborators (Babawale *et al.*, 2000) with some modifications. In brief, frozen sections were air-dried for 15 minutes at room temperature. Sections were permeabilised with acetone at -20°C for 15 minutes, followed by 40 μl of 0.1% Triton X-100/0.1%BSA/PBS solution for 10 minutes at room temperature. Sections were washed twice with 0.1%BSA/PBS solution and 40 μl of blocking buffer (5% normal human serum/0.1%BSA/PBS) was added. Tissue was incubated at 37°C for 30 minutes in a humid chamber. Sections were incubated overnight at 4°C with 25 μl primary antibodies: Occludin rabbit polyclonal (2.5 $\mu\text{g}/\text{ml}$; 71-1500; Thermo Fisher) and PECAM-1 mouse monoclonal (2.5 $\mu\text{g}/\text{ml}$; BBA7, R&D Systems), diluted in 0.3% Triton X-100/0.1%BSA/PBS. Sections were washed three times with 0.1% BSA/PBS, and incubated at 37°C for 2 hours with 25 μl secondary antibodies: Anti-Rabbit IgG FITC antibody produced in goat (F0382, Sigma, St. Louis, USA) and Anti-Mouse IgG TRITC antibody produced in goat (T5393, Sigma, St. Louis, USA), diluted in 0.1%BSA/PBS to 10 $\mu\text{g}/\text{ml}$. Finally, the slides were fixed onto coverslips covered with 4 μl of VECTASHIELD Antifade Mounting.

A Nikon fluorescence microscope (Nikon LaboPhot-2 fluorescence microscope, Nikon Instruments) was used to visualise the immunoreactivity of the placental tissue. A systematic random sampling of fields of view was adopted, and micrographs were taken with a Nikon DS-Fi1 digital camera. The number of vascular profiles showing immunoreactivity to Occludin and PECAM-1 was counted using unbiased “forbidden line” counting principle (Sciota, 2012) (Figure 2.13). No more

than 150 vascular profiles showing positive immunoreactivity were counted for each placenta to maintain efficiency.

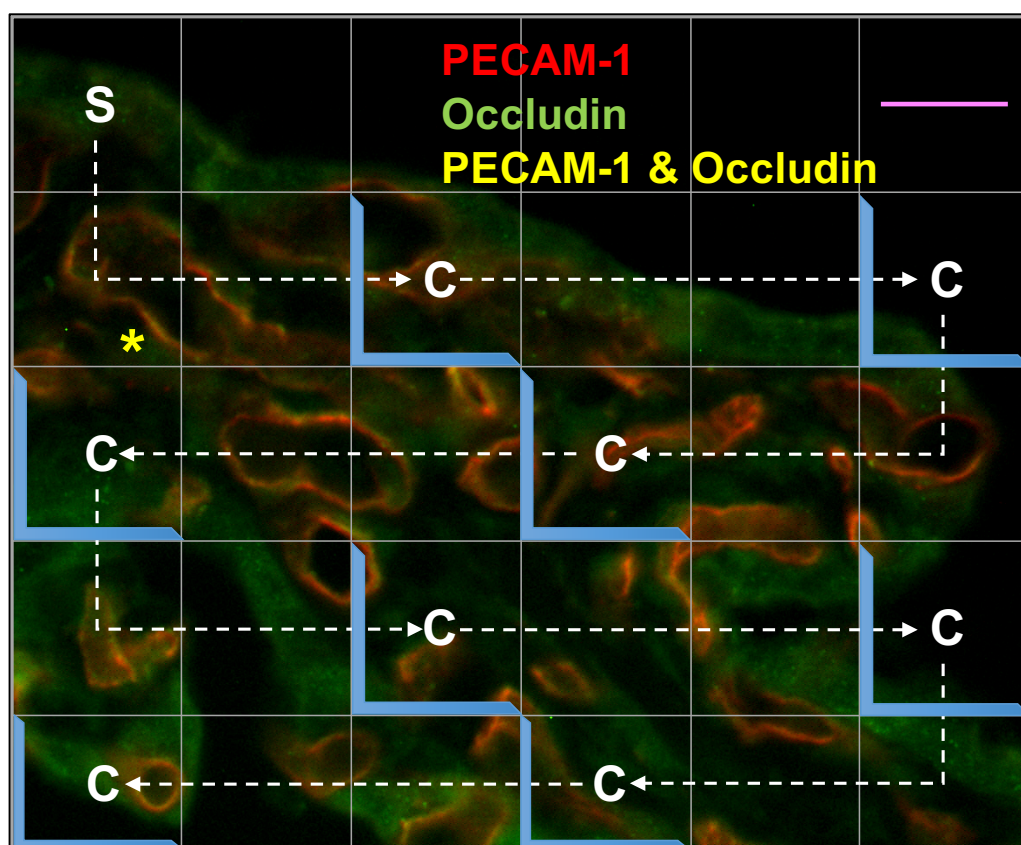


Figure 2.13. Systematic random sampling, “forbidden line” counting principle. A grid was superimposed on the micrograph, and counting started from the upper left area of the tissue (S). Every 3rd square was counted (C) after the start point. Counting continues following the pattern of the arrows. To avoid counting vessels twice, the “forbidden line” (light blue) method was used, where vessels on the forbidden line were not counted. Vessels were stained with PECAM-1 (red). Occludin (green). The presence of junctional occludin was counted when a bright yellow spot was located within the vessel membrane (yellow star). Scale bar= 20 μ m (purple).

2.22. STATISTICAL ANALYSIS

The data is expressed as means $\pm$ SEM. The data was tested for normality distribution by D’Agostino & Pearson test with a confidence interval of 95%. Statistical comparison between two groups were performed for parametric (independent samples *t*-test) and non-parametric datasets (Mann–Whitney test). Statistical analyses between three groups were performed for parametric (one-way ANOVA) and non-

parametric datasets (Kruskal-Wallis test). All statistics and graphs were calculated with GraphPad Prism 6.0 (La Jolla, USA); $p < 0.05$ was considered statistically significant.

CHAPTER 3

EXPRESSION OF JUNCTIONAL MOLECULES IN PLACENTAL VASCULATURE

3.1. INTRODUCTION

Previous studies in human placental tissue from diabetic pregnancies (T1DM, T2DM and GDM) have shown reduced immuno-positive vessels with junctional Occludin and VE-cadherin (Leach *et al.*, 2004) (Babawale *et al.*, 2000) (Baumüller *et al.*, 2015). These studies focused on protein localisation. Beyond phosphorylation events and translocation from junctions, alternative splicing of junctional molecules and hence differential expression of these genes splice variants and protein isoforms might be behind the reduced junctional location and increased barrier permeability found in these diabetic studies. In this chapter, our study aimed to elucidate *Occludin*, *VE-cadherin*, and *Plakoglobin* gene splice variants and protein isoforms expression levels in normal and GDM placental samples.

3.1.1. OCCLUDIN GENE AND PROTEIN STRUCTURES

Human *Occludin* gene (*OCN*) maps to two different genomic locations: a full-length copy located on chromosome 5q13.1 (68 Mb) (J. M. Gu *et al.*, 2008), that includes nine exons; and a partially duplicated truncated copy (*Occludin* pseudogene) located on the same chromosome 5q13.2 (70 Mb) (Kohaar *et al.*, 2010) that includes four exons. The first exon is entirely untranslated, and exon nine contains untranslated regions. As mentioned in Section 1.5.1, published literature suggests the presence of several *Occludin* gene splice variants. Here we focus on the predicted splice variants reported in NCBI and Ensembl databases (Pruitt *et al.*, 2014; Yates *et al.*, 2016) (Ensembl release 94 – October 2018) (Figure 3.1). These

records have been curated by NCBI staff and are reference standards in the RefSeqGene project (Pruitt *et al.*, 2014). Additionally, splice variants found in Ensembl database were derived from a gene source database: GENCODE Basic (Yates *et al.*, 2016).

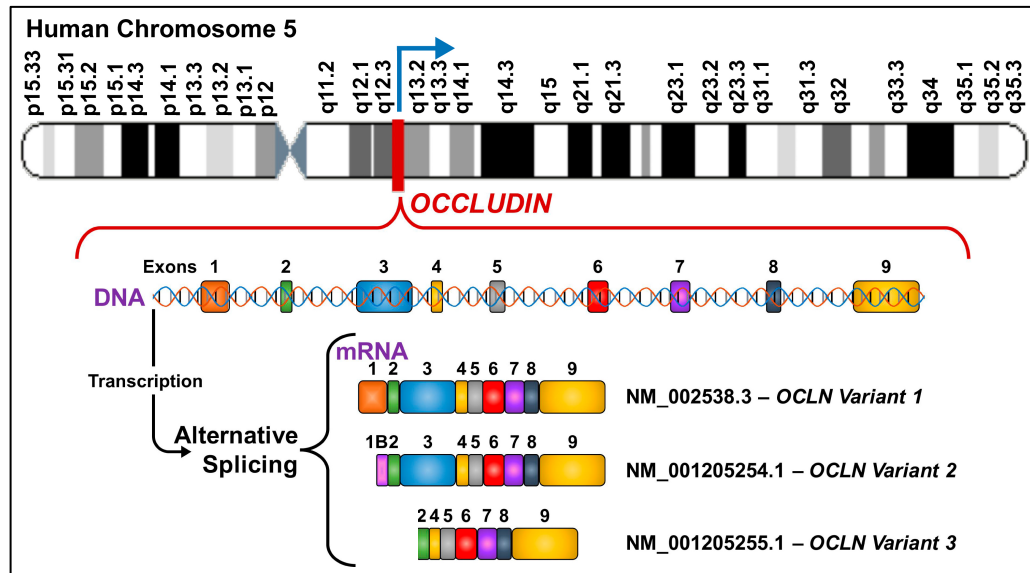


Figure 3.1. Schematic representation of Occludin gene localisation and splice variants. Occludin gene (OCLN) is localised on chromosome 5q13.1. This gene includes nine exons that, due to alternative splicing, gives rise to three splice variants. Exon sequences obtained from NCBI and Ensembl databases (Pruitt *et al.*, 2014; Yates *et al.*, 2016).

Occludin is a 59 kDa transmembrane protein that consists of five domains: N-terminal, four transmembrane (TM), two extracellular loops, an intracellular loop, and C-terminal domain (Figure 3.2A) (Förster, 2008; Yaffe *et al.*, 2012). Each domain helps mediate Occludin's maintenance and localisation in the TJs. For instance, the N-terminal domain plays a role in TJ structure and barrier function (Harhaj & Antonetti, 2004). The extracellular loops lead the insertion of Occludin into the TJs, and may also retain the protein in the membrane. The first extracellular loop contributes to intercellular adhesion by homo-dimerisation with Occludin from opposing cells (Yaffe *et al.*, 2012). While, the second extracellular loop helps mediate trans-epithelial electrical resistance (TER) (Bazzoni, 2006), and is essential for stable assembly of Occludin in the TJs (Feldman *et al.*, 2005). Furthermore, the four TM domains comprise the MARVEL motif; these

domains can induce and increase the resistance of the membrane (Yaffe *et al.*, 2012). Finally, the C-terminal domain binds to ZO-1 establishing a link between Occludin and the actin cytoskeleton (Mankertz *et al.*, 2002). The lack of the fourth TM and C-terminal domain affect Occludin ability to interact with ZO-1, preventing its localisation to the TJ (Cummins, 2012; Harhaj & Antonetti, 2004). *Occludin variant 1* and *variant 2* translate into the same functional protein Occludin isoform-A (Figure 3.2B); while *Occludin variant 3* translates into a smaller truncated version Occludin isoform-B (Figure 3.2C).

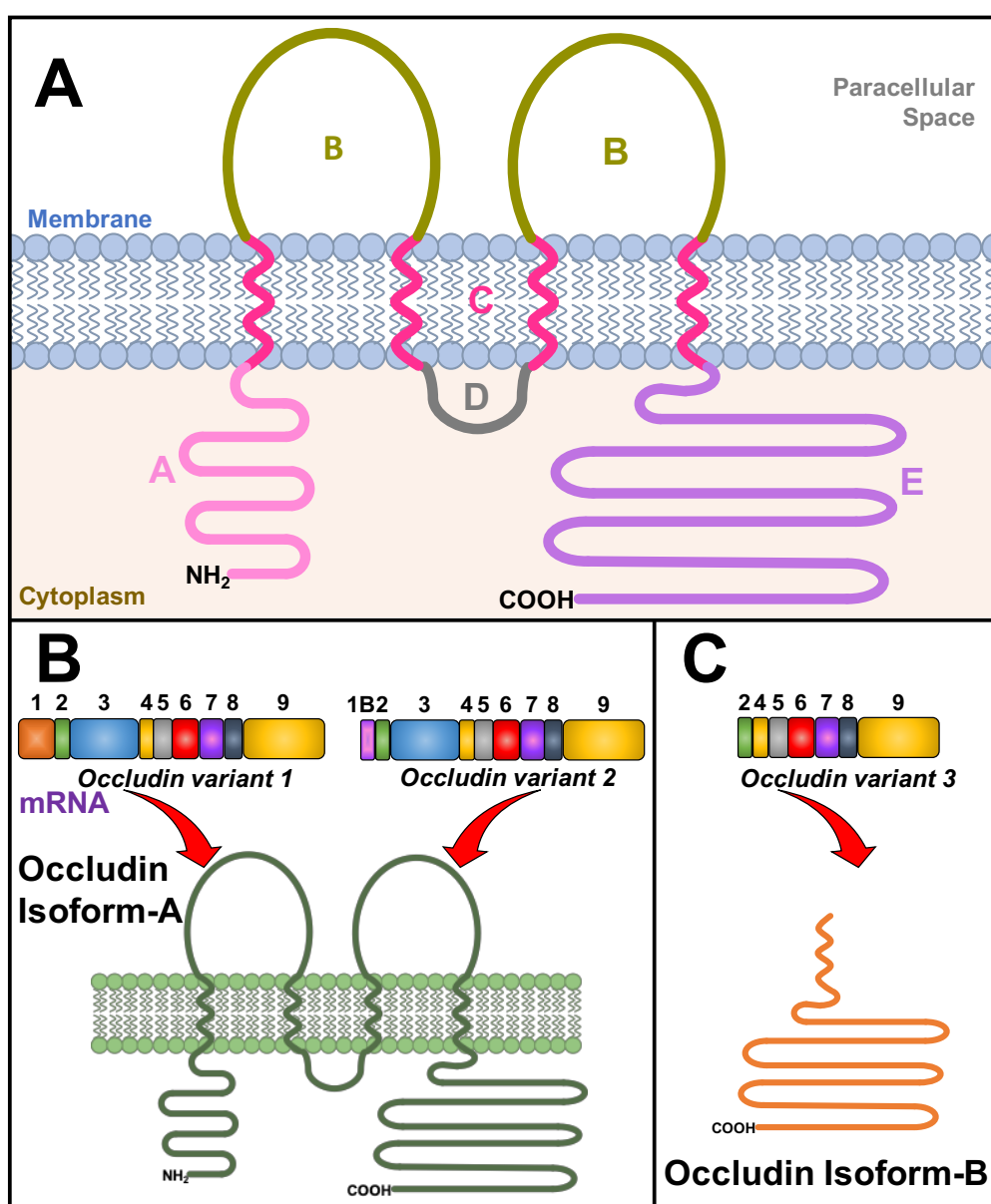


Figure 3.2. Schematic representation of Occludin protein. (A) Occludin presents five domains: N-terminal domain (A, pink), two extracellular domains (B,

olive), four transmembrane domains (C, fuchsia), intracellular loop (D, grey), and a C-terminal domain (E, purple). **(B)** Occludin isoform-A (~60 kDa) (green), fully functional membrane protein, is the product of the translation of *Occludin variants 1* or *2*. **(C)** Occludin isoform-B (~30 kDa) (orange), truncated, cytoplasmic protein, is the result of the translation of *Occludin variant 3*.

Several studies in diabetic tissue have shown that Occludin expression is affected and the integrity of the endothelial barrier is decreased compared to controls. For instance, lower Occludin protein levels and loss of junctional Occludin from the vessel's membranes were found in retinal tissue from streptozotocin-induced diabetic rats compared to controls (Antonetti *et al.*, 1998; Barber & Antonetti, 2003). The loss of Occludin was, in part, behind decreased integrity of the blood-retinal barrier, which is a characteristic of diabetic retinopathy. In human placenta, reduced levels of junctional Occludin were found in placental vessels from pregnancies complicated with GDM compared to controls (Babawale *et al.*, 2000). The effects of GDM on the gene expression of *Occludin* splice variants has not been studied in human placental vasculature.

3.1.2. VE-CADHERIN GENE AND PROTEIN STRUCTURES

Human *VE-cadherin* gene maps to chromosome 16q22.1. This gene includes 12 exons and several splice variants have been predicted. Here we focus on the expression of the four predicted VE-cadherin splice variants supported by NCBI and Ensembl databases (Pruitt *et al.*, 2014; Yates *et al.*, 2016) (Ensembl release 94 – October 2018) (Figure 3.3). The splice variants found in Ensembl database were derived from a gene source database: GENCODE Basic (Yates *et al.*, 2016). To our knowledge, these splice variants have not been reported so far in the literature. Each *VE-cadherin* variant is predicted to translate into a different protein.

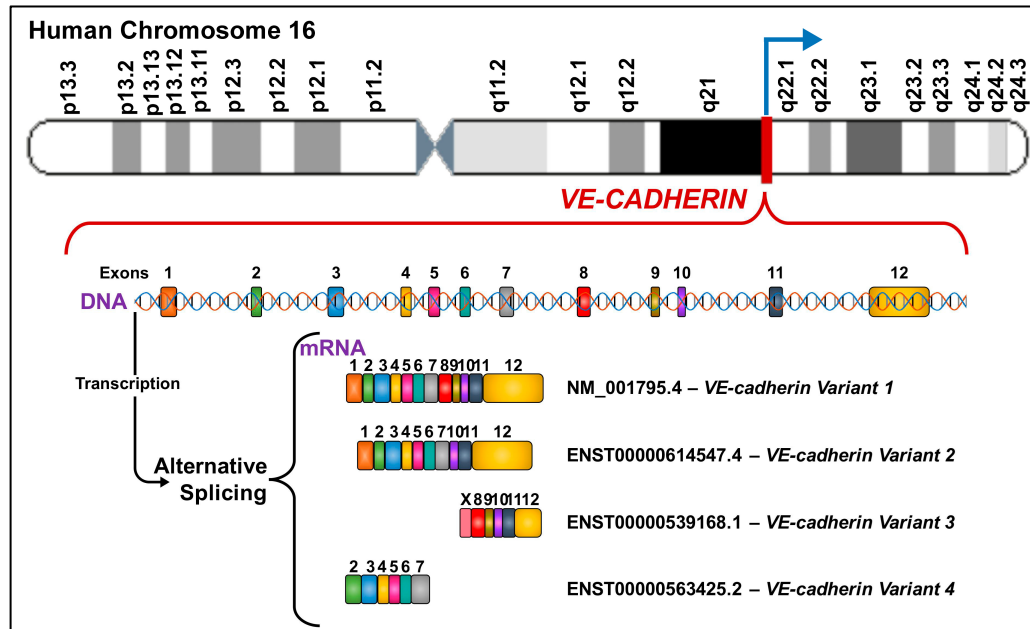


Figure 3.3. Schematic representation of *VE-cadherin* gene localisation and splice variants. *VE-cadherin* gene is localised on chromosome 16q22.1. This gene includes 12 exons that, due to alternative splicing, translates into four splice variants. *VE-cadherin* variant 3 presents an untranslated alternative exon at the start of the sequence (labelled as “X”). Exon sequences obtained from NCBI and Ensembl databases (Pruitt *et al.*, 2014; Yates *et al.*, 2016).

VE-cadherin is a 140 kDa type II cadherin protein that forms calcium-dependent homotypic bonds that connect apposed cells through the actin cytoskeleton via α - and β - catenins, and Plakoglobin (Kevil *et al.*, 1998). VE-cadherin presents four domains: extracellular cadherin (EC) domain, a transmembrane domain, juxtamembrane domain, and catenin binding domain (Figure 3.4A). The EC domain consists of five cadherin-like repeats that form a rigid structure that is stabilised by the binding of calcium ions to the intervening sequences located at the base of each domain (Vincent *et al.*, 2004). This domain mediates homophilic binding and adhesion between adjacent cells, these interactions can be *cis* (binding two adjacent cadherins of the same cell) or *trans* (between cadherins on adjacent cell). Moreover, the EC domain is important for proliferation. Previous studies of endothelial cells found that in the absence of the EC domain, cells proliferation was inhibited (Caveda *et al.*, 1996). On the other hand, the transmembrane domain constitutes an important part of flow signalling; the loss of this domain not only prevents VE-cadherin to anchor the membrane but also

changes subcellular distribution, protein clustering, and membrane micro-domain localisation (Coon *et al.*, 2015). Moreover, the two domains found in VE-cadherin's cytoplasmic tail interact with linker molecules to stabilise the cadherin and couple it to the actin cytoskeleton. The juxtamembrane domain contains a binding site for p120 and p0071; this binding stabilises the cadherin by preventing endocytosis (Xiao *et al.*, 2003). The catenin-binding domain will interact with β -catenin and Plakoglobin. Both of which are linked to α -catenin, that plays a role in coupling the cadherin tail to the actin cytoskeleton (Figure 3.4B). Translation of *VE-cadherin* splice variants leads to different protein isoforms (Figure 3.4C). VE-cadherin isoform 1 (~125 kDa) is a functional protein that consists of all the domains. VE-cadherin isoform 2 (~90 kDa) is missing one of the cadherin-like repeats (EC4). VE-cadherin isoform 3 (~30 kDa) presents one extracellular domain, plus the transmembrane and cytoplasmic domains. VE-cadherin isoform 4 (~40 kDa) presents only the first three EC domains.

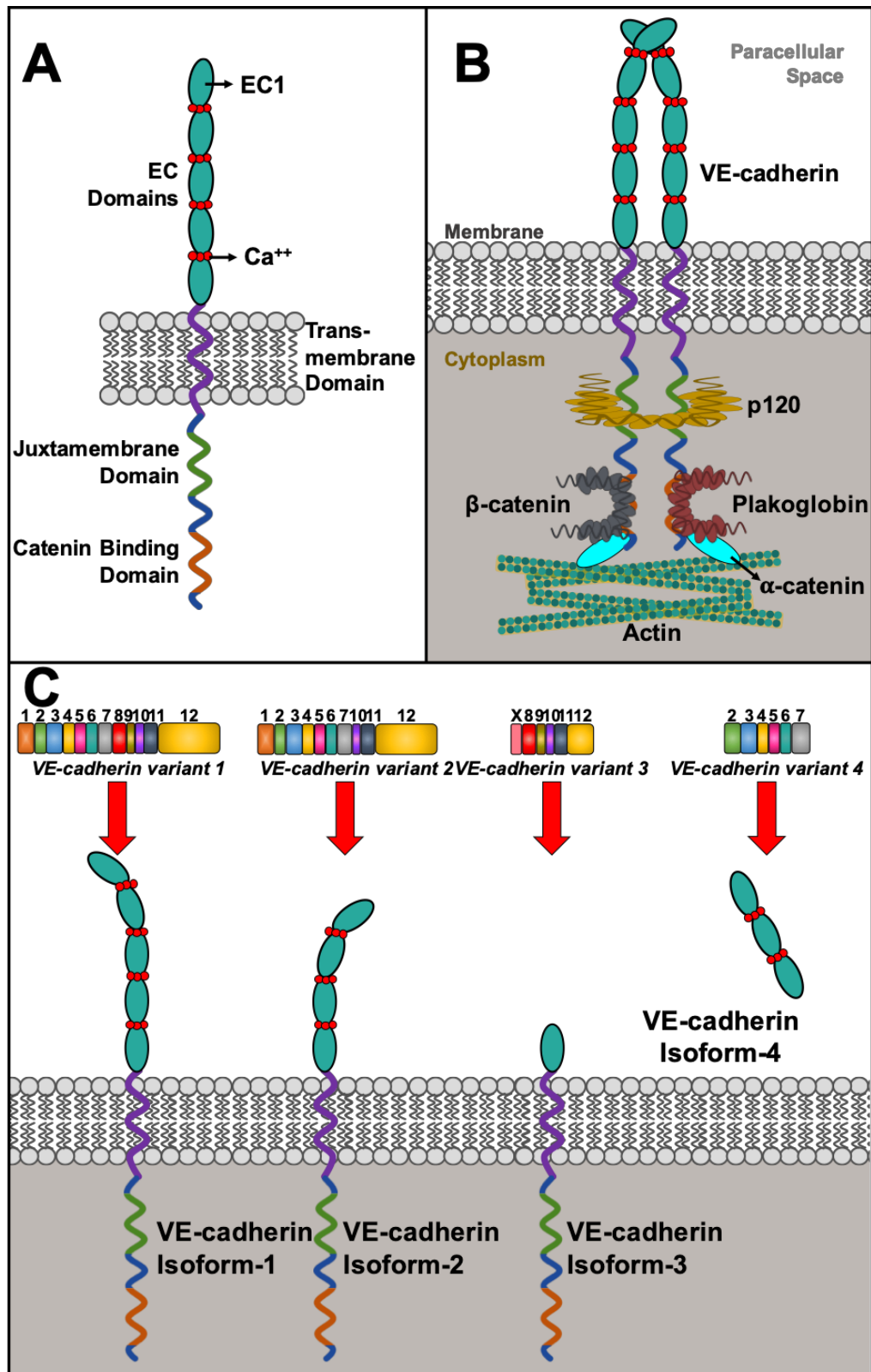


Figure 3.4. Schematic representation of VE-cadherin protein domains, protein interactions within AJs, and protein isoforms. (A) VE-cadherin presents four domains: Extracellular Cadherin (EC) domain, that consists of five cadherin repeats with Ca²⁺ binding sites between each other. A “transmembrane domain” (purple), a “juxtamembrane domain” (green) that binds p120-catenin, and a “catenin binding domain” (orange) that interacts with β-catenin and

Plakoglobin. **(B)** VE-cadherin interacts with different proteins to mediate cell-cell adhesion. VE-cadherin forms *cis* dimers by the lateral binding of cadherins within the plasma membrane of a single cell. p120 (yellow) is associated with the VE-cadherin juxtamembrane domain. β -catenin (grey) and Plakoglobin (brown) are associated with the catenin-binding domain, which in turn will bind to α -catenin (light blue) and to the actin cytoskeleton (green). **(C)** *VE-cadherin* gene splice variants translate into: VE-cadherin isoform 1 (~125 kDa), functional protein. VE-cadherin isoform 2 (~90 kDa), lacks one EC domain. VE-cadherin isoform 3 (~30 kDa), truncated protein, one EC domain, plus the transmembrane and cytoplasmic domains. VE-cadherin isoform 4 (~40 kDa), only three EC domains.

There are conflicting results about VE-cadherin expression in diabetic retinopathy. A study showed a loss of junctional VE-cadherin in retinal vessels and an increase permeability in retinal vasculature in streptozotocin-induced diabetic rats compared to controls (Navaratna *et al.*, 2007). On the contrary, VE-cadherin expression remained unchanged (results from gene and protein expression, plus immuno-localisation) and endothelial permeability was increased in human retinal endothelial cells (REC) cultured under hyperglycaemic conditions (25 mM glucose) compared to normal conditions (5mM glucose) (Saker *et al.*, 2014). In human placentae, junctional VE-cadherin levels were reduced in samples from diabetic pregnancies (T1DM, T2DM, GDM) compared to normal pregnancies (Babawale *et al.*, 2000) (Leach *et al.*, 2004) (Baumüller *et al.*, 2015). However, the effect of GDM on the gene expression of *VE-cadherin* splice variants has not been determined in human placental vasculature.

3.1.3. PLAKOGLOBIN GENE AND PROTEIN STRUCTURES

Human *Plakoglobin* gene (*JUP*) is mapped to chromosome 17q21.2 (Aberle *et al.*, 1995). This gene is a reverse strand transcript (transcription reads from right-to-left) and includes 14 exons. The first exon is entirely untranslated, and exon 14 contains untranslated regions. Here we focused on the expression of the seven predicted splice variants reported by NCBI and Ensembl databases (Pruitt *et al.*, 2014; Yates *et al.*, 2016) (Ensembl release 94 – October 2018) (Figure 3.5)). These records have been curated by NCBI (Pruitt *et al.*, 2014). To our knowledge, the gene expression of *Plakoglobin* splice variants has not been demonstrated. Variation within

Plakoglobin splice variants occurs in exons 1 and 14. As *Plakoglobin* translation sequence starts in exon two and finishes at the beginning of exon 14, all *Plakoglobin* splice variants translate into the same protein.

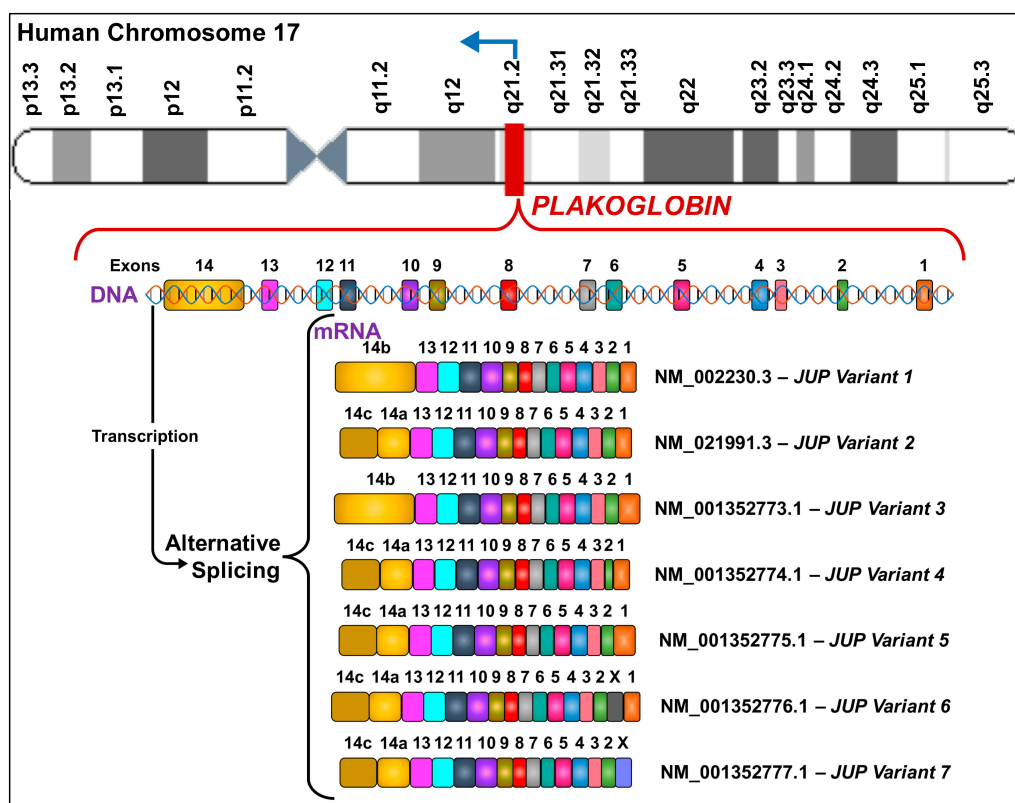


Figure 3.5. Schematic representation of *Plakoglobin* gene localisation and splice variants. *Plakoglobin* gene (*JUP*) is localised on chromosome 17q21.2, as a reverse stranded transcript. *JUP* presents 14 exons that, due to alternative splicing, translates into seven splice variants. Some variants present an alternative exon (labelled as “X”). *Plakoglobin* variants present two versions of exon 14: a complete sequence (14b) or a deletion of a central segment of exon 14 (14a + 14c). Exon sequences obtained from NCBI and Ensembl databases (Pruitt *et al.*, 2014; Yates *et al.*, 2016).

Plakoglobin is an 83 kDa protein that forms part of the armadillo protein family, sharing a 42-amino-acid motif repeated several times. Plakoglobin is formed by three domains: armadillo repeat domain, N-terminal domain, and C-terminal domain (Figure 3.6A). The armadillo repeat domain is composed by 12 repeats of ~42 amino acids each. This region folds into a superhelix composed of 36 small α -helices (3 per armadillo repeat). This armadillo complex mediates heterotypic interactions, including binding to cadherins. The N-terminal domain

mediates the binding of α -catenin to the actin cytoskeleton (Zhurinsky *et al.*, 2000). The C-terminal is a transactivation domain and is critical for controlling the armadillo repeat domain signalling (Williams *et al.*, 2000). This domain is less accessible to proteolysis, suggesting that this protein has a more compact structure (Figure 3.6B) (Solanas *et al.*, 2004). As previously mentioned, Plakoglobin is part of AJs and desmosomes. In AJs, Plakoglobin and β -catenin form mutually exclusive complexes with the cytoplasmic tail of VE-cadherin, providing a link to α -catenin and actin, and mediating cell adhesion (Figure 3.6C). In desmosomes, Plakoglobin mediates the link of desmosomal cadherins to desmoplakin and the intermediate filament cytoskeleton (Figure 3.6D). This binding blocks Plakoglobin's ability to bind to α -catenin, thereby inhibiting the interaction with actin (Williams *et al.*, 2000). Because of this dual targeting in epithelial cells, Plakoglobin is thought to play a key role in the cross-talk between AJs and desmosomes (Vincent *et al.*, 2004).

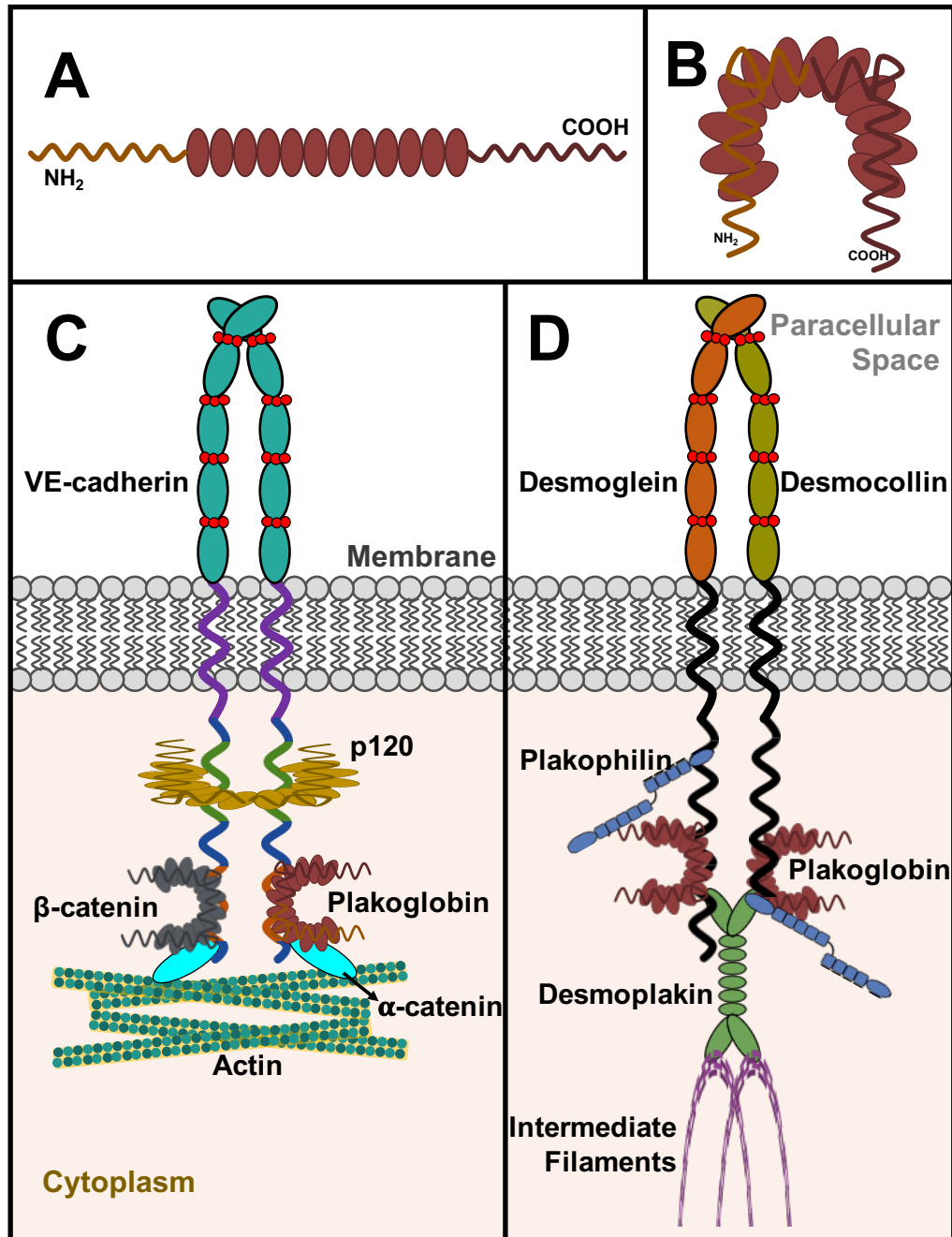


Figure 3.6. Schematic representation of Plakoglobin protein domains and protein interaction with AJs and desmosomes. (A) Plakoglobin presents three domains: armadillo repeat domain, that consists of 12 repeats of ~42 amino acid each, and binds to cadherins. An N-terminal domain (dark orange), and a C-terminal domain (brown). **(B)** Plakoglobin globular structure and domains. **(C)** In AJs, Plakoglobin binds to VE-cadherin to mediate cell-cell adhesion. Plakoglobin links VE-cadherin to α-catenin (light blue) and to the actin cytoskeleton. **(D)** In desmosomes, Plakoglobin binds to desmosomal cadherins: desmoglein (orange) and desmocollin (olive), mediating their interaction via plakophilin (blue) and desmoplakin (green) with intermediate filaments (purple) of the cytoskeleton.

In human placenta, a study showed stable expression of Plakoglobin protein in placental tissue between normal pregnancies and pregnancies complicated with preeclampsia (S. C. Lim *et al.*, 2009). Plakoglobin expression in human placenta from pregnancies complicated with diabetic is unknown.

3.1.4. HYPOTHESIS AND AIMS OF CHAPTER 3

During a GDM pregnancy, placental vessels present a differential expression of gene splice variants and protein isoforms of junctional molecules

To test this hypothesis, the aims of this chapter were:

- To compare the gene, protein and junctional expression of tight junctional molecule Occludin in the human placental tissue between normal and GDM pregnancies.
- To determine the gene expression of adherens junctional molecules VE-cadherin and Plakoglobin, in the human placental tissue of normal and GDM pregnancies.
- To define if treatment to control glucose levels (diet or metformin) counteracts the effects of GDM on the expression of junctional molecules.

3.2. METHODS

For detailed methodology see Chapter 2.

3.2.1. STUDY POPULATION

Pregnant women, who were schedule for elective C-section at term pregnancies (>37 weeks), were asked for written informed consent for placental tissue collection. Placental samples were collected from normal pregnancies ($n= 9$) used as a control, and pregnancies complicated with GDM where glucose levels were treated by a change of diet ($n= 7$) or by medication (metformin) ($n= 6$).

3.2.2. GENE EXPRESSION OF JUNCTIONAL PROTEINS

RNA isolation, reverse transcription and qPCR were performed as described in Sections 2.6, 2.7 and 2.9. Primers were designed for each gene. The most efficient primer concentration and cDNA dilution were found for each primer reaction (Table 3.1). Gene expression was analysed as described in Sections 2.11.

Table 3.1. Primer information of junctional molecules. Detailed information about *Occludin* (*OCLN*), *VE-cadherin* (*VE-cad*), and *Plakoglobin* (*JUP*) primers. Forward (F) and reverse (R) sequences, primer final concentration ([Final]), cDNA dilution (cDNA), annealing temperature (°C) and if 1mM betaine was needed for the specificity of the reaction (*).

Gene	Sequence	[Final]	cDNA	°C
<i>OCLN</i> <i>var1</i>	F: 5'- TGAATTGGTCACCGAGGGAG-3' R: 5'- TAAACCAATCTGCTGCGTCCTA-3'	600 ηM	1:8	60*
<i>OCLN</i> <i>var2</i>	F: 5'- GGCGAGCGGATTGGTTTAT-3' R: 5'- AAGGAGGTGGACTTTCAAGAGG-3'	600 ηM	1:64	58*
<i>OCLN</i> <i>var3</i>	F: 5'- CCTTACAGGCCTGATGAATTGC-3' R: 5'- TGTCCATCTTTCTTCGAGTTTTC-3'	300 ηM / 800 ηM	1:8	62*
<i>VE-cad</i> <i>var 1, 2, 3</i>	F: 5'- TGGTAGCCATCTTACTCTGCAT-3' R: 5'- GAGTTGAGCACCGACACATC-3'	600 ηM	1:12	60*
<i>VE-cad</i> <i>var 1, 2, 4</i>	F: 5'- CCAGCTTCACCATCAAAGTTCA-3' R: 5'- GGCAAAATACTCTTTCCCCTTC-3'	700 ηM	1:16	60
<i>JUP</i>	F: 5'- AGTACACGCTCAAGAAAACCAC-3' R: 5'- CTGACACACCAGGGCACAT-3'	600 ηM	1:12	60*

3.2.3. OCCLUDIN AND VE-CADHERIN PROTEIN EXPRESSION

Protein isolation and Western blot were performed as described in Sections 2.15 and 2.16. In brief, total protein (40 μg) was separated on a 4–20% SDS-PAGE (Bio-Rad, USA) by electrophoresis and transferred to a nitrocellulose membrane. Membranes were incubated overnight at 4°C with primary antibodies: polyclonal rabbit anti-Occludin (0.5 μg/ml; 71-1500; Thermo Fisher), or monoclonal rabbit anti-VE-cadherin (0.2 μg/ml;

2500; Cell Signaling Technology). Monoclonal mouse anti- β -actin (7 μ g/ml; A5316; Sigma) was used as loading control for each protein.

3.2.4. JUNCTIONAL OCCLUDIN IMMUNOSTAINING

Placental villous were stained for Occludin following the protocol described in Section 2.21.

3.2.5. STATISTICAL ANALYSIS

The data is expressed as means $\pm$ SEM. The data was tested for normality distribution by D'Agostino & Pearson test with a confidence interval of 95%. Results from the study population, protein expression and protein localisation presented a normal distribution. Hence, statistical comparison between two groups were performed by an independent samples *t*-test, and between three groups by one-way ANOVA. Results from gene expression failed to be normally distributed. Therefore, statistical comparison between two groups were performed by Mann-Whitney test, and between three groups by Kruskal-Wallis test.

3.3. RESULTS

3.3.1. COMPARISON OF MATERNAL CLINICAL PARAMETERS BETWEEN STUDY GROUPS

There was no significant difference in the maternal age ($t(19) = 1.39$, $p = 0.181$), gestational age ($t(19) = 1.72$, $p = 0.101$), or BMI ($t(19) = 1.86$, $p = 0.078$) between women with normal and GDM pregnancies (Table 2.1). Additionally, there was no significant differences between patients from normal and GDM pregnancies which glucose levels were maintained either by a change of diet or metformin in the maternal age ($F(2, 19) = 0.99$, $p = 0.3897$), gestational age ($F(2, 19) = 1.57$, $p = 0.236$), or BMI ($F(2, 19) = 2.144$, $p = 0.145$). However, the BMI from metformin-controlled GDM patients was significantly higher than normal patients ($t(13) = 2.33$, $p = 0.036$). Additionally, comparisons of glucose levels (HbA_{1c}) between diet-controlled and metformin-controlled GDM patients showed no significant

difference ($t(8) = 0.974$, $p = 0.359$) between groups. Even when GDM patient's BMI was high, their glycated haemoglobin levels (HbA_{1c}) showed controlled levels of glucose with both diet and metformin approaches (HbA_{1c} levels below 39.0 mmol/mol).

3.3.2. DIFFERENTIAL GENE EXPRESSION OF *OCLUDIN* IN PLACENTAL SAMPLES FROM DIET-CONTROLLED GDM PREGNANCIES

All three predicted *Occludin* splice variants reported in NCBI and Ensembl databases were found in normal placental samples: *Occludin variant 1*, *2*, and *3*. *Occludin variant 2* represents ~75% of total *Occludin* expression; followed by *Occludin variant 3* (~15%) and *Occludin variant 1* (~10%).

Occludin variant 1 gene expression was similar in placental samples between normal and GDM pregnancies ($U = 35.0$, $p = 0.126$) (Figure 3.7A). Additionally, there was no statistical significant difference in *Occludin variant 1* gene expression in placental samples from normal, diet-controlled and metformin-controlled GDM pregnancies ($H = 3.33$, $p = 0.194$) (Figure 3.7B). However, there was a tendency for *Occludin variant 1* expression to be lower in diet-controlled GDM samples than for normal placental samples ($U = 13.0$, $p = 0.055$).

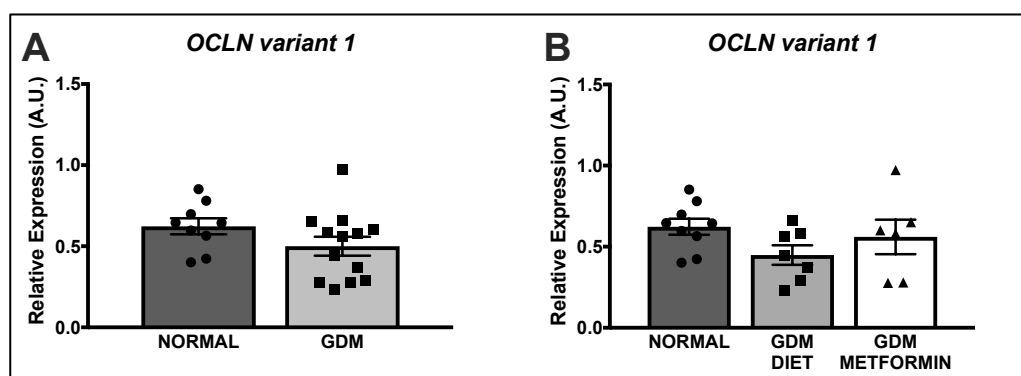


Figure 3.7. *Occludin variant 1* gene expression was similar between normal and GDM placental samples. Samples were taken from normal pregnancies ($n = 9$), and pregnancies complicated with GDM where glucose levels were treated with diet ($n = 7$) or metformin ($n = 6$). **(A)** *Occludin variant 1* expressions between normal and GDM placental samples. **(B)** *Occludin variant 1* expressions between

normal, diet-controlled and metformin-controlled GDM placental samples. Mean $\pm$ SEM. Kruskal Wallis test and Mann-Whitney post hoc test.

Occludin variant 2 gene expression was lower in placental samples from GDM pregnancies compared to control ($U = 27.0$, $p = 0.036$) (Figure 3.8A). Additionally, there was a statistically significant difference in *Occludin variant 2* gene expression in placental samples between normal, diet-controlled and metformin-controlled GDM pregnancies ($H = 6.68$, $p = 0.029$) (Figure 3.8B). *Occludin variant 2* expression was lower in diet-controlled GDM samples than in normal placental samples ($U = 9.0$, $p = 0.016$).

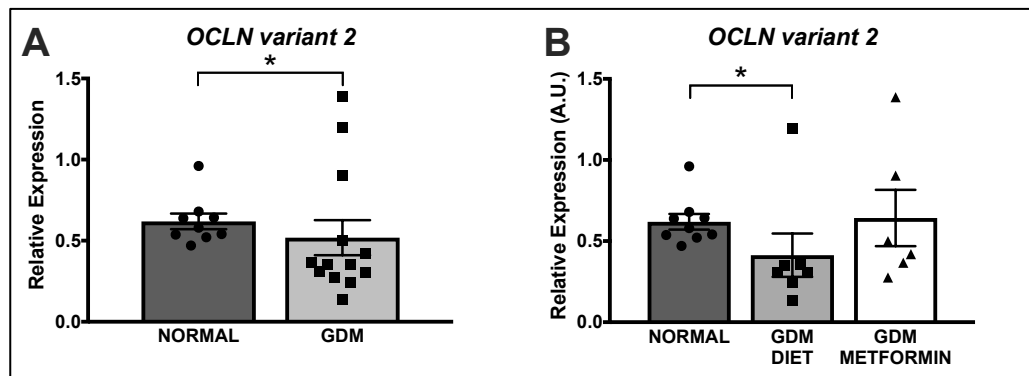


Figure 3.8. *Occludin variant 2* gene expression was lower in GDM placental samples compared to controls. Samples were taken from normal pregnancies ($n = 9$), and pregnancies complicated with GDM where glucose levels were treated with diet ($n = 7$) or metformin ($n = 6$). **(A)** *Occludin variant 2* expressions between normal and GDM placental samples. Mann-Whitney test ($*p < 0.05$) **(B)** *Occludin variant 2* expressions between normal, diet-controlled and metformin-controlled GDM placental samples. Mean $\pm$ SEM. Kruskal Wallis test and Mann-Whitney post hoc test ($*p < 0.05$).

Occludin variant 3 gene expression was similar in placental samples between normal and GDM pregnancies ($U = 28.0$, $p = 0.089$) (Figure 3.9A). Furthermore, there was no statistically significant difference in *Occludin variant 3* gene expression in placental samples from normal, diet-controlled and metformin-controlled GDM pregnancies ($H = 4.40$, $p = 0.110$) (Figure 3.9B). However, *Occludin variant 3* expression was higher in diet-controlled GDM samples than for normal placental samples ($U = 8.0$, $p = 0.020$).

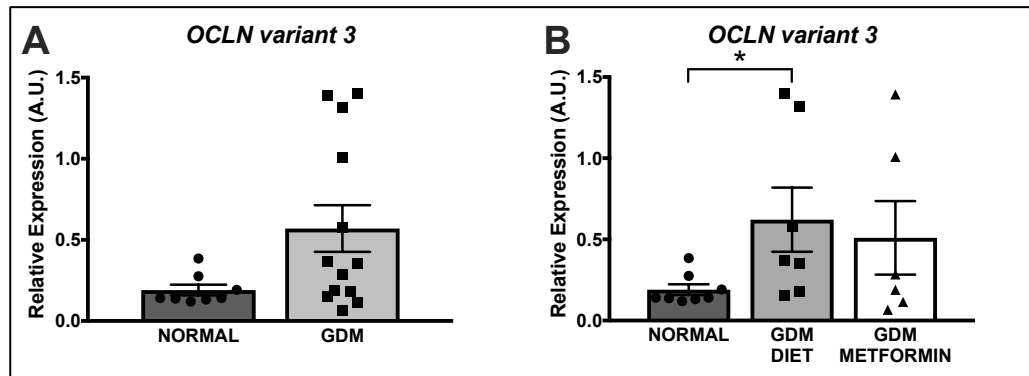


Figure 3.9. Occludin variant 3 gene expression was higher in diet-controlled GDM placental samples compared to controls. Samples were taken from normal pregnancies ($n = 9$), and pregnancies complicated with GDM where glucose levels were treated with diet ($n = 7$) or metformin ($n = 6$). **(A)** Occludin variant 3 expressions between normal and GDM placental samples. **(B)** Occludin variant 3 expressions between normal, diet-controlled and metformin-controlled GDM placental samples. Mean $\pm$ SEM. Kruskal Wallis test and Mann-Whitney post hoc test ($*p < 0.05$).

3.3.3. STABLE *VE-CADHERIN* GENE EXPRESSION IN PLACENTAL SAMPLES FROM NORMAL AND GDM PREGNANCIES

The four *VE-cadherin* predicted splice variants reported in NCBI and Ensembl databases were detected in normal placental samples: *VE-cadherin variants 1, 2, 3* and *4*. Primers detected more than one variant at the time, so *VE-cadherin* expression was analysed in groups.

VE-cadherin variants 1, 2 and *3* gene expression in placental samples was similar between normal and GDM pregnancies ($U = 30.0$, $p = 0.152$) (Figure 3.10A). Likewise, *VE-cadherin variants 1, 2* and *4* gene expression in placental samples was similar between normal and GDM pregnancies ($U = 47.0$, $p = 0.882$) (Figure 3.10B). Furthermore, *VE-cadherin variants 1, 2* and *3* gene expression was similar in placental samples from normal, diet-controlled and metformin-controlled GDM pregnancies ($H = 2.25$, $p = 0.335$) (Figure 3.10C). Similarly, *VE-cadherin variants 1, 2* and *4* gene expression was similar in placental samples from normal, diet-controlled and metformin-controlled GDM pregnancies ($H = 0.109$, $p = 0.955$) (Figure 3.10D).

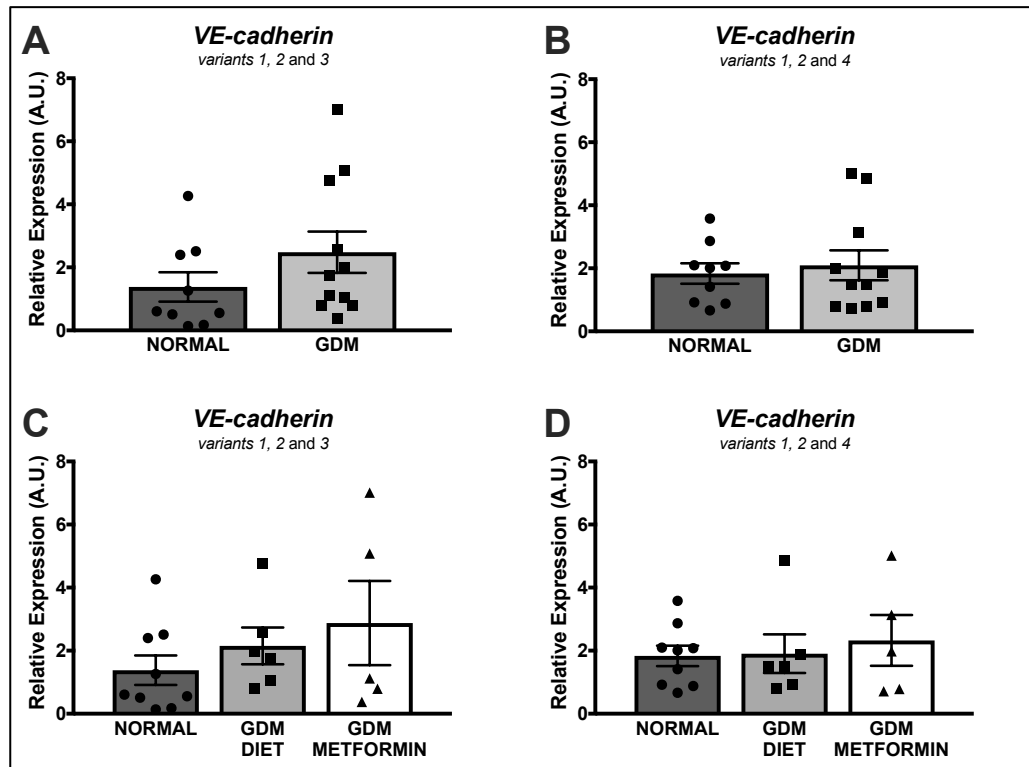


Figure 3.10. *VE-cadherin* variants 1, 2, 3, and 4 gene expression was similar between normal and GDM placental tissue. Samples were taken from normal pregnancies ($n = 9$), and pregnancies complicated with GDM where glucose levels were treated with diet ($n = 7$) or metformin ($n = 6$). *VE-cadherin* variants 1, 2 and 3 expressions between normal and GDM (A); and normal, diet-controlled and metformin-controlled GDM placental samples (C). *VE-cadherin* variants 1, 2 and 4 expressions between normal and GDM (B); and normal, diet-controlled and metformin-controlled GDM placental samples (D). Mean $\pm$ SEM. Kruskal Wallis test and Mann-Whitney post hoc test.

3.3.4. HIGHER *PLAKOGLOBIN* GENE EXPRESSION IN PLACENTAL SAMPLES FROM DIET-CONTROLLED GDM PREGNANCIES

As *Plakoglobin* splice variants translate into the same protein, primers were designed to amplify the coding region and so detect all the splice variants. *Plakoglobin* gene expression was similar between placental samples from normal and GDM pregnancies ($U = 24.0$, $p = 0.056$) (Figure 3.11A). Furthermore, *Plakoglobin* gene expression was similar between placental samples from normal, diet-controlled and metformin-controlled GDM pregnancies ($H = 3.93$, $p = 0.141$) (Figure 3.11B). However, *Plakoglobin* expression was higher in diet-controlled GDM samples than for normal placental samples ($U = 10.0$, $p = 0.049$).

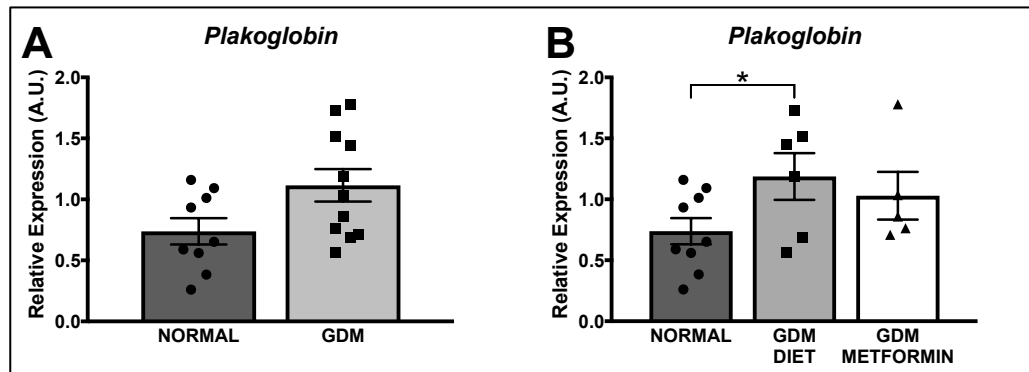


Figure 3.11. *Plakoglobin* gene expression was higher in diet-controlled GDM placental samples compared to controls. Samples were taken from normal pregnancies ($n = 9$), and pregnancies complicated with GDM where glucose levels were treated with diet ($n = 7$) or metformin ($n = 6$). **(A)** *Plakoglobin* expression between normal and GDM placental samples. **(B)** *Plakoglobin* expression between normal, diet-controlled and metformin-controlled GDM placental samples. Mean $\pm$ SEM. Kruskal Wallis test and Mann-Whitney post hoc test (* $p < 0.05$).

3.3.5. DOWNREGULATION OF OCCLUDIN PROTEIN EXPRESSION IN PLACENTAL SAMPLES FROM DIET-CONTROLLED GDM PREGNANCIES

Two Occludin protein isoforms were detected in normal placental samples (Figure 3.12). Occludin isoform-A represents the 83% of total Occludin protein expression (Figure 3.12C).

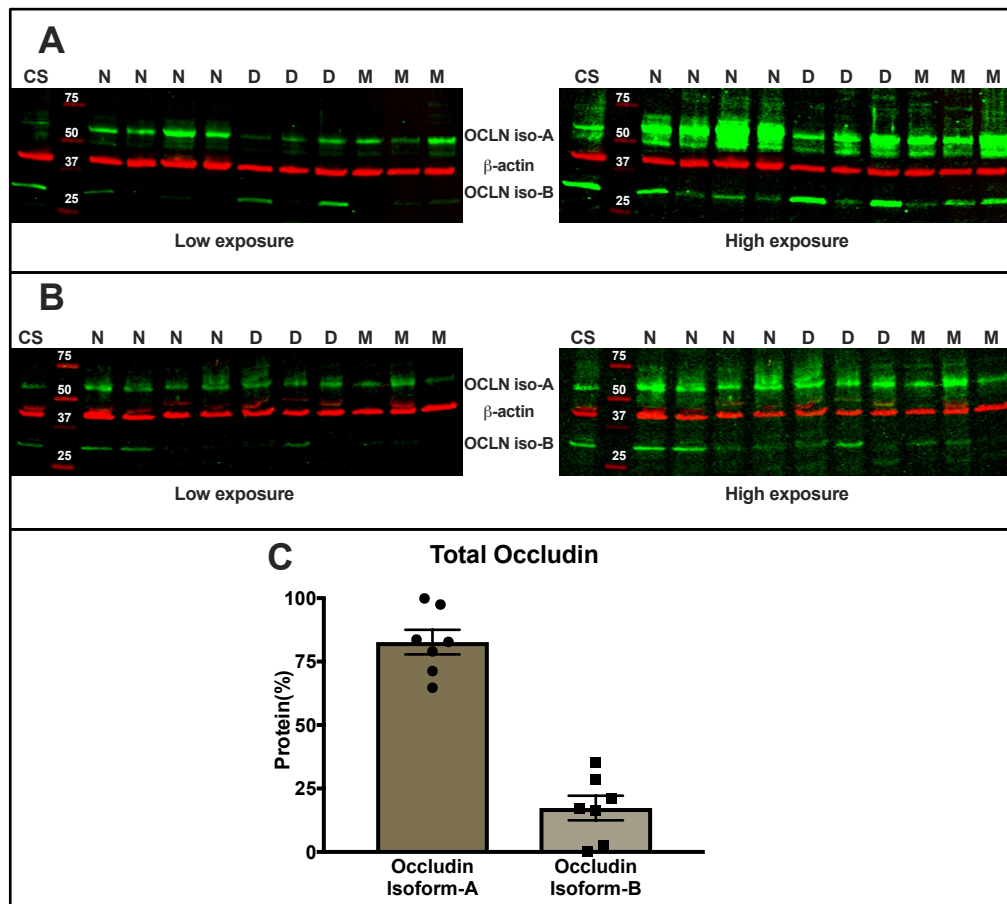


Figure 3.12. Occludin protein expression in human placental tissue. Samples were taken from normal pregnancies ($n = 9$), and pregnancies complicated with GDM where glucose levels were treated with diet ($n = 7$) or metformin ($n = 6$). **(A and B)** Western Blot bands of Occludin isoform-A (~60 kDa; green) and isoform-B (~30 kDa; green) from normal (N), diet-controlled (D), and metformin-controlled (M) GDM placental samples. β -actin (~40 kDa; red) used as a loading control. A control sample (CS) was run in each gel to control for variation between gels. Two pictures (low and high exposure) were taken from each gel to quantify both Occludin isoforms. **(C)** Percentage of Occludin isoforms in normal placental samples. Mean $\pm$ SEM.

Occludin isoform-A expression was similar between placental samples from normal and GDM pregnancies ($t(18) = 1.24$, $p = 0.231$) (Figure 3.13A). Occludin isoform-A expression was unchanged in placental samples from normal, diet-controlled and metformin-controlled GDM pregnancies ($F(2, 17) = 2.04$, $p = 0.161$) (Figure 3.13B). However, Occludin isoform-A expression for diet-controlled GDM pregnancies was significantly lower than normal pregnancies ($t(12) = 2.30$, $p = 0.040$).

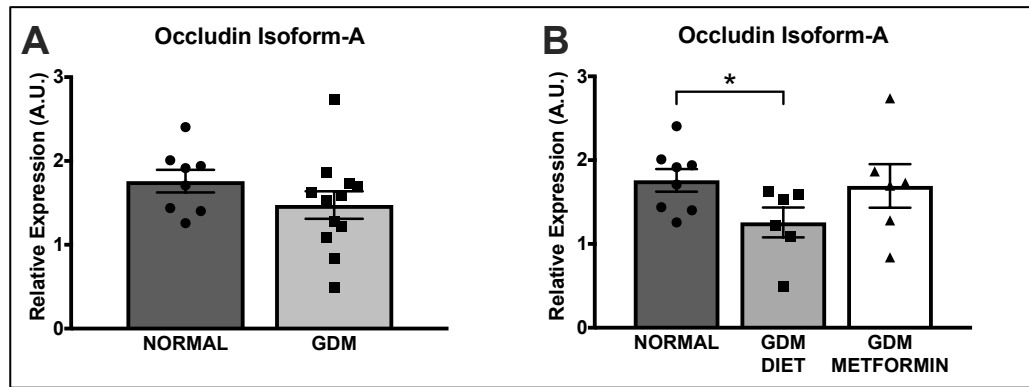


Figure 3.13. Occludin isoform-A protein expression was lower in diet-controlled GDM placental tissue compared to controls. Samples were taken from normal pregnancies ($n = 9$), and pregnancies complicated with GDM where glucose levels were treated with diet ($n = 7$) or metformin ($n = 6$). **(A)** Occludin isoform-A expression between normal and GDM placental samples. **(B)** Occludin isoforms-A expression between normal, diet-controlled and metformin-controlled GDM placental samples. Mean $\pm$ SEM. One-way ANOVA and post hoc Unpaired t-test ($*p < 0.05$).

Occludin isoform-B expression was similar in placental samples from normal and GDM pregnancies ($t(17) = 0.24, p = 0.808$) (Figure 3.14A). Furthermore, Occludin isoform-B expression was unchanged in placental samples from normal, diet-controlled and metformin-controlled GDM pregnancies ($F(2, 16) = 0.03, p = 0.967$) (Figure 3.14B).

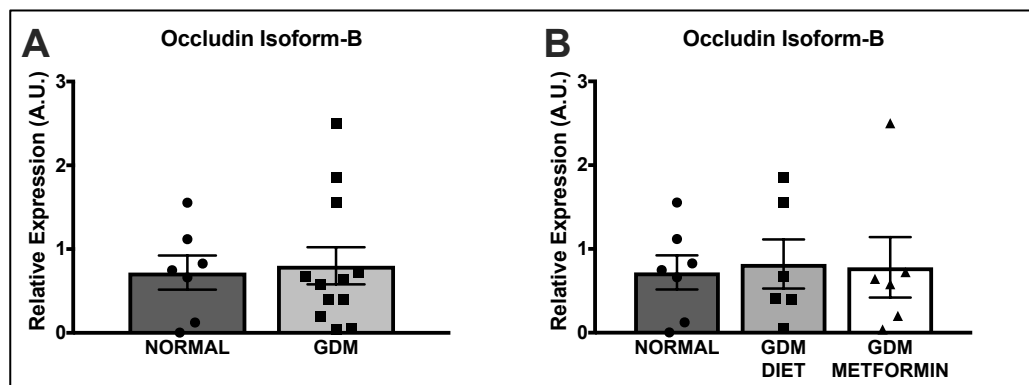


Figure 3.14. Occludin isoform-B protein expression was similar between normal and GDM placental tissue. Samples were taken from normal pregnancies ($n = 9$), and pregnancies complicated with GDM where glucose levels were treated with diet ($n = 7$) or metformin ($n = 6$). **(A)** Occludin isoform-B expression between normal and GDM placental samples. **(B)** Occludin isoforms-B expression between normal, diet-controlled and metformin-controlled GDM placental samples. Mean $\pm$ SEM. One-way ANOVA and post hoc Unpaired t-test.

3.3.6. STABLE EXPRESSION OF VE-CADHERIN PROTEIN EXPRESSION IN PLACENTAL SAMPLES BETWEEN NORMAL AND GDM PREGNANCIES

Three VE-cadherin protein isoforms were detected in placental samples from normal and GDM pregnancies (Figure 3.15A). In placental samples from normal pregnancies, VE-cadherin isoform 1 represents the 66% of total VE-cadherin protein expression, followed by isoform 2 (26%) and isoform 3 (8%) (Figure 3.15B).

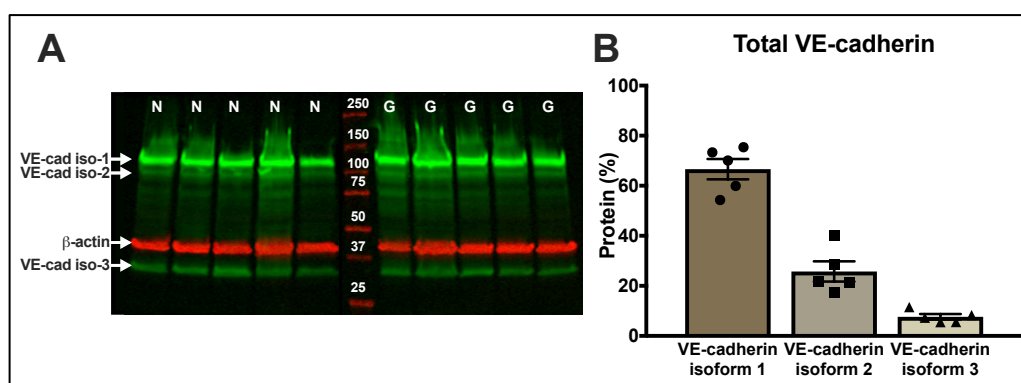


Figure 3.15. VE-cadherin protein expression in human placental tissue. Samples were taken from normal pregnancies ($n= 5$), and pregnancies complicated with GDM ($n= 5$). **(A)** Western Blot bands of VE-cadherin isoform-1 (~125 kDa), isoform-2 (~90 kDa) and isoform-3 (~30 kDa) from normal (N), and gestational diabetic (G) placental samples. β -actin (~40 kDa; red) used as a loading control. **(B)** Percentage of VE-cadherin isoforms in normal placental samples. Mean $\pm$ SEM.

VE-cadherin isoform 1 expression was similar in placental samples from normal and GDM pregnancies ($t(8) = 0.40, p = 0.698$) (Figure 3.16A). Similar results were obtained for VE-cadherin isoform 2 ($t(8) = 0.26, p = 0.800$) (Figure 3.16B), and isoform 3 ($t(8) = 0.35, p = 0.732$) (Figure 3.16C). Correspondingly, the expression of total VE-cadherin protein (adding the three isoforms) was similar between normal and GDM placental samples ($t(8) = 0.03, p = 0.978$) (Figure 3.16D).

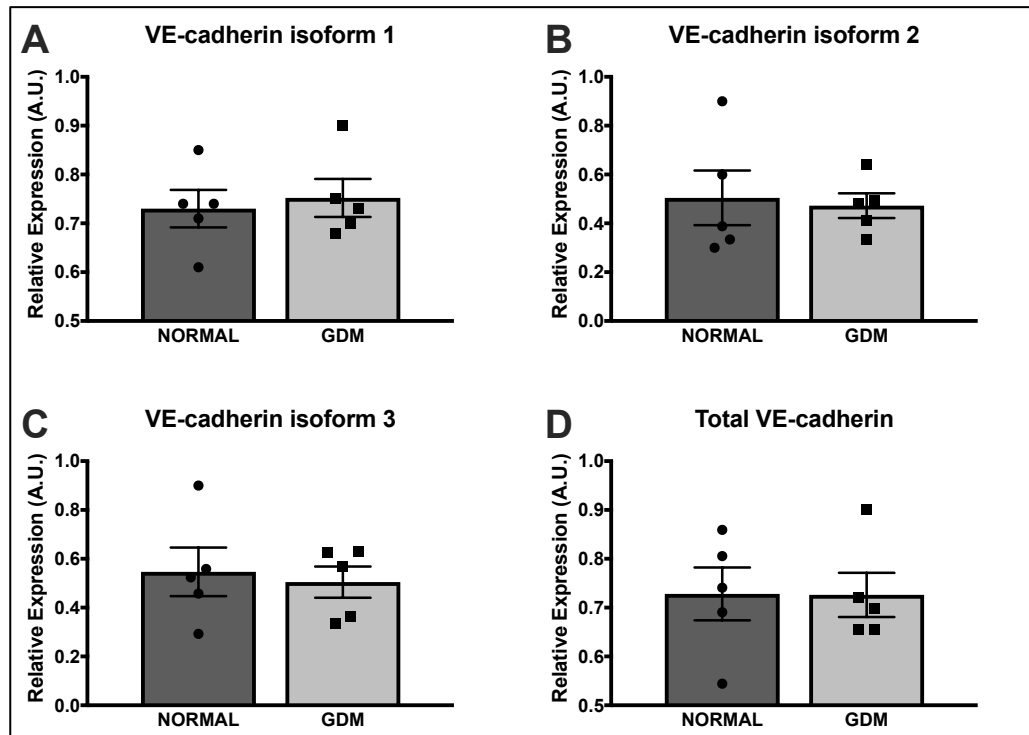


Figure 3.16. VE-cadherin protein expression was similar between normal and GDM placental tissue. Samples were taken from normal pregnancies ($n=5$), and pregnancies complicated with GDM ($n=5$). Protein expression of VE-cadherin isoform 1 (A) isoform 2 (B) and isoform 3 (C) between normal and GDM placental samples. (D) Total VE-cadherin protein expression between normal and GDM placental samples. Mean $\pm$ SEM. Unpaired t-test

3.3.7. DOWNREGULATION IN JUNCTIONAL OCCLUDIN LOCALISATION IN PLACENTAL SAMPLES FROM DIET-CONTROLLED GDM PREGNANCIES

A total of 451 micrographs were taken from placental samples (Figure 3.17). An average of 12 micrographs per sample were used for the quantification of junctional Occludin ($n=222$ micrographs). Junctions were counted (between 100-150 junctions per sample; $n=2837$ junctions), and junctional occupancy of Occludin was determined by the percentage of immuno-positive vessels. PECAM-1 (red) was used as a control for endothelial cell-cell adhesion (Figure 3.17A, D, G). Occludin (green) was found in some tight junctions (Figure 3.17B, E, H). Double localisation of PECAM-1 and Occludin (yellow) was quantified for each sample (Figure 3.17C, F, I).

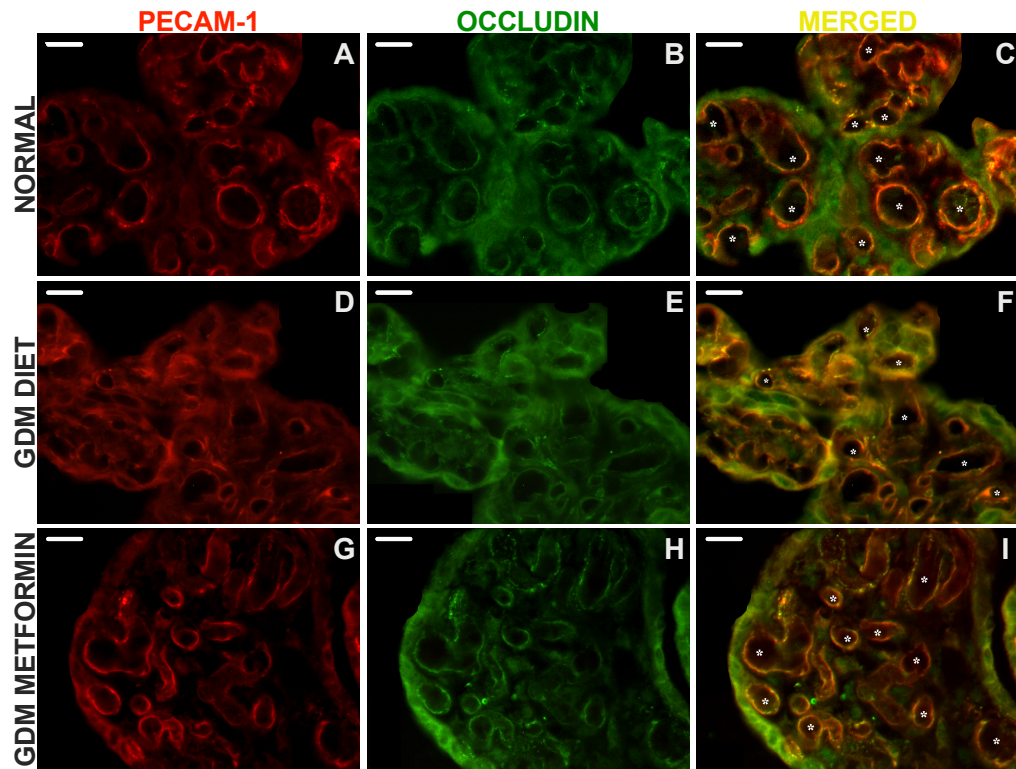


Figure 3.17. Representative micrographs of Occludin localisation in human placental tissue from normal and GDM pregnancies. Samples were taken from normal pregnancies ($n=6$), and pregnancies complicated with GDM where glucose levels were maintained with diet ($n=6$) or metformin ($n=6$). Samples were double-stained with PECAM-1 (red; panels A, D, and G) and Occludin (green; panels B, E, and H). Panels C, F, and I show double-staining with PECAM-1 and Occludin (yellow merged), stars show junctional Occludin in vessels' paracellular clefts. **(C)** Normal placental micrograph, junctional Occludin can be visualised in all junctions (11/11). **(F)** Diet-controlled GDM micrograph, only seven of 11 vascular profiles presented Occludin in their junctions. **(I)** Metformin-controlled GDM micrograph, junctional Occludin was found in most of the vessels (10/11). Scale bars= 20 μm .

Junctional Occludin localisation was similar between placental samples from normal and GDM pregnancies ($t(16) = 1.98$, $p = 0.065$) (Figure 3.18A). However, junctional Occludin localisation showed significant differences between placental samples from normal, diet-controlled and metformin-controlled GDM pregnancies ($F(2, 15) = 5.57$, $p = 0.015$) (Figure 3.17B). Furthermore, junctional Occludin localisation for diet-controlled GDM patients was significantly lower than normal patients ($t(10) = 3.58$, $p = 0.005$) and metformin-controlled patients ($t(10) = 2.24$, $p = 0.049$).

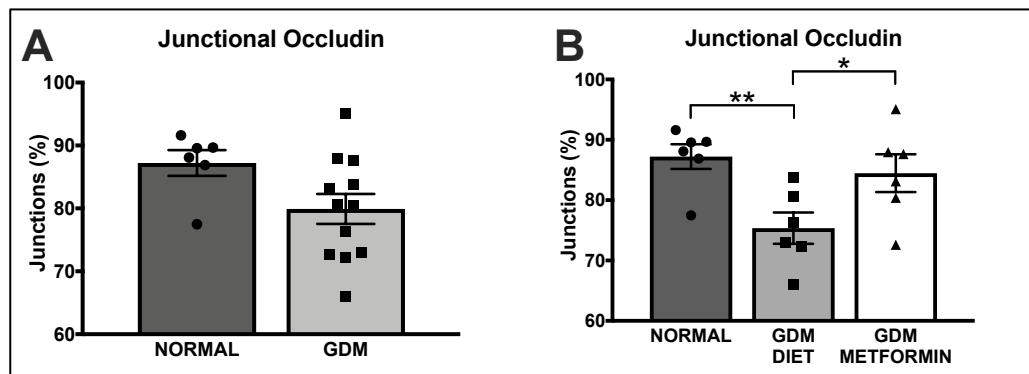


Figure 3.18. Lower junctional Occludin localisation in diet-controlled- GDM placental tissue. Samples were taken from normal pregnancies ($n= 6$), and pregnancies complicated with GDM where glucose levels were maintained with diet ($n= 6$) or metformin ($n = 6$). **(A)** Comparison of the percentage of junctions with Occludin between normal and GDM placental samples. **(B)** Comparison of the percentage of junctions with Occludin between normal, diet-controlled and metformin-controlled GDM placental samples. Mean $\pm$ SEM. One-way ANOVA and post hoc Unpaired t-test. (* $p < 0.05$; ** $p < 0.01$).

3.4. DISCUSSION

Our GDM study groups presented controlled hyperglycaemic levels in the last trimester of pregnancy (HbA_{1c} levels below 39.0 mmol/mol), suggesting that both methods of maintaining glucose levels were successful. Nevertheless, our results revealed that GDM influences Occludin gene and protein expression, as well as the protein localisation in the membranes of the placental vasculature. This effect was only visible in patients whose hyperglycaemia was controlled by a change of diet. Occludin expression and localisation were similar between placental samples from normal pregnancies and samples from GDM patients who were taking medication to control glucose levels (metformin-controlled GDM).

Considering maternal medical records, the GDM group was subdivided depending on the method used to control glucose levels. In general, women treated only with diet are usually at lower risk of complications related to a GDM pregnancy, while women who need medication to control glucose levels present a higher risk and will be managed in a more intense setting (McCance, 2015). Our results showed a

differential expression and localisation of Occludin in placental samples from diet-controlled GDM pregnancies compared to controls. Even when the women with GDM pregnancies presented controlled glucose levels through their last trimester, it seems like there are other factors that are influencing the gene expression in placental vasculature.

According to NICE guidelines and QMC practice, pregnant women will get an oral glucose tolerance test during the late second trimester (between 24 and 28 weeks of gestation). However, if pregnant women present a BMI above 30 kg/m² (obese), they will be tested for GDM earlier during pregnancy (before 24 weeks). Given that women from our diet-controlled GDM group were not obese, GDM would be detected after 24 weeks. In this case, hyperglycaemic insults would have been undetected for a longer period of time in diet-controlled GDM group compared to the metformin-controlled GDM group. This time difference could be behind the downregulation of Occludin our study found in placental tissue from diet-controlled GDM pregnancies. In this regard, a perfusion study using human placental samples from non-diabetic pregnancies showed that acute exposure to hyperglycaemic conditions (15 mM D-glucose) for 3-hours in the maternal circulation was enough to decrease the percentage of junctional VE-cadherin and increase vascular permeability in placental vessels compared to perfusion with euglycemic conditions (5 mM D-glucose) (Sciota, 2012).

During a GDM pregnancy, maternal hyperglycaemia will result in fetal hyperglycaemia and hyperinsulinemia to compensate for the high glucose levels (Pedersen's hypothesis). The use of metformin during a GDM pregnancy could have regulated the levels of circulating insulin due to the controlling actions of metformin through the activation of AMPK and enhancement of insulin sensitivity (Rena *et al.*, 2017). Nevertheless, as mentioned before, women from the diet-controlled GDM group could have experienced longer periods of undetected hyperglycaemia insults which could have been accompanied by hyperinsulinemia. A pilot study from our

group used perfusion of placental samples from normal pregnancies to show that the exposure to hyperinsulinemia (25 mU/mL) in the fetal circulation for 20 minutes results in increased junctional disruption (loss of β -catenin) and vascular leakage (Lucas *et al.*, 2008). To our knowledge, there is no published information regarding the effects of fetal hyperinsulinemia over Occludin expression and localisation in placental vasculature. Future work should consider maternal and fetal glucose and insulin levels from pregnancies complicated with GDM, as well as the effect of hyperinsulinemia over Occludin expression and localisation in the endothelial barrier.

We confirmed the presence of the three predicted *Occludin* gene splice variants, reported by NCBI and Ensembl databases, in human placental vessels. Our results showed that *Occludin variant 2* represents ~75% of total *Occludin* expression in placental samples from normal pregnancies. Additionally, we found that the expression of this variant had a 1.5-fold decrease in diet-controlled GDM samples (~60% of total *Occludin* expression) compared to control. As *Occludin variant 2* seems to be responsible for most *Occludin* gene expression, a change in this variant's expression could bring downstream repercussions. In this regard, we found a 1.4-fold decrease in protein expression of Occludin isoform-A in diet-controlled GDM samples compared to normal samples. Occludin isoform-A is a fully functional protein that oversees the stability of the membrane. A lower expression of this isoform might lead to changes in barrier integrity of vessels in diet-controlled GDM samples. Consequently, we found a 1.2-fold decrease localisation of junctional Occludin in the vessels of diet-controlled GDM samples compared to controls. This result was consistent with a previous study where junctional Occludin levels were reduced in insulin-controlled GDM placental vasculature (Babawale *et al.*, 2000). Additionally, several studies have confirmed that diabetes affects Occludin expression and junctional localisation in the tight junctions leading to decreased barrier integrity. Studies have shown lower Occludin protein levels and increased BBB permeability in cerebral tissue from

streptozotocin-induced diabetic rats compared to controls (Chehade *et al.*, 2002; Yoo *et al.*, 2016). Likewise, studies revealed lower Occludin protein levels and loss of junctional Occludin from the vessel's membranes of retinas from streptozotocin-induced diabetic rats compared to controls (Antonetti *et al.*, 1998) (Barber & Antonetti, 2003). This loss of Occludin was, in part, behind decreased integrity of the blood-retinal barrier, which is a characteristic of diabetic retinopathy. It remains to be established if Occludin downregulation in placental vasculature from GDM pregnancies is due to a hyperglycemic environment.

Our results showed that *Occludin variant 3* represents ~15% of total *Occludin* gene expression in placental samples from normal pregnancies. *Occludin variant 3* expression was 3.3-fold higher in diet-controlled GDM samples compared to samples from normal pregnancies (~30% of total *Occludin* gene expression). As *Occludin variant 2* decreased its expression, we could speculate that the rise in expression of *Occludin variant 3* may be a compensatory mechanism. *Occludin variant 3* translates into the truncated protein Occludin isoform-B. To our knowledge, there is no published information regarding the functionality of this isoform. However, as it lacks the extracellular loops and N-terminal domains, we hypothesise that Occludin isoform-B will not maintain the structure of the tight junctions. Nevertheless, the increase in *Occludin variant 3* gene expression did not translate into an increase in Occludin isoform-B protein expression. Further studies are needed to determine the localisation of Occludin isoform-B in the endothelial cells.

Our study confirmed the expression of four predicted *VE-cadherin* gene splice variants and three VE-cadherin protein isoforms in human placental tissue. Our results showed that VE-cadherin gene and protein expression were unchanged in placental samples between normal and GDM pregnancies. In agreement with our results, studies have found no change on VE-cadherin gene and protein expression in diabetic conditions. For instance, VE-cadherin protein expression was similar in human placental

tissue from pregnancies complicated with T1DM when compared to non-diabetic placental tissue (Leach *et al.*, 2004). Likewise, VE-cadherin gene and protein expression were similar between human retinal endothelial cells cultured under hyperglycaemic (25 mM glucose) and normoglycaemic conditions (5 mM glucose) (Saker *et al.*, 2014). Nevertheless, studies have shown decreased junctional VE-cadherin localisation in the placental vessels of T1DM (Leach *et al.*, 2004), insulin-controlled GDM (Babawale *et al.*, 2000), and T2DM (Baumüller *et al.*, 2015) when compared to non-diabetic placental samples. Similarly, perfusion studies of human placentae from non-diabetic pregnancies have shown that high glucose perfusion (15 mM glucose for 3-hours) of the maternal circulation results in a loss of junctional VE-cadherin and increased vascular permeability (Sciota, 2012). These results suggest that the loss of junctional VE-cadherin in endothelial vessels from diabetic studies is independent of the VE-cadherin gene or protein expression. Previous studies have shown that VEGF-A and inflammatory mediators, induce VE-cadherin phosphorylation and endothelial cell-cell dissociation (Dejana *et al.*, 2008; Esser *et al.*, 1998). In human placenta, studies have found increased tyrosine-phosphorylation in tissue from T1DM and GDM pregnancies compared to normal pregnancies (results from protein expression and immuno-localisation) (Babawale *et al.*, 2000; Leach *et al.*, 2004). A recent study also confirmed the effect of VEGF-A over VE-cadherin localisation in endothelial membranes (Pang *et al.*, 2017). HUVEC and placental vessels exposed to VEGF-A_{165a} presented increased vascular leakage and reduced junctional VE-cadherin. The loss of VE-cadherin from the membranes and increased vascular leakage could be reverted if the endothelial cells were treated with VEGF-A_{165b} afterwards, returning barrier stability and junctional VE-cadherin to normal levels.

Our results showed that *Plakoglobin* gene expression had a 1.6-fold increase in diet-controlled GDM placental samples. To our knowledge, this is the first report of *Plakoglobin* gene expression in a diabetic environment. Nonetheless, reports have determined that Plakoglobin protein expression is affected in diabetic conditions. A proteome study showed

downregulation of Plakoglobin protein levels in the pancreas of streptozotocin-induced diabetic rats compared to controls (Jiang *et al.*, 2011). Likewise, lower Plakoglobin protein levels were found in patients undergoing coronary artery bypass surgery with uncontrolled diabetes ($\text{HbA}_{1c} > 75 \text{ mmol/mol}$) when compared to controlled diabetic ($\text{HbA}_{1c} < 53 \text{ mmol/mol}$) and non-diabetic patients ($\text{HbA}_{1c} < 42 \text{ mmol/mol}$) (Feng *et al.*, 2016). This downregulation of Plakoglobin protein levels is contradictory with our upregulation of *Plakoglobin* gene levels. It is important to highlight that Plakoglobin forms part of AJ and desmosomes. Thus, future work should determine if the increase *Plakoglobin* gene expression found in diet-controlled GDM placental samples will translate into an increase of Plakoglobin protein expression, and if these Plakoglobin protein will contribute to either AJ or desmosomes structure in GDM placental samples.

It is known that metformin can cross the placenta, as similar plasma concentrations have been found in the maternal and fetal circulation (Charles *et al.*, 2006). Multiple studies have focused on the outcome of offspring from pregnancies treated by metformin. These studies have found no apparent concern on short-term (Butalia *et al.*, 2017; Moore *et al.*, 2007; Rowan *et al.*, 2008), or long-term effects of fetal exposure to metformin (Battin *et al.*, 2015; Ijäs *et al.*, 2015; Rowan *et al.*, 2011). However, a study showed that offspring born to mothers with metformin-controlled GDM pregnancies were larger (weight, BMI, and waist to height ratio) than insulin-controlled GDM offspring (9 years after birth) (Rowan *et al.*, 2018). Up until now, this is the longest follow up study published. Therefore, more studies should focus on the long-term outcome of fetal exposure to metformin; especially because effects on childhood obesity are unlikely to manifest until 9 years of age (Priya & Kalra, 2018). Our study showed no differences in neonatal weight between normal and metformin-controlled GDM pregnancies. Additionally, we found that metformin-controlled GDM placental samples presented stable expression of Occludin gene and protein levels, while diet-controlled GDM samples showed differential expression of Occludin. It is important to highlight that both GDM groups presented

controlled levels of glucose. Hence, our results suggest a regulatory effect of metformin over Occludin expression in GDM pregnancies. This regulatory effect could be independent of the glucose pathway.

There are two mechanisms by which metformin acts: activation of AMP-activated protein kinase (AMPK) (G. Zhou *et al.*, 2001), and reduction of cyclic AMP (cAMP) levels and PKA (cAMP-dependent protein kinase) phosphorylation levels (Miller *et al.*, 2013). Activation of AMPK will inhibit gluconeogenesis, promote fatty acid oxidation, and decrease lipid synthesis (Y. Li *et al.*, 2011). Furthermore, recent studies have shown that metformin and AMPK are involved in the maintenance of endothelial paracellular barrier. For instance, rat brain capillary endothelial cells (RBECs) cultured with metformin showed up-regulation of BBB functions (resistance and permeability) via AMPK activation (F. Takata *et al.*, 2013). Additionally, in the BBB, human brain microvascular endothelial cell (HBMEC) cultured under hyperglycaemic (25 mM D-glucose) conditions presented increased permeability, reduced protein expression of Occludin, ZO-1 and claudin-5, increased levels of NAD(P)H oxidase subunits, and reduced AMPK phosphorylation levels (C. Liu *et al.*, 2012). In the presence of high glucose, activation of AMPK upregulated the expression of TJ molecules and recovered endothelial integrity by inhibition of NAD(P)H oxidase (C. Liu *et al.*, 2012). Furthermore, HUVEC cultured under hyperglycaemic (25 mM D-glucose) (HG) conditions, lipopolysaccharides (1 ug/ml) (LPS), or both (HG + LPS) showed downregulation of AMPK phosphorylation levels, increase paracellular permeability, and increased VE-cadherin phosphorylation levels; upregulation of AMPK abolished these effects (Yan Yang *et al.*, 2017). With this in mind, it is possible that the placental vasculature from metformin-controlled pregnancies presents higher levels of activated AMPK which could be regulating the expression and localisation of Occludin and maintaining the stability of the endothelial barrier. In this regard, the diet-controlled GDM group did not receive any help to activate AMPK and hence, the downregulation of Occludin could not be rescued. Further work

is needed to determine the levels of phosphorylated AMPK in placental vasculature from normal and GDM pregnancies.

In conclusion, we confirmed our hypothesis that placental vasculature from pregnancies complicated with GDM present differential expression of Occludin gene splice variants and protein isoforms. We confirmed the expression of predicted gene splice variants for *Occludin* and *VE-cadherin*, as well as their predicted protein isoforms. We determined that the lower expression of Occludin was only found in diet-controlled GDM samples, suggesting that metformin may be restoring Occludin expression in GDM pregnancies. Further work is needed to establish if a change in the expression of angiogenic factors is behind the decreased barrier integrity in GDM placentae (Chapter 4). Additionally, further work is needed to determine the effect of a hyperglycaemic environment in the expression of Occludin (Chapter 5). Finally, further work is needed to establish if post-translational modifications (phosphorylation) or epigenetic factors (promoter methylation, microRNAs) are behind the decreased Occludin expression in diet-controlled GDM placentae (Chapter 6).

CHAPTER 4

EXPRESSION OF ANGIOGENIC FACTORS IN PLACENTAL VASCULATURE

4.1. INTRODUCTION

Placental angiogenesis plays an essential role in fetal development and pregnancy outcome as placental transport of nutrients and waste is dependent on vascular development. During the last trimester of pregnancy, a balance between branching and non-branching angiogenesis controls the formation of terminal villi which will mediate blood flow towards the fetus. In T1DM pregnancies, placental vessels are characterised for increased angiogenesis, altered junctional maturity, and increased leakiness (Leach *et al.*, 2004, 2009; Mayhew, 2002). In GDM pregnancies, placental vessels are also characterised by increased villous immaturity. However, placental angiogenesis results are contradictory for GDM. Some studies have found increased angiogenesis in GDM placental samples (Jirkovská *et al.*, 2002; Teasdale, 1981); while others have found stable levels of angiogenesis (Babawale *et al.*, 2000; Calderon *et al.*, 2007; Madazli *et al.*, 2008; Mayhew, 2002). It is important to highlight that these angiogenic factors are also considered permeability factors due to their interactions with junctional molecules. Hence, this study compared the gene expression of *VEGF-A*, *Ang-1*, and *Ang-2*; and their respective receptors: *VEGFR-2* and *Tie-2* in placental samples from normal and GDM pregnancies. Our study also determined the gene expression of *MMP-9* in placental samples, due to the contribution of this metalloproteinase to angiogenesis and maintenance of the endothelial membrane.

4.1.1. VEGF-A GENE AND PROTEIN STRUCTURES

The human *VEGF-A* gene is mapped to chromosome 6p21.1 (Vincenti *et al.*, 1996). This gene includes eight exons that, due to alternative splicing of exons 6 and 7, transcribes into several splice variants: *VEGF-A₁₂₁*, *VEGF-A₁₄₅*, *VEGF-A₁₄₈*, *VEGF-A₁₆₅*, *VEGF-A₁₈₃*, *VEGF-A₁₈₉* and *VEGF-A₂₀₆* (Neufeld *et al.*, 1996) (Figure 4.1). Additionally, Bates and collaborators identified a new *VEGF-A* splice variant: *VEGF-A_{165b}* which is generated by alternative splicing of exon 8 (Bates *et al.*, 2002). This change in exon 8 can also occur in conjunction with splicing of exons 6 and 7. Therefore, *VEGF-A* mRNA splicing generates two families of proteins: *VEGF-A_{xxx}* (pro-angiogenic) and *VEGF-A_{xxxb}* (anti-angiogenic), xxx is the number of amino acids of the mature protein (Perrin *et al.*, 2005). Previous studies have found that endothelial cells express more than one *VEGF-A* variant simultaneously. This difference in expression is tissue specific, for instance, higher levels of *VEGF-A₁₂₁*, *VEGF-A₁₆₅*, and *VEGF-A₁₈₉* have been found in human kidneys, liver, heart, capillary haemangiomas, myometrium, and placenta (Neufeld *et al.*, 1996) (Chesnokov *et al.*, 2018) (Zygalaki *et al.*, 2008). Moreover, a change in expression of *VEGF-A* variants has been found in different diseases. For example, in hepatocellular carcinoma, *VEGF-A₁₈₉* expression is reduced while *VEGF-A₁₂₁* expression is increased (Chesnokov *et al.*, 2018). This change in expression of *VEGF-A₁₈₉* and *VEGF-A₁₂₁* is correlated with tumour metastasis. Likewise, in diabetic heart vessels, *VEGF-A₁₂₁* and *VEGF-A₁₆₅* mRNA levels were upregulated compared to normal patients (Zygalaki *et al.*, 2008).

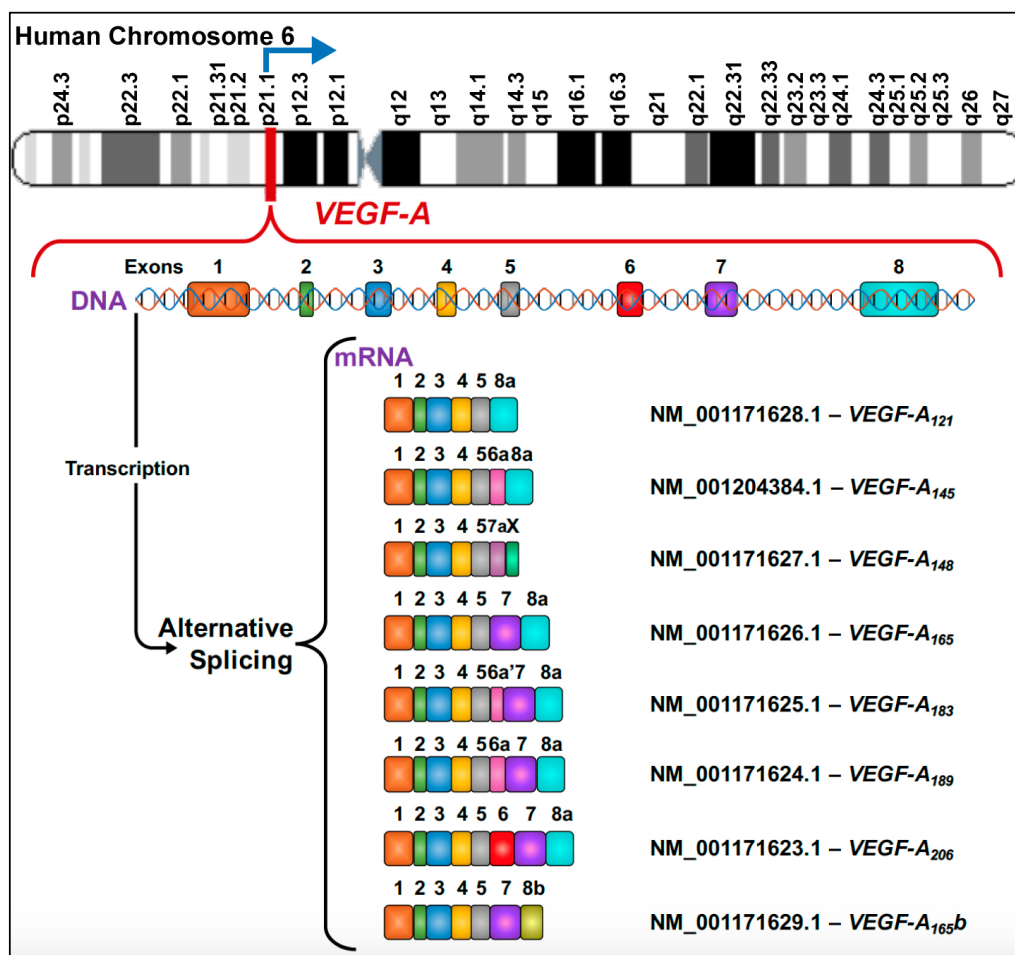


Figure 4.1. Schematic representation of Vascular endothelial growth factor – A (VEGF-A) gene localization and splice variants. VEGF-A is localized on chromosome 6p21.1. This gene includes eight exons that, due to alternative splicing, translate into several splice variants. Exons 6 and 7 are affected by splicing events. A change in exon 8 distal splice site selection gives rise to the anti-angiogenic VEGF-A family (VEGF-A_{xxx}b). Exon sequences obtained from NCBI and Ensembl databases (Pruitt *et al.*, 2014; Yates *et al.*, 2016).

At a protein level, VEGF-A is a disulphide-bonded dimeric glycoprotein that presents three domains: VEGFR binding domain which binds to and activates both receptors (VEGFR-1 and VEGFR-2); heparin binding domain; and C-terminal domain (Figure 4.2A). The VEGF-A protein isoforms differ between them by their size and their heparin and heparan-sulfate binding ability (Figure 4.2B). For instance, VEGF-A₁₂₁, the shortest isoform, lacks the heparin binding site, which makes this binding weakly or non-existent. Therefore, VEGF-A₁₂₁ is freely diffusible in the surrounding extracellular milieu. While the longer isoforms (VEGF-A₁₆₅, VEGF-A₁₈₉ and VEGF-A₂₀₆) display an increasing binding to heparin-rich extracellular

matrix. VEGF-A₁₈₉, and VEGF-A₂₀₆ present a longer heparin binding domain. This domain will interact with the extracellular matrix by heparin sulphate binding properties, keeping these isoforms immobile at the cell surface or extracellular matrix (Delcombel *et al.*, 2013). Additionally, VEGF-A_{xxx}b variants present a different C-terminal domain, which is the result of exon 8b translation, and will give the anti-angiogenic properties of this VEGF isoform.

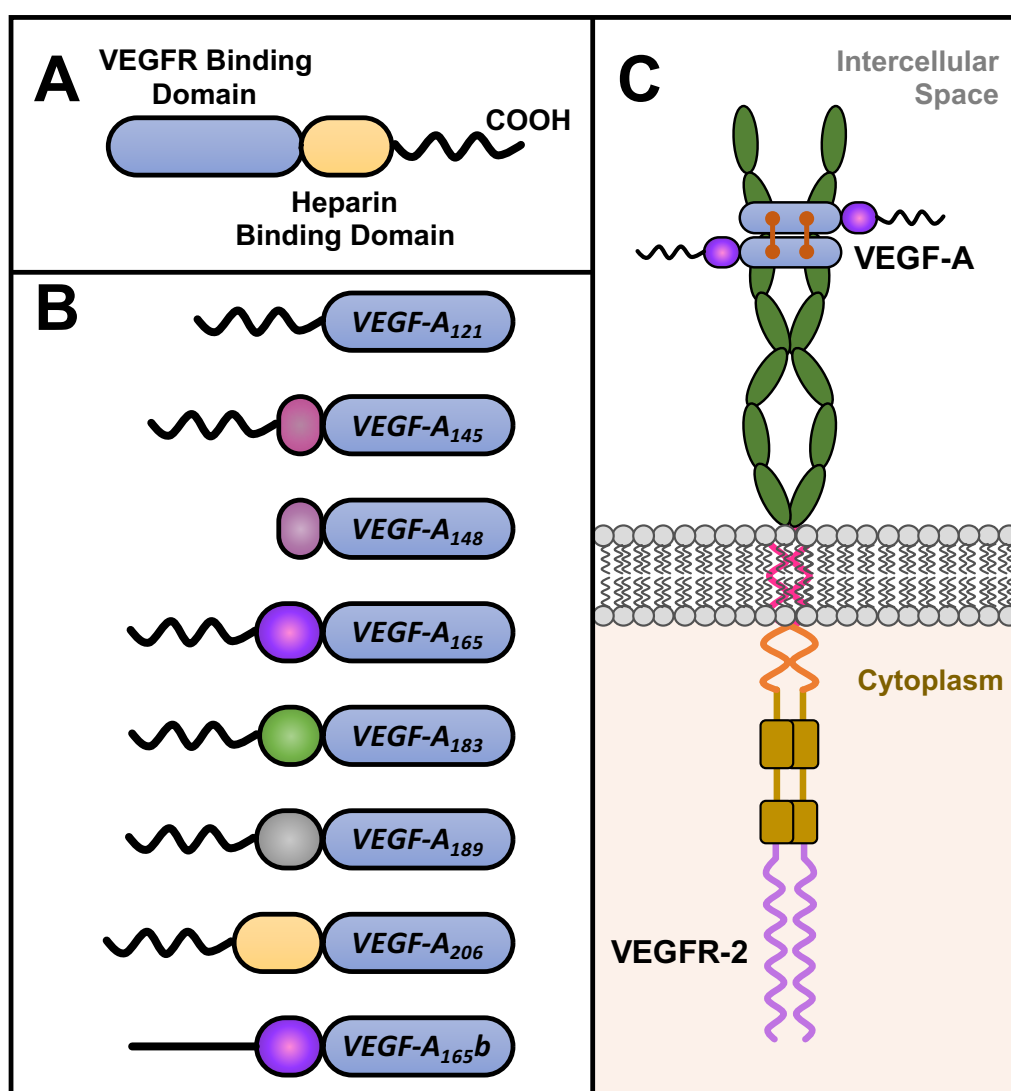


Figure 4.2. Schematic representation of VEGF-A protein domains and protein interaction with its receptor VEGFR-2. (A) VEGF-A presents three domains: VEGFR binding domain (blue), heparin binding domain (yellow), and a C-terminal domain (black). (B) VEGF-A protein splice isoforms. (C) VEGF-A proteins are disulphide-crosslinked (dark orange) antiparallel homodimers. VEGF-A binding to one of its receptors, VEGFR-2, by its binding domain.

VEGF-A is not only a mayor angiogenic factor, it also regulates paracellular permeability through phosphorylation of junctional proteins. VEGF-A induces the phosphorylation of VE-cadherin (Dejana *et al.*, 2008; Esser *et al.*, 1998; Pang *et al.*, 2017), and Occludin (Antonetti *et al.*, 1999; Harhaj *et al.*, 2006; Sundstrom *et al.*, 2009). These phosphorylation events will lead to internalisation of the junctional proteins and reduction of barrier stability.

VEGF-A expression has been determined in human placental tissue from pregnancies complicated with diabetes. Studies have shown that VEGF-A expression is different between types of diabetes. For instance, studies in T1DM placental samples have shown increased VEGF-A protein levels and increased angiogenesis when compared to controls (Dubova *et al.*, 2012; Leach *et al.*, 2004). On the contrary, studies have shown reduced (Meng *et al.*, 2016), or stable (Gauster *et al.*, 2012; Lappas, 2014; Lygnos *et al.*, 2006; Madazli *et al.*, 2008; Troncoso *et al.*, 2017) VEGF-A gene and protein expression in samples (placental tissue, or maternal and cord plasma levels) from normal and GDM pregnancies.

4.1.2. VEGF RECEPTOR -2 GENE AND PROTEIN STRUCTURES

Human *VEGFR-2* gene is located on chromosome 4q12, and includes 30 protein coding exons (Terman *et al.*, 1991). Due to alternative splicing, two variants have been described: *VEGFR-2* and a soluble *VEGFR-2* (*sVEGFR-2*) (Figure 4.3). *sVEGFR-2* is present in various tissues as the skin, heart, spleen, kidney, ovary, and in plasma (Koch & Claesson-Welsh, 2012). *VEGFR-2* is expressed most prominently in vascular endothelial cells and their embryonic precursors, with highest levels during embryonic vasculogenesis and angiogenesis, and during pathological processes associated with neovascularization (tumour angiogenesis).

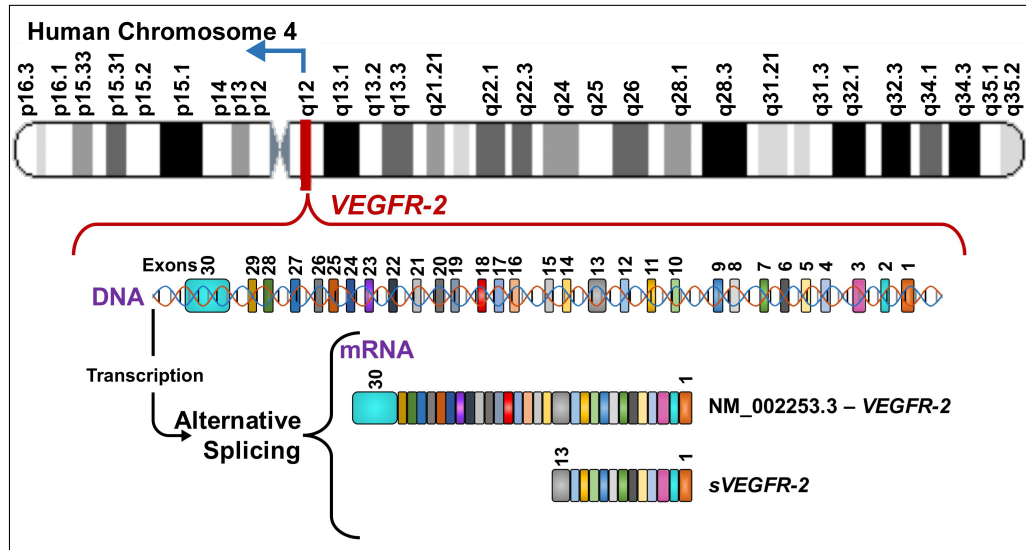


Figure 4.3. Schematic representation of *Vascular endothelial growth factor receptor –2 (VEGFR-2)* gene localization and splice variants. *VEGFR-2* is localized on chromosome 4q12. This gene includes 30 exons that, due to alternative splicing, translate into two splice variants. Exon sequences obtained from NCBI and Ensembl databases (Pruitt *et al.*, 2014; Yates *et al.*, 2016). *sVEGFR-2* sequence obtained from (Albuquerque *et al.*, 2009).

VEGFR-2 protein consists of five domains: an extracellular domain (seven immunoglobulin (Ig)-like loops), a short transmembrane domain, a juxtamembrane domain, a kinase domain (split by a kinase insert), and a C-terminal domain (Figure 4.4A) (Shibuya *et al.*, 1999). Dimeric VEGF-A binds to the second and third extracellular Ig-like domains of VEGFR-2. This binding induces the receptor's dimerization, which is stabilized by low-affinity homotypic interactions between the seven Ig-like domains (Figure 4.4B) (Ruch *et al.*, 2007). This interaction allows autophosphorylation of specific intracellular tyrosine residues from the kinase and the C-terminal domains. These phosphorylations will activate intracellular signalling cascades resulting in proliferation, migration, survival and increased permeability (Holmes *et al.*, 2007).

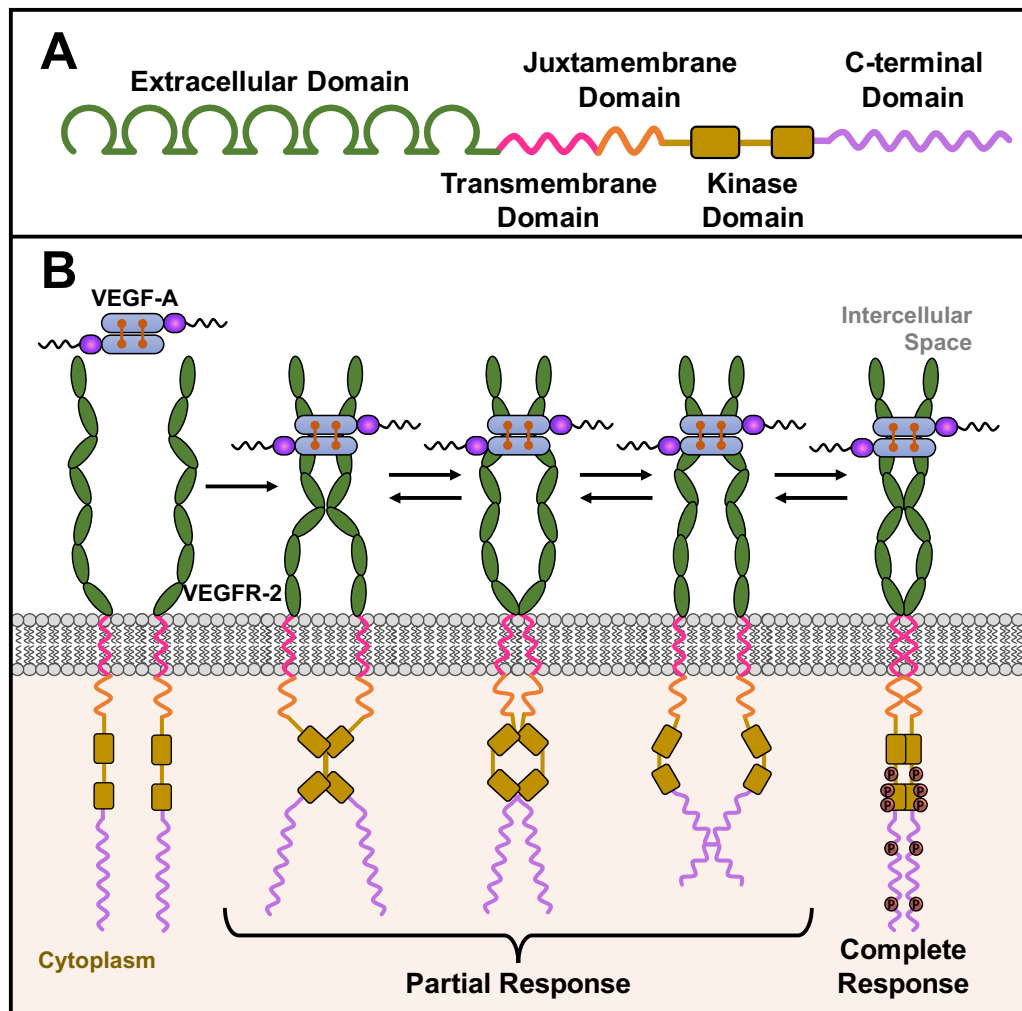


Figure 4.4. Schematic representation of VEGFR-2 protein domains and protein activation after binding to VEGF-A. (A) VEGFR-2 presents five domains: Extracellular Domain, with seven immunoglobulin (Ig)-like loops (green); Transmembrane Domain (pink); Juxtamembrane Domain (orange); Kinase Domain, split by a kinase insert (gold); and a C-terminal domain (purple). **(B)** VEGFR-2 activation. VEGF-A binds to the 2nd and 3rd Ig-like loops. Partial response: VEGFR-2 dimerization and stabilization by low-affinity homotypic interactions between the Ig-like domains. Complete response: Receptor interactions at the 2nd, 3rd, 4th, and 7th Ig-like loops, plus autophosphorylation (red circles: P) at specific tyrosine residues.

In pregnancies complicated with diabetes, studies have shown contradictory results regarding VEGFR-2 expression. For instance, studies have shown stable *VEGFR-2* gene expression (Janota *et al.*, 2003), while VEGFR-2 protein localisation was increased (Dubova *et al.*, 2012) in placental samples from T1DM pregnancies compared to controls. Likewise, studies determined that *VEGFR-2* gene expression was higher (Marini *et al.*, 2008), similar (Lappas, 2014), and lower (Meng *et al.*, 2016; Troncoso *et al.*, 2017)

in placental samples from GDM pregnancies compared to controls. VEGFR-2 protein expression was found to be lower (Marini *et al.*, 2008; Meng *et al.*, 2016), and higher (Troncoso *et al.*, 2017) in placental samples from GDM pregnancies compared to normal pregnancies.

4.1.3. ANGIOPOIETIN (ANG)-1 AND ANG-2 GENE AND PROTEIN STRUCTURES

Human *Ang-1* gene (*ANGPT1*) is located on chromosome 8q23.1, while *Ang-2* (*ANGPT2*) is located on chromosome 8p23.1. Both molecules share a ~60% homology (Davis *et al.*, 1996; Maisonpierre *et al.*, 1997). Alternative splicing events result in four different splice variants for *Ang-1*, and two splice variants for *Ang-2* (Huang *et al.*, 2000; I. Kim *et al.*, 2000) (Figure 4.5).

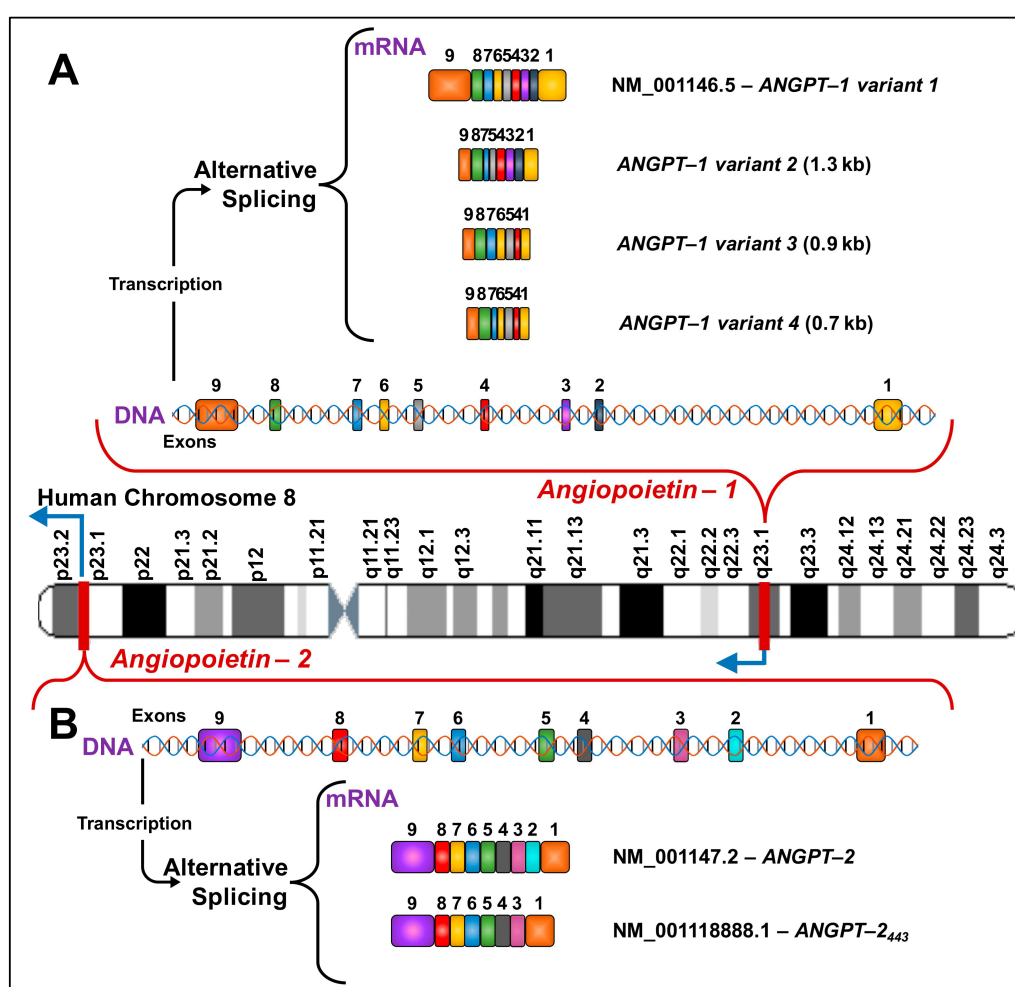


Figure 4.5. Schematic representation of Angiopoietin -1 and -2 gene localization and splice variants. *Angiopoietin -1* and *-2* genes (*ANGPT1* and *ANGPT2*) are localized on chromosome 8. **(A)** *Angiopoietin-1* gene (*ANGPT1*) is

localized on chromosome 8q23.1. *ANGPT1* includes nine exons that, due to alternative splicing, translate into four splice variants (Huang *et al.*, 2000). **(B)** *Angiopoietin-2* gene (*ANGPT2*) is localized on chromosome 8p23.1. *ANGPT2* includes nine exons that, due to alternative splicing, translate into two splice variants (I. Kim *et al.*, 2000). Exon sequences obtained from NCBI and Ensembl databases (Pruitt *et al.*, 2014; Yates *et al.*, 2016).

Angiopoietin proteins are formed by three domains: N-terminal super-clustering domain (SCD); coiled-coil domain (CCD), which is responsible for ligand homo-oligomerization; and C-terminal fibrinogen-related domain (FReD), which mediates Tie-2 oligomerization and activation (Figure 4.6A). Ang-1 and Ang-2 differ from each other by a region within the FReD (X. Yu *et al.*, 2013). Ang-1 and Ang-2 bind Tie-2 at the first Ig-like and EGF-like domains of Tie-2. Ang-1 is a multimer, held together by the CCD and disulphide crosslink (Davis & Yancopoulos, 1999). This multimerization is essential for activation of Tie-2; Ang-1 must be present as a tetramer or higher order structure to stimulate the receptor (K. T. Kim *et al.*, 2005). Ang-2 also exists as a homodimer by a disulphide linking. However, multimerization of Ang-2 is not needed for Tie-2 inactivation (K. T. Kim *et al.*, 2005). Ang-1 isoforms 1 (1.5 kb) and 2 (1.3 kb) bind the receptor and induce its autophosphorylation; while isoforms 3 (0.9 kb) and 4 (0.7 kb) also bind to Tie-2, but do not induce autophosphorylation (Figure 4.6B) (Huang *et al.*, 2000). Likewise, Ang-2 isoforms (Ang-2 and Ang-2₄₄₃) bind to the Tie-2 receptor but do not induce its autophosphorylation (Figure 4.6C) (I. Kim *et al.*, 2000).

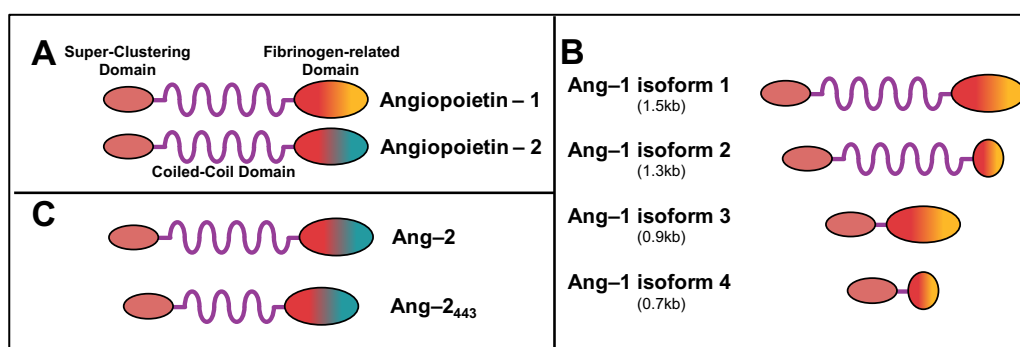


Figure 4.6. Schematic representation of Angiopoietin-1 and -2 protein domains and isoforms. **(A)** Angiopoietin proteins present three domains: Super-Clustering Domain (SCD) (pink); Coiled-Coil Domain (CCD) (purple); and a C-

terminal Fibrinogen-related domain (FReD) (red). Angiopoietins share a high percentage of similarities within these domains; however, they differ in a region on their FReD: Ang-1 (red and orange) while Ang-2 (red and turquoise) **(B)** Representation of Ang-1 isoforms as reported by Huang (Huang *et al.*, 2000). Ang-1 isoform 1 and 2 present a complete CCD that helps maintain oligomerization. Additionally, their FReD will bind to Tie-2 receptor and induce its autophosphorylation. Ang-1 isoforms 3 and 4, lack the CCD (no oligomerisation), will bind Tie-2 receptor but do not activate it. **(C)** Representation of Ang-2 isoforms as described by Kim (I. Kim *et al.*, 2000). Ang-2₄₄₃ presents a shorter CCD. Both Ang-2 isoforms bind to Tie-2 receptor but do not induce its autophosphorylation.

Ang-1 is considered an anti-permeability factor (Fukuhara *et al.*, 2010; Ngok *et al.*, 2012; Thurston *et al.*, 2000). Studies have shown that Ang-1 induces *Occludin* gene expression (Hori *et al.*, 2004), inhibits Occludin phosphorylation and promotes Occludin interaction with ZO-1 to stabilise the TJs of the BBB (Siddiqui *et al.*, 2015). Furthermore, a study showed that Ang-1 can inhibit VEGF effects over VE-cadherin phosphorylation and internalisation in endothelial cells (Gavard *et al.*, 2008).

The expression of Ang-1 and Ang-2 have been compared in samples between normal and diabetic pregnancies. Studies have shown that Ang-1 gene and protein expression is not affected by diabetes. For instance, similar levels of *Ang-1* gene expression (placental tissue from T1DM pregnancies) (Janota *et al.*, 2003), Ang-1 protein levels (maternal plasma from T1DM pregnant women) (Loukovaara *et al.*, 2004), and Ang-1 protein levels (fetal-cord plasma from GDM pregnancies) (Giri *et al.*, 2013) were found compared to controls. Moreover, studies have shown contradictory results regarding Ang-2 expression in diabetic pregnancies compared to controls. For instance, studies have found unchanged *Ang-2* gene expression levels in placental tissue (Janota *et al.*, 2003), and lower Ang-2 protein levels in maternal plasma (Loukovaara *et al.*, 2004) from T1DM pregnancies compared to controls. In GDM pregnancies, *Ang-2* gene expression was found to be higher in placental samples (Radaelli *et al.*, 2003) and HUVEC isolated (Giri *et al.*, 2013) from GDM pregnancies

compared to controls. Ang-2 protein levels in fetal-cord plasma were similar to normal pregnancies (Giri *et al.*, 2013).

4.1.4. TIE-2 RECEPTOR GENE AND PROTEIN STRUCTURES

Human *Tie-2* gene is located on chromosome 9p21.2, and includes 23 protein coding exons (Dumont, Anderson, *et al.*, 1994). There is a report of three *Tie-2* gene splice variants due to alternative splicing (Jin *et al.*, 2008) (Figure 4.7). However, no further information has been reported.

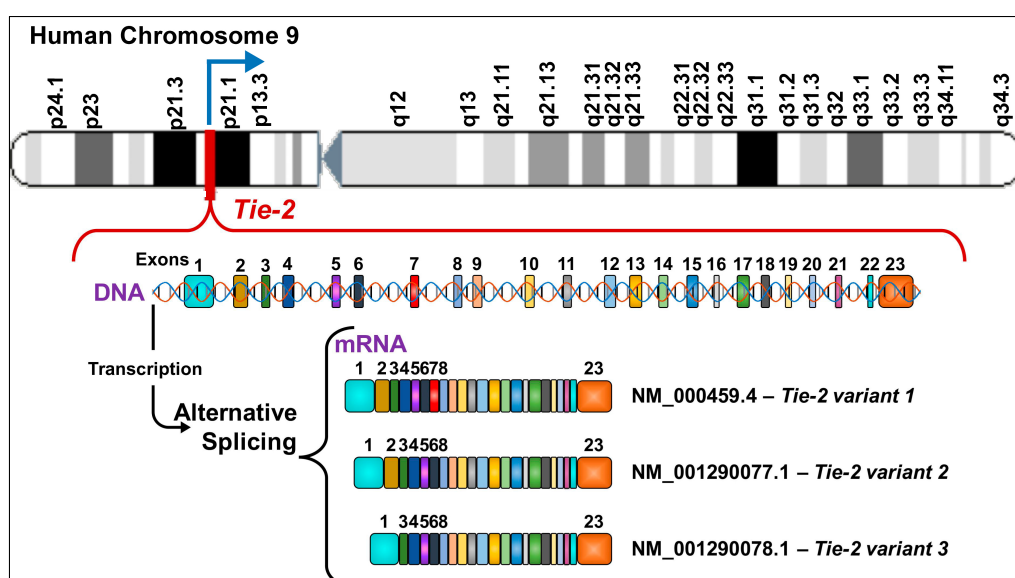


Figure 4.7. Schematic representation of *Tie - 2* gene localization and splice variants. *Tie - 2* gene is localized on chromosome 9p21.2. It includes 23 exons that, due to alternative splicing, translate into three splice variants. Exon sequences obtained from NCBI and Ensembl databases (Pruitt *et al.*, 2014; Yates *et al.*, 2016).

Tie-2 protein is formed by five domains: immunoglobulin (Ig)-like domain (two Ig-like motifs) flanking epidermal growth factor (EGF)-like domain (three EGF-like repeats); fibronectin type III domain (three repeats); transmembrane domain; a split tyrosine kinase domain (Figure 4.8A) (Sato *et al.*, 1993). Ang-1 and Ang-2 bind to the first Ig-like loop and the EGF-like domain (Fiedler *et al.*, 2003). Ang-1 activates the receptor, while Ang-2 inactivates it (Figure 4.8B). The tyrosine kinase domain is responsible for intracellular signal transduction. Once Ang-1 binds *Tie-2*, the receptor dimerizes and autophosphorylates. Following *Tie-2*

activation, intracellular signalling pathways are activated, resulting in cell migration, cell survival, inflammation and permeability.

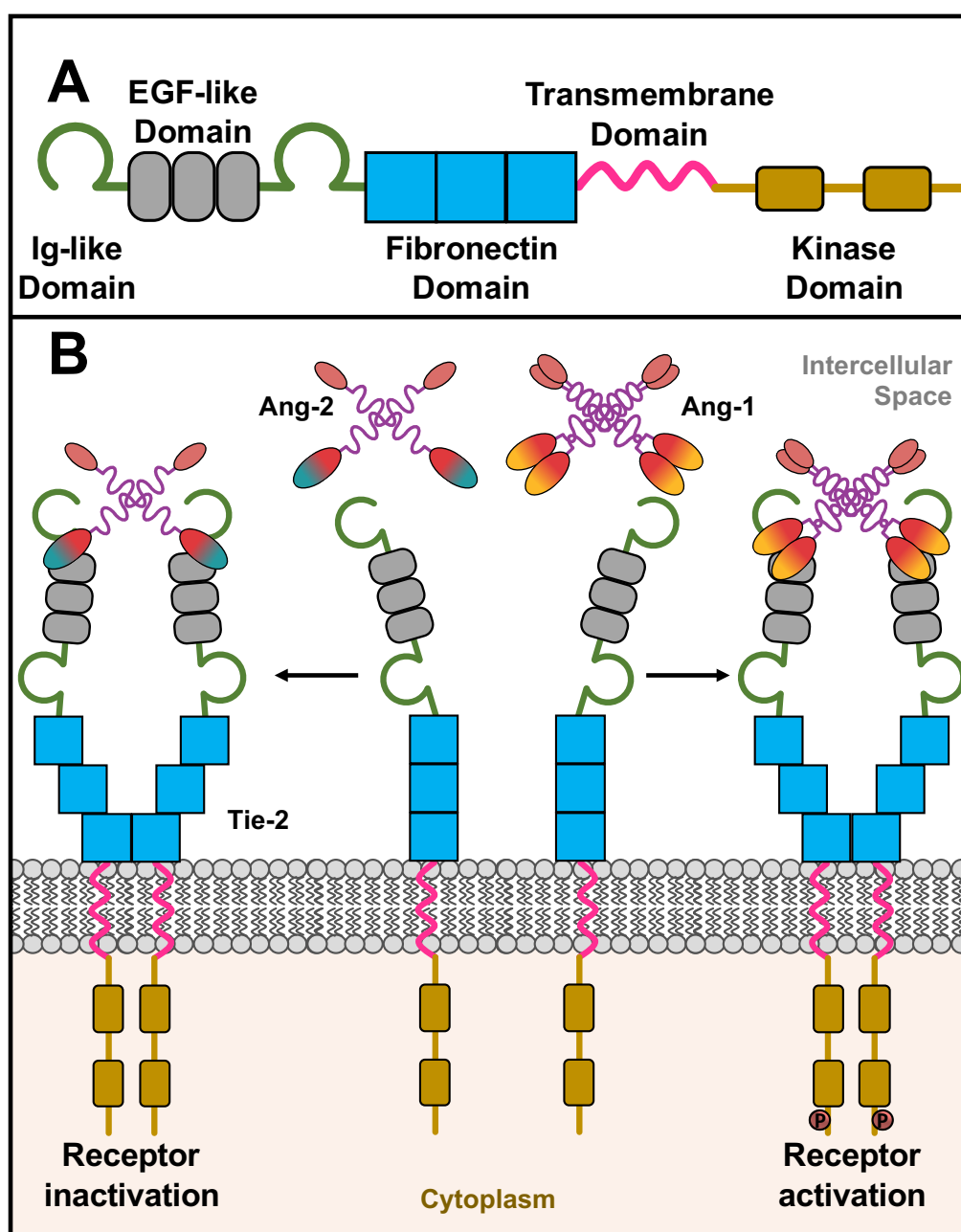


Figure 4.8. Schematic representation of Tie-2 receptor protein domains and protein activation. (A) Tie-2 receptor present five domains: two immunoglobulin (Ig)-like domains (green); three epidermal growth factor (EGF)-like domain repeats (grey); three fibronectin type III domain repeats (blue); transmembrane domain (pink); a split tyrosine kinase domain (gold). (B) Tie-2 receptor binds to both Angiopoietins (Ang). Receptor activation (left panel): a tetramer of Ang-1 (red and orange) binds to Tie-2 receptor and induce its autophosphorylation. Receptor inactivation (right panel): a dimer of Ang-2 (red and turquoise) binds to Tie-2 receptor but does not induce its autophosphorylation.

Studies with pre-gestational diabetes pregnancies have found that Tie-2 gene expression (Janota *et al.*, 2003) and protein localisation (Vuorela & Halmesmäki, 2006) in placental samples was similar between diabetic and normal samples. To our knowledge, there is no published information regarding Tie-2 expression in placental samples from GDM pregnancies.

4.1.5. MMP – 9 GENE AND PROTEIN STRUCTURES

Human *MMP-9* gene is located on chromosome 20q13.12 and includes 13 coding exons (Huhtala *et al.*, 1991) (Figure 4.9A). There are no reports of expression nor predicted splice variants for *MMP-9* gene. At a protein level, MMP-9 is formed by six domains: signal peptide domain, pro-peptide domain, fibronectin-like domain, catalytic domain, collagen V-like domain, C-terminal hemopexin domain (Figure 4.9B). The signal peptide domain is cleaved during transport through the secretory pathway. The presence of the fibronectin domain allows the binding and processing of denatured collagen or gelatine (Gomis-Rüth, 2009). The pro-peptide domain maintains MMP-9 in an inactive state through a cysteine switch mechanism. Activation of the enzyme occurs when the interaction between the active site zinc molecule and a cysteine in the pro-domain is disrupted, rendering the active site accessible (Figure 4.9C) (Vu & Werb, 1998). Once activated, MMP-9 will be involved in the breakdown of the extracellular matrix contributing to angiogenesis, cell migration, and embryonic development. Furthermore, MMP-9 proteolytic activities cause a downregulation of Occludin and VE-cadherin protein levels and increase in paracellular permeability (Caron *et al.*, 2005; Giebel *et al.*, 2005; Navaratna *et al.*, 2007).

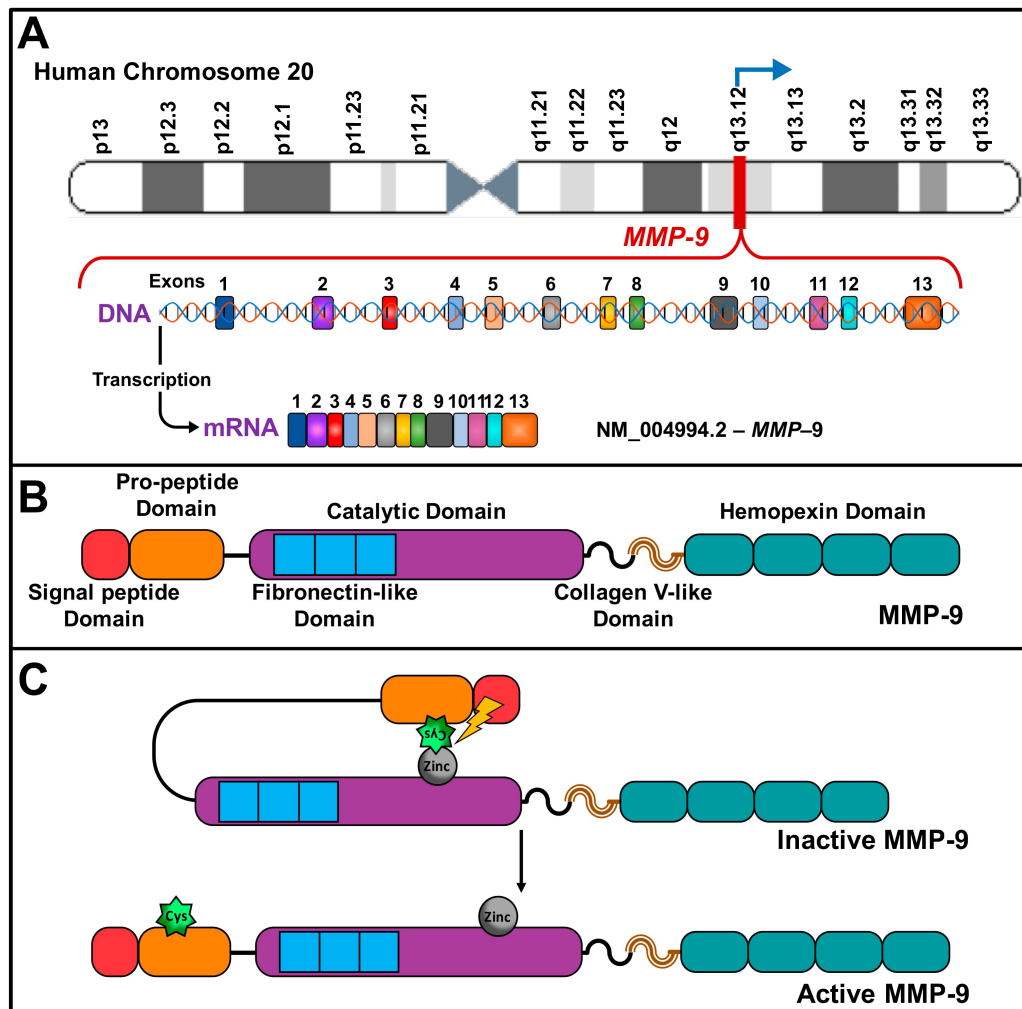


Figure 4.9. Schematic representation of matrix metalloproteinase (MMP)-9 gene and protein. (A) *MMP-9* gene is localized on chromosome 20q13.12. Exon sequences obtained from NCBI and Ensembl databases (Pruitt *et al.*, 2014; Yates *et al.*, 2016). (B) *MMP-9* protein presents six domains: signal peptide domain (red), pro-peptide domain (orange), fibronectin-like domain (blue), catalytic domain (purple), collagen V-like domain (brown), C-terminal hemopexin domain (green). (C) Activation of *MMP-9* occurs by disruption of the interaction between the zinc molecule from the catalytic domain and the cysteine in the pro-peptide domain (yellow thunderbolt).

Studies in diabetic pregnancies have determined *MMP-9* protein expression and enzymatic activity. However, there is no published information about *MMP-9* gene expression for this study group. A study found no difference in *MMP-9* protein expression in placental samples between T2DM pregnancies and controls (Capobianco *et al.*, 2012). Furthermore, *MMP-9* enzymatic activity was higher in placental samples from T1DM (Pustovrh *et al.*, 2000) and T2DM (Capobianco *et al.*, 2012).

pregnancies when compared to non-diabetic placental samples. Finally, a study showed that MMP-9 enzymatic activity was lower in GDM placental samples compared to controls (Pustovrh *et al.*, 2000).

4.1.6. HYPOTHESIS AND AIMS OF CHAPTER 4

During a GDM pregnancy, there is an increased expression of angiogenic factors that could explain the downregulation of Occludin expression and localisation in placental vasculature

To test this hypothesis, the aims of this chapter were:

- To compare VEGF-A gene and protein expression in human placental tissue of normal and GDM pregnancies
- To quantify the gene expression of the angiopoietin system (*Tie-2*, *Ang-1*, and *Ang-2*) in human placental tissue of normal and GDM pregnancies
- To determine the gene expression of a matrix metalloproteinase (*MMP-9*) in human placental tissue of normal and GDM pregnancies
- To define if treatment to control glucose levels (diet or metformin) counteracts the effects of GDM on the expression of angiogenic factors.

4.2. METHODS

For detailed methodology see Chapter 2.

4.2.1. STUDY POPULATION

Pregnant women, who were scheduled for elective C-section at term pregnancies (>37 weeks), were asked for written informed consent for placental tissue collection. Placental samples were collected from normal pregnancies ($n= 9$) used as a control, and pregnancies complicated with GDM where glucose levels were treated by a change of diet ($n= 7$) or by medication (metformin) ($n= 6$).

4.2.2. GENE EXPRESSION OF ANGIOGENIC FACTORS

RNA isolation, reverse transcription and qPCR were performed as described in Sections 2.6, 2.7 and 2.9. Primers were designed for each gene. The most efficient primer concentration and cDNA dilution was determined for each primer reaction (Table 4.1). Gene expression was analysed in placental samples as described in Sections 2.11.

Table 4.1. Primer information of angiogenic factors. Detailed information about *VEGF-A*, *VEGR-2*, *Tie-2* receptor, *Ang-1*, *Ang-2* and *MMP-9* primers. Forward (F) and reverse (R) sequences, primer final concentration ([Final]), cDNA dilution (cDNA), annealing temperature (°C) and if 1mM betaine was needed for the specificity of the reaction (*).

Gene	Sequence	[Final]	cDNA	°C
<i>VEFG-A</i>	F: 5'- ATCTTCAAGCCATCCTGTGTGC -3' R: 5'- TATGTGCTGGCCTTGGTGAG -3'	700 nM	1:8	60*
<i>VEGFR-2</i>	F: 5'- GGCCCCTGATTATACTACACCA -3' R: 5'- AATGTAGTCTTTGCCATCCTGC -3'	600 nM	1:16	60*
<i>Tie-2</i>	F: 5'- ATCTCACTGCTTGGACCCCTTAG -3' R: 5'- GCTGAGGAGTGTGTAATGTTGG -3'	500 nM	1:8	60
<i>Ang-1</i>	F: 5'- GGAACCGAGCCTATTCACAGTA -3' R: 5'- CTGCTCTGTTTTCTGCTGTC -3'	200 nM	1:8	58
<i>Ang-2</i>	F: 5'- GACCACGAGACTTGAACCTTCAG -3' R: 5'- TGTCTTCCATAGCTAGCACCTT -3'	300 nM	1:16	60
<i>MMP-9</i>	F: 5'- CGCTACCACCTCGAACTTTG -3' R: 5'- CCGGCACTGAGGAATGATCTAA -3'	500 nM	1:16	54*

4.2.3. VEGF-A PROTEIN EXPRESSION

Protein isolation and Western blot were performed as described in Sections 2.15 and 2.16. Briefly, placental total protein (20 µg) was separated on a 4–20% SDS-PAGE (Bio-Rad, USA) by electrophoresis and transferred to a nitrocellulose membrane. Membranes were incubated overnight at 4°C with primary antibody polyclonal rabbit anti-VEGF-A (3.6 µg/ml; ab183100; Abcam). Monoclonal mouse anti-β-actin (7 µg/ml; A5316; Sigma) was used as loading control for each protein.

4.2.4. STATISTICAL ANALYSIS

The data is expressed as means $\pm$ SEM. The data was tested for normality distribution by D'Agostino & Pearson test with a confidence interval of 95%. Results from the study population presented a normal distribution. Hence, statistical comparison between two groups was performed by independent samples *t*-test, and between three groups by one-way ANOVA. Results from gene expression presented failed to be normally distributed. Therefore, statistical comparison between two groups were performed by Mann-Whitney test, and between three groups by Kruskal-Wallis test.

4.3. RESULTS

4.3.1. COMPARISON OF NEONATAL CLINICAL PARAMETERS BETWEEN STUDY GROUPS

There was no significant difference in the placental weight ($t(16) = 1.03$, $p = 0.181$), baby weight ($t(19) = 0.65$, $p = 0.523$), or placental efficiency ($t(15) = 0.74$, $p = 0.469$) between normal and GDM pregnancies (Table 2.1). Furthermore, there were no significant differences in the placental weight ($F(2, 15) = 0.63$, $p = 0.548$), baby weight ($F(2, 18) = 0.23$, $p = 0.798$), or placenta efficiency ($F(2, 15) = 0.69$, $p = 0.520$) between normal, diet-controlled GDM and metformin-controlled GDM pregnancies.

4.3.2. DOWNREGULATION OF VEGF-A PROTEIN EXPRESSION IN DIET-CONTROLLED GDM SAMPLES; NO CHANGE IN *VEGF-A* GENE EXPRESSION

Using a specific pair of primers, the expression of the different *VEGF-A* gene splice variants was studied in placental samples. Total *VEGF-A* gene expression was similar between normal and GDM placental samples ($U = 37.0$, $p = 0.370$) (Figure 4.10A). Furthermore, there was no statistical significant difference in *VEGF-A* gene expression in placental samples from normal, diet-controlled and metformin-controlled GDM pregnancies ($H = 2.72$, $p = 0.267$) (Figure 4.10B).

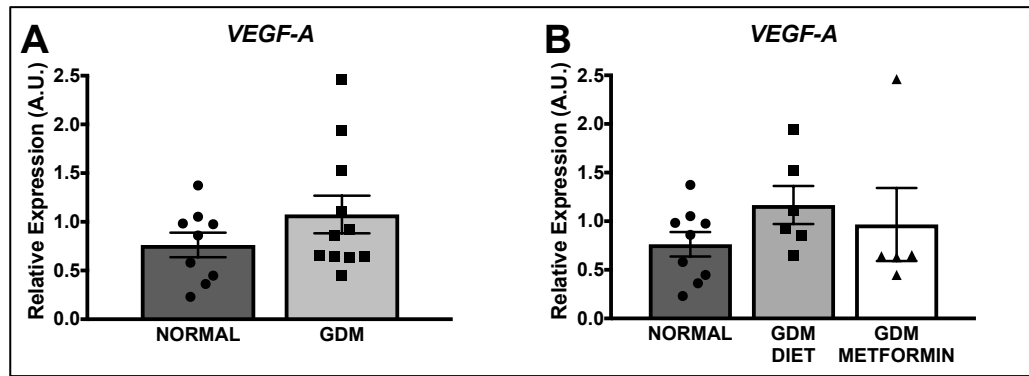


Figure 4.10. VEGF-A gene expression was similar between normal and GDM placental samples. Placental samples were taken from normal pregnancies ($n=9$), and pregnancies complicated with GDM where glucose levels were managed with diet ($n=7$) or metformin ($n=6$). **(A)** VEGF-A gene expression between normal and GDM placental samples. **(B)** VEGF-A gene expression between normal, diet-controlled and metformin-controlled GDM placental samples. Mean $\pm$ SEM. Kruskal Wallis test and Mann-Whitney post hoc test.

Similarly, an antibody that detects all VEGF-A protein variants was used to quantify their expression. Figure 4.11C shows the western blots used for the quantification. There was no significant difference in VEGF-A protein expression ($t(18) = 1.89, p = 0.075$) in placental samples between normal and GDM pregnancies (Figure 4.11A). VEGF-A protein expression was unchanged between placental samples from normal, diet-controlled and metformin-controlled GDM pregnancies ($F(2, 17) = 2.66, p = 0.099$). However, VEGF-A protein expression for diet-controlled GDM pregnancies was significantly lower than normal pregnancies ($t(12) = 2.25, p = 0.044$); the VEGF-A protein expression of metformin-controlled GDM pregnancies did not differ from normal or diet-controlled GDM pregnancies (Figure 4.11B).

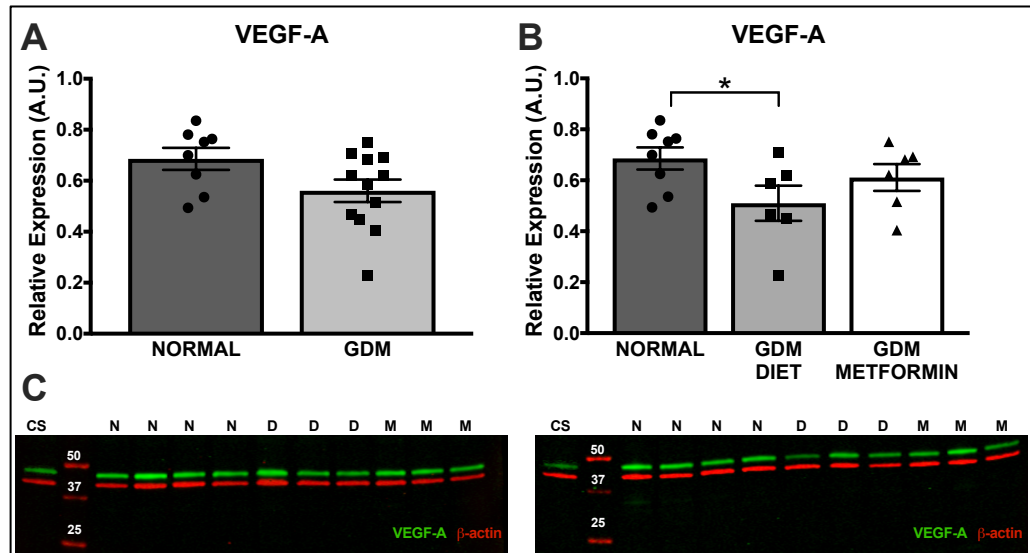


Figure 4.11. Lower VEGF-A protein expression in diet-controlled GDM placental samples compared to controls. Placental samples were taken from normal pregnancies ($n=9$), and pregnancies complicated with GDM where glucose levels were managed with diet ($n=7$) or metformin ($n=6$). **(A)** VEGF-A protein expression between normal and GDM placental samples. **(B)** VEGF-A protein expression between normal, diet-controlled and metformin-controlled GDM placental samples. **(C)** Western blot bands of VEGF-A (~45 kDa; green) from normal (N), diet-controlled (D), and metformin-controlled (M) GDM placental samples. A control sample (CS) was run in each gel to control for variation between gels. β -actin (~40 kDa; red) used as a loading control. Mean $\pm$ SEM. One-way ANOVA and post hoc Unpaired t-test (* $p < 0.05$).

4.3.3. SIMILAR *VEGFR-2* GENE EXPRESSION IN PLACENTAL SAMPLES BETWEEN NORMAL AND GDM PREGNANCIES

VEGFR-2 gene expression was similar between normal and GDM placental samples ($U = 38.0$, $p = 0.412$) (Figure 4.12A). Furthermore, there was no statistical significant difference in *VEGFR-2* gene expression in placental samples from normal, diet-controlled GDM, and metformin-controlled GDM placental samples ($H = 2.74$, $p = 0.264$) (Figure 4.12B). Likewise, *VEGFR-2* gene expression was similar between samples from diet-controlled GDM pregnancies and normal pregnancies ($U = 15.0$, $p = 0.181$).

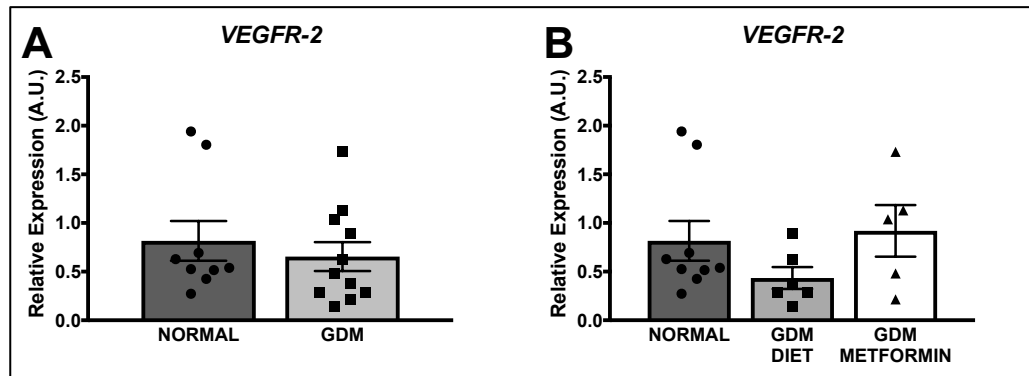


Figure 4.12. *VEGFR-2* gene expression was similar between normal and GDM placental samples. Placental samples were taken from normal pregnancies ($n=9$), and pregnancies complicated with GDM where glucose levels were managed with diet ($n=7$) or metformin ($n=6$). **(A)** *VEGFR-2* expression between normal and GDM placental samples. **(B)** *VEGFR-2* expression between normal, diet-controlled and metformin-controlled GDM placental samples. Mean $\pm$ SEM. Kruskal Wallis test and Mann-Whitney post hoc test.

4.3.4. SIMILAR *Tie-2* GENE EXPRESSION IN PLACENTAL SAMPLES BETWEEN NORMAL AND GDM PREGNANCIES

Total *Tie-2* gene expression was similar between normal and GDM placental samples ($U = 44.0$, $p = 0.710$) (Figure 4.13A). Additionally, there was no statistically significant difference in *Tie-2* gene expression between normal, diet-controlled GDM, and metformin-controlled GDM placental samples ($H = 4.36$, $p = 0.112$) (Figure 4.13B). Similarly, there was no statistical difference in *Tie-2* gene expression between samples from diet-controlled GDM pregnancies and normal pregnancies ($U = 12.0$, $p = 0.088$).

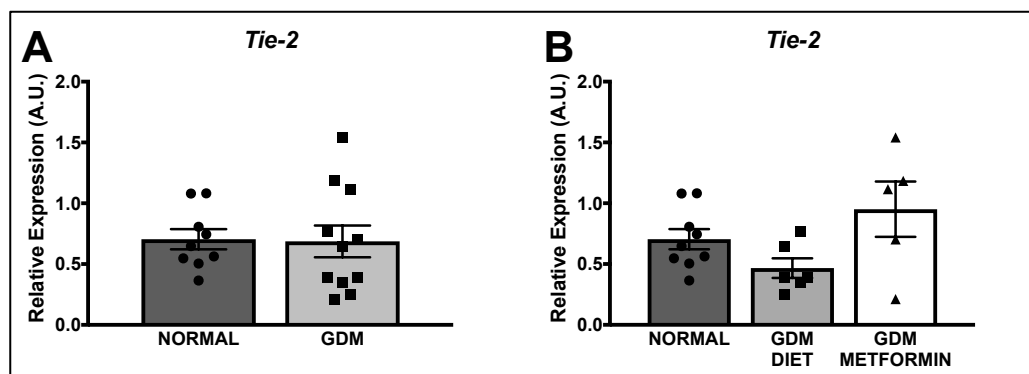


Figure 4.13. *Tie-2* receptor gene expression was similar between normal and GDM placental samples. Placental samples were taken from normal pregnancies ($n=9$), and pregnancies complicated with GDM where glucose levels were managed with diet ($n=7$) or metformin ($n=6$). **(A)** *Tie-2* expression between

normal and GDM placental samples. **(B)** *Tie-2* expression between normal, diet-controlled and metformin-controlled GDM placental samples. Mean $\pm$ SEM. Kruskal Wallis test and Mann-Whitney post hoc test.

4.3.5. SIMILAR *ANG-1* AND *ANG-2* GENE EXPRESSION IN PLACENTAL SAMPLES BETWEEN NORMAL AND GDM PREGNANCIES

Total *Ang-1* gene expression was similar in placental samples from normal and GDM pregnancies ($U = 28.0$, $p = 0.079$) (Figure 4.14A). Furthermore, there was no statistically significant difference in *Ang-1* gene expression between normal, diet-controlled GDM, and metformin-controlled GDM placental samples ($H = 1.95$, $p = 0.193$) (Figure 4.14B). Likewise, there was no statistical significant difference in *Ang-1* gene expression in placental samples from normal pregnancies compared to diet-controlled GDM samples ($U = 19.0$, $p = 0.097$) or metformin-controlled GDM samples ($U = 9.0$, $p = 0.081$).

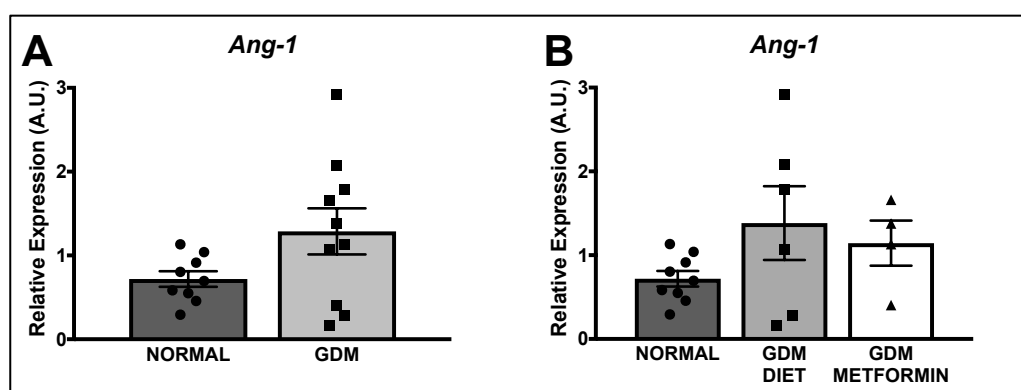


Figure 4.14. *Angiopoietin-1* gene expression was similar between normal and GDM placental samples. Placental samples were taken from normal pregnancies ($n = 9$), and pregnancies complicated with GDM where glucose levels were managed with diet ($n = 7$) or metformin ($n = 6$). **(A)** *Ang-1* expression between normal and GDM placental samples. **(B)** *Ang-1* expression between normal, diet-controlled and metformin-controlled GDM placental samples. Mean $\pm$ SEM. Kruskal Wallis test and Mann-Whitney post hoc test.

Total *Ang-2* gene expression was similar between normal and GDM placental samples ($U = 37.0$, $p = 0.599$) (Figure 4.15A). Additionally, there was no statistically significant difference in *Ang-2* gene expression between normal, diet-controlled GDM, and metformin-controlled GDM placental samples ($H = 1.21$, $p = 0.540$) (Figure 4.15B).

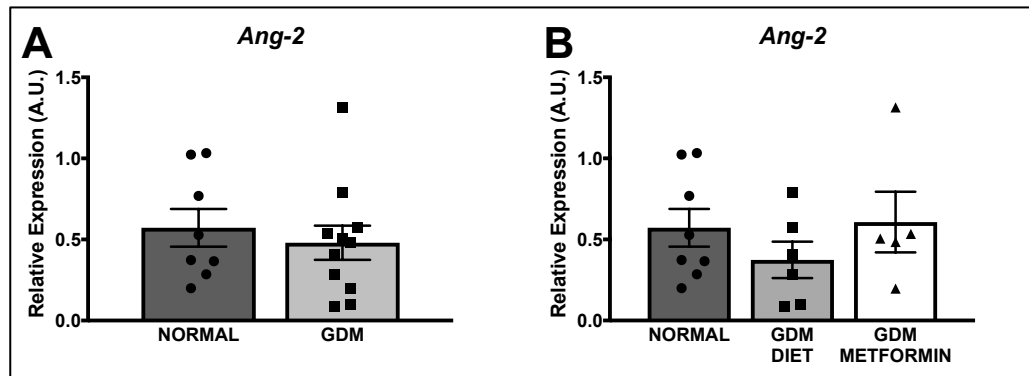


Figure 4.15. Angiopoietin-2 gene expression was similar between normal and GDM placental samples. Placental samples were taken from normal pregnancies ($n=9$), and pregnancies complicated with GDM where glucose levels were managed with diet ($n=7$) or metformin ($n=6$). **(A)** *Ang-2* expression between normal and GDM placental samples. **(B)** *Ang-2* expression between normal, diet-controlled and metformin-controlled GDM placental samples. Mean $\pm$ SEM. Kruskal Wallis test and Mann-Whitney post hoc test.

4.3.6. SIMILAR *MMP-9* GENE EXPRESSION IN PLACENTAL SAMPLES BETWEEN NORMAL AND GDM PREGNANCIES

MMP-9 gene expression was similar between normal and GDM placental samples ($U = 49.0$, $p = 0.754$) (Figure 4.16A). Furthermore, there was no statistically significant difference in *MMP-9* gene expression between normal, diet-controlled GDM, and metformin-controlled GDM placental samples ($H = 1.95$, $p = 0.398$) (Figure 4.16B). Likewise, there was no statistical significant difference in *MMP-9* gene expression between placental samples from normal and diet-controlled GDM pregnancies ($U = 16.0$, $p = 0.117$).

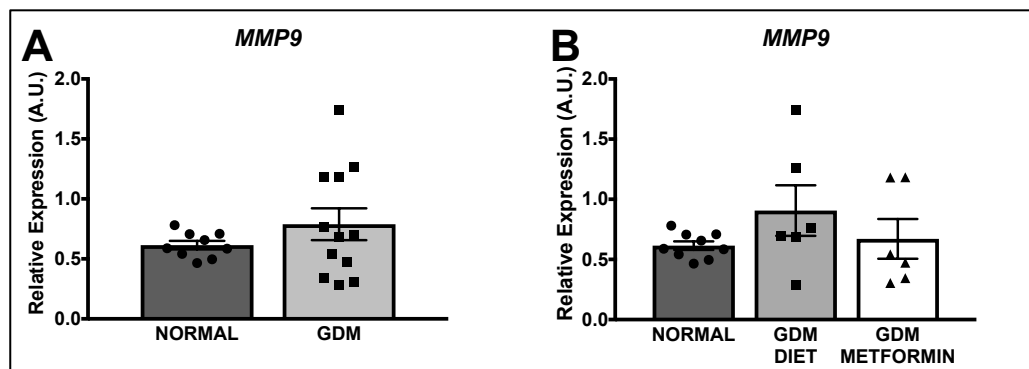


Figure 4.16. *MMP-9* gene expression was similar between normal and GDM placental samples. Placental samples were taken from normal pregnancies ($n=9$), and pregnancies complicated with GDM where glucose levels were managed with diet ($n=7$) or metformin ($n=6$). **(A)** *MMP-9* expression between normal and GDM placental samples. **(B)** *MMP-9* expression between normal, diet-controlled and metformin-controlled GDM placental samples. Mean $\pm$ SEM. Kruskal Wallis test and Mann-Whitney post hoc test.

4.4. DISCUSSION

There are contradictory results regarding the levels of angiogenesis in placenta from pregnancies complicated with GDM. Some studies have found increased angiogenesis levels in GDM placental samples (Jirkovská *et al.*, 2002; Teasdale, 1981); while others have found stable levels of angiogenesis (Babawale *et al.*, 2000; Calderon *et al.*, 2007; Madazli *et al.*, 2008; Mayhew, 2002) compared to controls. Our results showed a decrease in VEGF-A protein expression in placental samples from diet-controlled GDM pregnancies compared to control. In contrast, the gene expression levels of *VEGF-A*, *Ang-1*, *Ang-2*, *VEGFR-2*, *Tie-2*, and *MMP-9* remained stable between GDM and normal placental samples. Our results agree with previous studies and confirm that placental angiogenesis levels are stable between normal and GDM pregnancies.

Placental efficiency measures the effectiveness by which the placenta delivered nutrients to ensure the development of the fetus. Therefore, placental efficiency depends on surface area of exchange, thickness of the exchange barrier, blood flow through the uterine and feto-placental vasculature, and the transporter proteins in the trophoblast plasma membranes (Angiolini *et al.*, 2006; Myatt, 2006). Placental efficiency can be increased by branching angiogenesis, providing more arterio-venous circuits and returning fetal blood to the umbilical cord (Burton *et al.*, 2009; Myatt, 2006). Previous studies have shown no difference in placental efficiency between normal and GDM pregnancies (Babawale *et al.*, 2000; Muralimanoharan *et al.*, 2016). These results are in agreement with our study as no significant differences were found in placental efficiency between normal and GDM pregnancies. Furthermore, studies have found

the same pattern for T1DM placentae, no significant differences were found in baby weight, placental weight, or placental efficiency between normal and T1DM pregnancies (Leach *et al.*, 2004; Mayhew, 2002). However, these studies also found increased placental angiogenesis due to longitudinal growth of the vessels in T1DM samples compared to control (Leach *et al.*, 2004; Mayhew, 2002). With this in mind, it is clear that placental efficiency is not a reliable measure of placental angiogenesis. Future studies should use stereological measures of placental volume, surface area and lengths of peripheral villi and their capillaries to assert placental angiogenesis levels in samples from pregnancies complicated with GDM.

VEGF-A is one of the most important angiogenic factors as it is essential for embryonic vasculature formation. VEGF-A also regulates paracellular permeability through phosphorylation of VE-cadherin (Dejana *et al.*, 2008; Esser *et al.*, 1998; Pang *et al.*, 2017) and Occludin (Antonetti *et al.*, 1999; Harhaj *et al.*, 2006; Sundstrom *et al.*, 2009). For this reason, we determined the expression of VEGF-A in placental samples from normal and GDM pregnancies. Our results showed a 1.3-fold decrease of total VEGF-A protein expression, while total *VEGF-A* gene expression was unchanged, in placental samples from diet-controlled GDM pregnancies compared to controls. Our results agree with a previous study that showed reduced VEGF-A protein expression in placental samples from diet-controlled GDM compared to controls (Meng *et al.*, 2016). Conversely, studies have found unchanged VEGF-A expression between samples (placental tissue, maternal and fetal-cord plasma) from normal, diet-controlled GDM (Lygnos *et al.*, 2006; Madazli *et al.*, 2008), and insulin-controlled GDM (Lappas, 2014) pregnancies. Nevertheless, studies in T1DM placental samples have shown increased VEGF-A protein levels and increased angiogenesis when compared to controls (Dubova *et al.*, 2012; Leach *et al.*, 2004). These results suggest that each type of diabetes has a different effect over VEGF-A expression in placental vasculature. Furthermore, our results suggest that downregulation of junctional Occludin localisation was not due to VEGF-A expression in placental samples from diet-controlled GDM pregnancies.

As previously mentioned, our study showed that the downregulation of VEGF-A protein expression was only present in diet-controlled GDM placental samples. Placental samples from metformin-controlled GDM pregnancies showed similar VEGF-A protein expression than controls. Recent studies have suggested that metformin affects the angiogenic pathway. However, there are conflicting results regarding the effect of metformin over VEGF-A expression. For instance, no effect over VEGF-A expression was found when trophoblast cells (Sw.71) were cultured with or without metformin (0.5 mM) (Han *et al.*, 2015). Reduced expression of VEGF-A was found in breast epithelial cells (MCF10A-ER-Src) cultured with metformin (0.1 mM) compared to controls (Hirsch *et al.*, 2013). Likewise, in human studies, the use of metformin in obese T2DM patients (mean daily dosage 1.4 g) (Ersoy *et al.*, 2008) and women with polycystic ovary syndrome (mean daily dosage 1.7 g) (Tang *et al.*, 2006) showed a reduction of VEGF-A protein expression compared to controls. On the contrary, HUVEC cultured with metformin (10mM) presented higher VEGF-A expression than controls (Dallaglio *et al.*, 2014). This increase on VEGF-A expression is due to metformin AMPK activation (Dallaglio *et al.*, 2014; Ouchi *et al.*, 2005). Considering the cell culture studies, low doses of metformin (0.1 mM) reduce VEGF-A expression, while high doses (10 mM) increase VEGF-A expression. In human studies, the use of up to 1.7 g/day of metformin generated a reduction of VEGF-A expression. Metformin doses ranging from 0.5 to 2.5 g/day have been used to treat women with GDM (Priya & Kalra, 2018). Hence, information regarding the daily dose of the women from our metformin-controlled GDM group could help us elucidate metformin's involvement in the unchanged levels of VEGF-A found in placental samples from the metformin-controlled GDM group. Additionally, further work is needed to confirm that metformin's effect over VEGF-A expression is dosage dependent.

VEGFR-2 is the principal VEGF-A receptor on endothelial cells. Our study showed unchanged *VEGR-2* gene expression levels in placental

samples between GDM and normal pregnancies. Contradictory results for *VEGR-2* gene expression have been reported between placental samples from normal and GDM pregnancies. Unchanged *VEGR-2* gene expression was found in placental samples from insulin-controlled GDM pregnancies compared to controls (Lappas, 2014); while lower *VEGR-2* gene expression was found in placental samples from diet-controlled GDM pregnancies compared to controls (Meng *et al.*, 2016). The latter results disagree with our findings; this could be due to sample size per group. Meng compared the expression of 20 samples per group, while our study used lower sample numbers (normal $n=9$, diet-controlled GDM $n=7$). A smaller sample size could be behind the lack of significance found by our study. Furthermore, as the binding of VEGF-A to VEGFR-2 triggers angiogenesis; it is important to determine the effects of GDM in VEGFR-2 protein expression and localisation in the endothelial membrane of GDM placental samples. Considering our results of lower VEGF-A protein expression in diet-controlled GDM samples; we could speculate that a differential expression of VEGFR-2 will not promote placental angiogenesis in diet-controlled GDM pregnancies compared to normal pregnancies.

The angiopoietin/Tie-2 system is involved in two antagonistic processes: vessel maturation (Ang-1 + Tie-2) and vessel remodelling (Ang-2 + Tie-2). Furthermore, several studies have determined that Ang-1 promotes the stabilisation of the vasculature by induction of *Occludin* gene expression (Hori *et al.*, 2004), inhibition of Occludin tyrosine phosphorylation (Siddiqui *et al.*, 2015), or by inhibition of VEGF effect over VE-cadherin (Gavard *et al.*, 2008). Therefore, we determined the gene expression of both Angiopoietins (*Ang-1* and *Ang-2*) and their receptor (*Tie-2*) in placental samples from normal and GDM pregnancies. Our results showed similar expression of *Ang-1* in placental samples from normal and GDM pregnancies. In agreement with our results, several studies have found that Ang-1 gene and protein expression was unaffected in placental tissue (T1DM) (Janota *et al.*, 2003), maternal plasma (T1DM) (Loukovaara *et al.*, 2004), and fetal-cord plasma (GDM) (Giri *et al.*, 2013) between diabetic and

normal samples. Further work is needed to determine Ang-1 protein expression, and Ang-1 role in Occludin downregulation in placental samples from diet-controlled GDM pregnancies.

Our results showed similar gene expression of *Ang-2* in placental samples from normal and GDM pregnancies. In disagreement with our results, studies have found higher *Ang-2* gene expression in placental samples from insulin-controlled GDM pregnancies (Radaelli *et al.*, 2003) and HUVEC isolated from diet-controlled GDM pregnancies (Giri *et al.*, 2013) compared to controls. These contradictory results could depend on the hypoxia levels of each GDM pregnancy, as Ang-2 belongs to the hypoxia sensitive proangiogenic factors (Pichiule *et al.*, 2004). During a diabetic pregnancy, maternal hyperglycaemia results in fetal hyperglycaemia and hyperinsulinemia. Increased levels of insulin will stimulate fetal metabolism and subsequently enhance fetal oxygen demand, leading to chronic fetal hypoxia (Björk & Persson, 1984). Moderate degrees of fetal hypoxia have been found in umbilical cord blood samples from GDM pregnancies compared to non-diabetic pregnancies (Taricco *et al.*, 2009). Therefore, we could speculate that the higher expression of *Ang-2* gene found in previous studies were the result of increased hypoxia conditions in those diabetic pregnancies. Further work is needed to determine hypoxia conditions of GDM pregnancies, and the expression of the angiopoietin system, to have a better understanding of the influence of GDM in placental angiogenesis.

Our results showed that *Tie-2* gene expression was similar between normal and GDM placental samples. To our knowledge, this is the first study to investigate *Tie-2* gene expression in placental samples from GDM pregnancies. Studies showed that Tie-2 gene and protein expression were unchanged in placental tissue from T1DM compared to controls (Janota *et al.*, 2003; Vuorela & Halmesmäki, 2006). Further work is needed to determine the protein expression and localisation of Tie-2, in placental samples from GDM and normal pregnancies

MMP-9 plays an essential role in the maintenance and remodelling of the extracellular matrix during angiogenesis. Additionally, MMP-9 is involved in the regulation of the endothelial paracellular barrier. MMP-9 proteolytic activity degrades Occludin (Caron *et al.*, 2005; Giebel *et al.*, 2005), and VE-cadherin (Navaratna *et al.*, 2007), leading to an increase in barrier permeability. Our study compared the gene expression of *MMP-9* between placental samples from normal and GDM pregnancies. To our knowledge, this is the first report of *MMP-9* gene expression in placental samples from GDM pregnancies. Our results showed unchanged *MMP-9* gene expression between normal and GDM placental samples. Previous studies have determined MMP-9 proteolytic activity in placental samples. These studies found that MMP-9 activity in placental samples was lower in diet-controlled GDM (Pustovrh *et al.*, 2000), while higher in T1DM (Pustovrh *et al.*, 2000) and T2DM (Capobianco *et al.*, 2012) compared to controls. It is impossible to compare our *MMP-9* gene expression results to MMP-9 protein activity results from these studies. Furthermore, an enzymatic activity assay would have been more relevant to determine if MMP-9 was responsible for the downregulation of Occludin protein levels found in placental samples from diet-controlled GDM pregnancies. However, considering our results (*MMP-9* stable gene expression) and literature (MMP-9 lower enzymatic activity), we could suggest that there is lower remodelling of the extracellular matrix by MMP-9 in GDM placentae. Therefore, lower degradation of junctional molecules by MMP-9. Further work is needed to determine MMP-9 protein levels and enzymatic activity in GDM placental samples.

In conclusion, based on our results we can reject our hypothesis that there is an increase in expression of angiogenic factors in diet-controlled GDM placental samples. In fact, there was a downregulation of total VEGF-A protein expression in diet-controlled GDM samples. In contrast, placental samples from metformin-controlled GDM pregnancies did not present any change in VEGF-A protein expression compared to controls. This suggests

that metformin may be restoring VEGF-A protein expression. Further work is needed to determine if epigenetic factors (promoter methylation, microRNAs), or post-translational modifications (phosphorylation) are behind the decreased Occludin expression found in diet-controlled GDM placentae (Chapter 6).

CHAPTER 5

EFFECT OF AN ACUTE HYPERGLYCAEMIC INSULT IN THE EXPRESSION OF OCCLUDIN

5.1. INTRODUCTION

The results in chapter 3 showed that Occludin expression was downregulated in placental samples from diet-controlled GDM pregnancies compared to control. Maternal hyperglycaemia is a hallmark of GDM women. Furthermore, even when HbA_{1c} values showed controlled glucose levels in our GDM women (HbA_{1c} < 5.6%), periods of hyperglycaemia can go undetected. For instance, women with diet-controlled GDM pregnancies, who presented a mean HbA_{1c} of 5.1%, showed hyperglycaemic insults (glucose level > 8 mM) while being monitored by a Continuous Glucose Monitoring (CGM) System (R. Chen *et al.*, 2003; Jovanovic, 2000). Moreover, a previous study determined that perfusion of placental vasculature from non-diabetic pregnancies with an acute hyperglycaemia insult (15 mM glucose for 3 hours) in the maternal circulation, results in increased vascular permeability and reduced junctional VE-cadherin localisation in vessels compared to controls (5 mM glucose perfusion) (Sciota, 2012).

Therefore, this study wanted to determine if the cause of Occludin downregulation was the hyperglycaemic milieu. To test this, placental chorionic villus explants were used as an *ex vivo* model of acute hyperglycaemia (15 mM D-glucose). This glucose concentration was chosen based on previous findings in T1DM pregnant women (Kerssen *et al.*, 2004). Kerssen showed that women's postprandial blood glucose concentrations can be as high as 15 mM before returning to normal.

5.1.1. PLACENTAL CHORIONIC VILLUS EXPLANTS AS AN *EX VIVO* MODEL

Multiple studies have used placental explants as an *ex vivo* model to study the impact of a diabetic environment. For instance, placental explants under hyperglycaemic conditions (15mM glucose for 24-hours) showed a lower junctional VE-cadherin location compared to controls (Sciota, 2012), similar to that found in T1DM placental pregnancies (Leach *et al.*, 2004). Additionally, placental explants under hyperglycaemic conditions (25 mM glucose for 48h) presented lower *AMPK α* gene expression compared to controls, this downregulation was also found in T1DM placental samples (Hulme *et al.*, 2018). Placental explants from normal pregnancies under hyperglycaemic conditions (> 10 mM D-glucose) showed increased triglyceride content, reduced fatty acid oxidation (Visiedo *et al.*, 2013), and reduced placental growth hormone (Patel *et al.*, 1995) compared to controls.

5.1.2. OCCLUDIN EXPRESSION IN HYPERGLYCAEMIC CONDITIONS

Several studies have used cell culture techniques to determine the effect of glucose on Occludin expression. For instance, rat retinal endothelial cells cultured under 30 mM D-glucose for more than 24-hours showed downregulation of Occludin protein expression, reduced junctional Occludin localisation, and increased membrane permeability compared to controls (Tien *et al.*, 2013). Likewise, rhesus monkey retinal fovea vascular endothelial cells (M. Li *et al.*, 2017), and human retinal pigmented epithelial cells (Ved *et al.*, 2017) cultured under high glucose (> 30mM D-glucose) for over 24-hours showed lower Occludin protein expression and junctional localisation compared to controls. These studies showed that Occludin expression can be affected under hyperglycaemic conditions. To our knowledge, there are no published studies that used placental explants under hyperglycaemic conditions and determined Occludin expression and localisation.

5.1.3. HYPOTHESIS AND AIMS OF CHAPTER 5

A hyperglycaemic environment causes downregulation of Occludin expression and localisation in placental endothelium

To test these hypotheses, the aims of this chapter were:

- To recreate a hyperglycaemic environment from a GDM pregnancy
- To compare *Occludin* gene expression in an *ex vivo* model of hyperglycaemia and normoglycaemia.
- To determine Occludin junctional localisation in placental explants under hyperglycaemic conditions.

5.2. METHODS

For detailed methodology see Chapter 2.

5.2.1. STUDY POPULATION

Women with uncomplicated pregnancies, who were scheduled for elective C-section at term pregnancies (>37 weeks), were asked for written informed consent for placental tissue collection ($n=8$).

5.2.2. PLACENTAL CHORIONIC VILLUS EXPLANTS

Placentae were collected within 15 minutes of delivery. Chorionic villus explants were obtained following the recommendations of Sciota (Sciota, 2012), as described in Section 2.5. A schematic representation of this experiment is shown in Figure 5.1. Briefly, a total of 24 explants were obtained per placenta; explants were incubated in explant media (M199, 1% penicillin/streptomycin, 15 mM dextran, 0.5% BSA, 26 mM sodium bicarbonate, 5000 IU/ml heparin) at 37°C humidified incubator with 5% CO₂ in air for 24 hours. Afterwards, these explants were divided in three conditions: normoglycaemia control (5 mM D-glucose), hyperglycaemia control (5 mM D-glucose and 10 mM L-glucose), and hyperglycaemia (15 mM D-glucose) and were further incubated for 3- or 24-hours. Finally, explants were stored in RNeasy lysis buffer ($n=4$ per condition, per placenta) for gene

expression, and 1% PFA ($n= 4$ per condition, per placenta) for immunostaining.

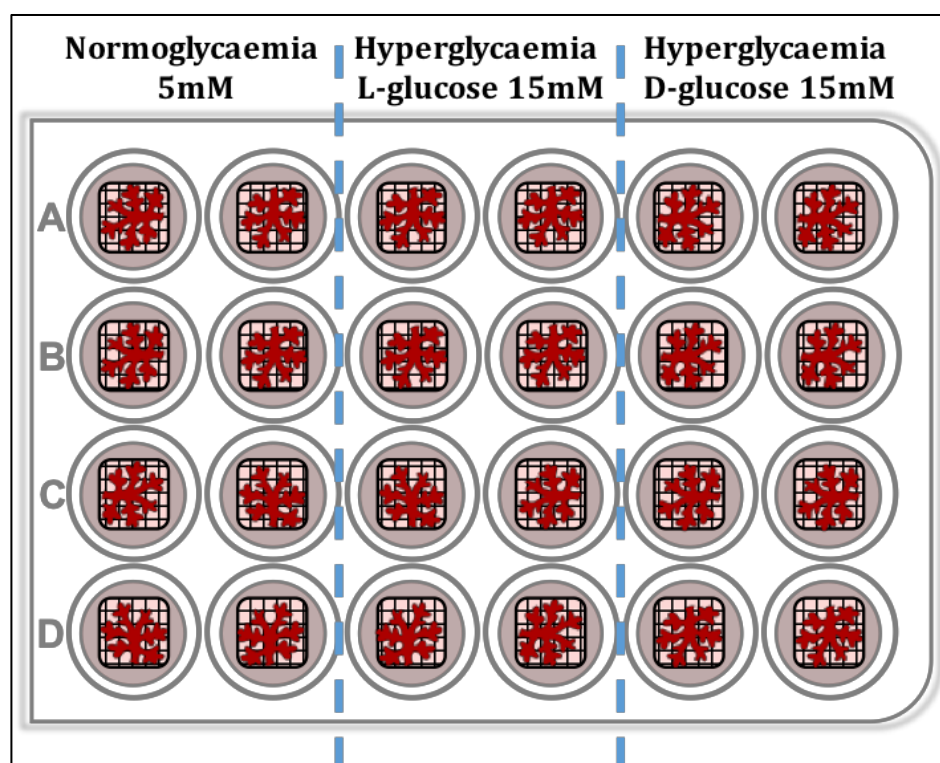


Figure 5.1. Schematic representation of placental chorionic villus explants experiment under treatment media. Explants were incubated in explant media at 37°C with 5% CO₂ for 24 hours; followed by treatment media at 37°C with 5% CO₂ for 3- or 24- hours.

5.2.3. *OCLUDIN* GENE EXPRESSION IN PLACENTAL CHORIONIC VILLUS EXPLANTS

RNA isolation, reverse transcription and qPCR were performed as described in Section 3.2.2.

5.2.4. JUNCTIONAL OCLUDIN IMMUNOSTAINING IN CHORIONIC VILLUS EXPLANTS

Chorionic villus were stained for Occludin following the protocol described in Section 2.21.

5.2.5. STATISTICAL ANALYSIS

The data is expressed as means $\pm$ SEM. The data was tested for normality distribution by D'Agostino & Pearson test with a confidence interval of 95%. Statistical comparisons were performed for parametric

datasets between two groups (independent samples *t*-test), and three groups (one-way ANOVA).

5.3. RESULTS

5.3.1. STUDY GROUP

Placental samples from uncomplicated pregnancies ($n = 8$) were obtained for chorionic villus explants experiment. The clinical details of the women are detailed in Table 5.1. Maternal BMI was considered healthy ($\leq 25.0 \text{ kg/m}^2$).

Table 5.1. Clinical characteristics of the women whose placentae were used for *ex vivo* model of hyperglycaemia. BMI, body mass index. Data reported as mean $\pm$ the standard deviation (SD).

Characteristic	Explants ($n = 8$)
Maternal age, years, mean (SD)	33.13 (3.00)
Maternal BMI, kg/m^2 , mean (SD)	24.38 (3.62)
Gestational age at delivery, weeks, mean (SD)	39.38 (0.34)
Placental weight, g, mean (SD)	596.40 (161)
Baby weight, kg, mean (SD)	3.66 (0.51)
Placenta efficiency, fetal/placental weight ratio, mean (SD)	6.41 (1.40)

5.3.2. OCCLUDIN GENE EXPRESSION AND LOCALISATION WERE NOT AFFECTED BY A CHANGE IN OSMOLARITY OF THE HYPERGLYCAEMIC ENVIRONMENT

L-glucose was used as a hyperglycaemic control because it cannot be used by cells as a source of energy. Hence, L-glucose acted as a control for osmolality. *Occludin variant 1* gene expression was similar between normoglycaemia (5 mM D-glucose) and hyperglycaemia (15 mM L-glucose) control conditions ($t(11) = 1.08$, $p = 0.301$) (Figure 5.2A). Likewise, *Occludin variant 1* gene expression was similar between normoglycaemia and hyperglycaemia conditions, independent from the duration (3- or 24-hours) of the exposure ($F(2, 10) = 0.536$, $p = 0.601$) (Figure 5.2B). *Occludin variant 2* gene expression was unchanged between normoglycaemia and

hyperglycaemia control conditions ($t(11) = 0.686, p = 0.507$) (Figure 5.2C). Similarly, *Occludin variant 2* gene expression was similar between normoglycaemia and 3- or 24-hours exposure to hyperglycaemic control conditions ($F(2, 10) = 1.11, p = 0.366$) (Figure 5.2D). *Occludin variant 3* gene expression was unchanged between normoglycaemia and hyperglycaemia control conditions ($t(10) = 1.03, p = 0.326$) (Figure 5.2E). Moreover, *Occludin variant 3* gene expression was similar between normoglycaemia and short or long exposure to hyperglycaemic control conditions ($F(2, 9) = 0.485, p = 0.631$) (Figure 5.2F).

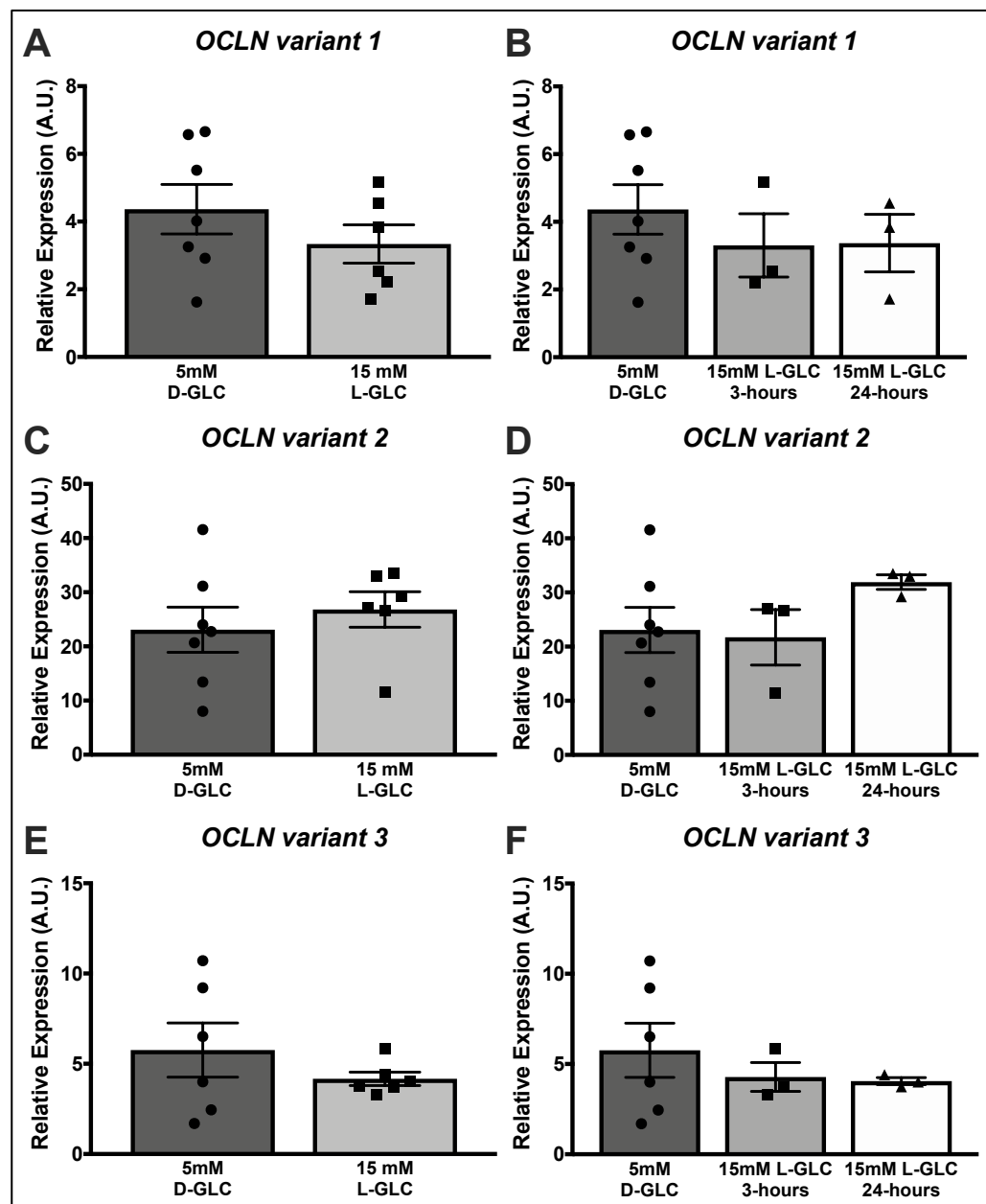


Figure 5.2. Occludin variants 1, 2, and 3 gene expression was similar in human placental chorionic villus explants subjected to normoglycaemic (5mM D-glucose) and hyperglycaemic (15 mM L-glucose) control conditions. Chorionic explants were taken from normal pregnancies ($n=8$). Occludin variants 1, 2, and 3 expression was similar between normoglycaemic (5 mM D-glucose) and hyperglycaemic (15 mM L-glucose) control conditions (**A, C, E**). Occludin variants 1, 2, and 3 expression was unchanged between normoglycaemic and short (3-hours) or long (24-hours) exposure to hyperglycaemic control conditions (**B, D, F**). Mean $\pm$ SEM of eight independent experiments, each performed in replicates ($n=4$). One-way ANOVA and post hoc Unpaired t-test. OCLN= Occludin; D-GLC= D-glucose; L-GLC= L-glucose.

Junctional Occludin localisation in placental chorionic villus explants was similar between normoglycaemic and hyperglycaemic (L-glucose) controls control conditions ($t(13) = 0.014$, $p = 0.989$) (Figure 5.3A). Additionally, junctional Occludin localisation was unchanged between normoglycaemia and short or long exposure to hyperglycaemic control conditions ($F(2, 12) = 1.15$, $p = 0.348$) (Figure 5.3B). As no statistical differences were found, the expression of both glycaemic control conditions were used as “normoglycaemic control” to compare with results from hyperglycaemic conditions (15 mM D-glucose).

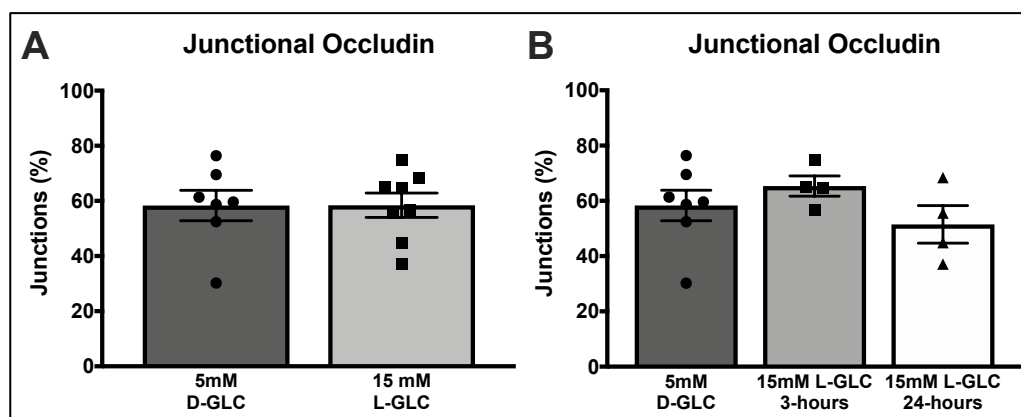


Figure 5.3. The junctional localisation of Occludin was similar in fetoplacental vessels subjected to normoglycaemic (5mM D-glucose) and hyperglycaemic (15 mM L-glucose) control conditions. Chorionic explants were taken from normal pregnancies ($n=8$). **(A)** Junctional Occludin localisation was similar between normoglycaemic (5 mM D-glucose) and hyperglycaemic (15 mM L-glucose) control conditions. **(B)** Junctional Occludin localisation was unchanged between normoglycaemic and short (3-hours) or long (24-hours) exposure to hyperglycaemic control conditions. Mean $\pm$ SEM of eight independent

experiments, each performed in replicates ($n = 4$). One-way ANOVA and post hoc Unpaired t-test. D-GLC= D-glucose; L-GLC= L-glucose.

5.3.3. OCCLUDIN GENE EXPRESSION AND JUNCTIONAL LOCALISATION WERE NOT AFFECTED BY A HYPERGLYCAEMIC INSULT INDEPENDENT OF THE DURATION OF THE EXPOSURE

Occludin variant 1 expression was similar between explants under normoglycaemic (5 mM D-glucose) and hyperglycaemic (15 mM D-glucose) conditions ($t(19) = 0.969$, $p = 0.345$) (Figure 5.4A). Additionally, *Occludin variant 1* gene expression was unchanged between normoglycaemic conditions and short (3-hours) or long (24-hours) of hyperglycaemic conditions ($F(2, 18) = 0.713$, $p = 0.504$) (Figure 5.4B). Likewise, there was no statistical significance in *Occludin variant 1* gene expression between placental explants cultured under control conditions (5 mM D-glucose) and under a short exposure (3-hours) ($t(15) = 1.19$, $p = 0.252$) or long exposure (24-hours) ($t(15) = 0.288$, $p = 0.777$) to hyperglycaemic conditions (15 mM D-glucose).

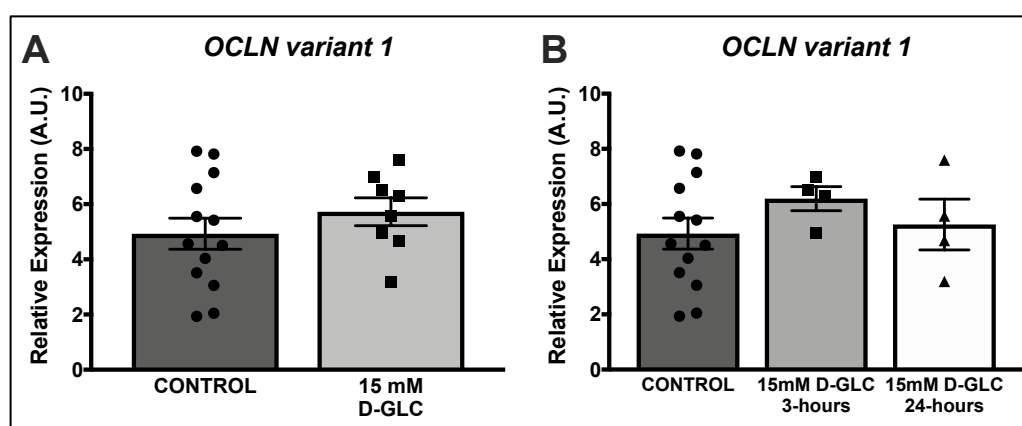


Figure 5.4. *Occludin variant 1* expression was similar in human placental chorionic villus explants subjected to normoglycaemic (5mM D-glucose) and hyperglycaemic (15 mM D-glucose) conditions. Chorionic explants were taken from normal pregnancies ($n = 8$). **(A)** *Occludin variant 1* gene expression was similar between controls (5 mM D-glucose) and hyperglycaemic conditions (15 mM D-glucose). **(B)** *Occludin variant 1* gene expression was unchanged between control and short (3-hours) or long (24-hours) exposure to hyperglycaemic (15 mM D-glucose) conditions. Mean $\pm$ SEM of eight independent experiments, each performed in replicates ($n = 4$). One-way ANOVA and post hoc Unpaired t-test. OCLN= Occludin; D-GLC= D-glucose.

Occludin variant 2 gene expression was unchanged between explants under normoglycaemic (5 mM D-glucose) and hyperglycaemic (15 mM D-glucose) conditions (t (19) = 0.488, p = 0.631) (Figure 5.5A). Likewise, *Occludin variant 2* gene expression was similar between normoglycaemic conditions and short (3-hours) or long (24-hours) of hyperglycaemic conditions (F (2, 18) = 0.797, p = 0.466) (Figure 5.5B). Furthermore, no statistical difference was found in *Occludin variant 2* gene expression between placental explants cultured under normoglycaemic conditions (5 mM D-glucose) and under a short exposure (3-hours) (t (15) = 1.03, p = 0.321) or long exposure (24-hours) (t (15) = 0.312, p = 0.759) to hyperglycaemic conditions (15 mM D-glucose).

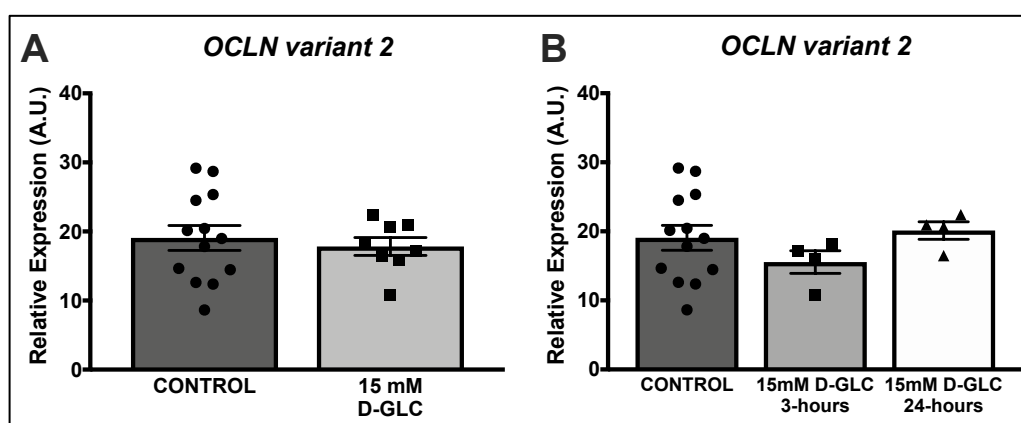


Figure 5.5. *Occludin variant 2* expression was similar in human placental chorionic villus explants subjected to normoglycaemic (5mM D-glucose) and hyperglycaemic (15 mM D-glucose) conditions. Chorionic explants were taken from normal pregnancies (n = 8). **(A)** *Occludin variant 2* gene expression was similar between controls (5 mM D-glucose) and hyperglycaemic conditions (15 mM D-glucose). **(B)** *Occludin variant 2* gene expression was unchanged between control and short (3-hours) or long (24-hours) exposure to hyperglycaemic (15 mM D-glucose) conditions. Mean $\pm$ SEM of eight independent experiments, each performed in replicates (n = 4). One-way ANOVA and post hoc Unpaired t -test. OCLN= Occludin; D-GLC= D-glucose.

Occludin variant 3 expression was similar between explants under normoglycaemic (5 mM D-glucose) and hyperglycaemic (15 mM D-glucose) conditions (t (19) = 0.726, p = 0.477) (Figure 5.6A). Furthermore, *Occludin variant 3* expression was unchanged between normoglycaemic conditions and short (3-hours) or long (24-hours) of hyperglycaemic conditions (F (2,

18) = 1.49, $p = 0.251$) (Figure 5.6B). Likewise, *Occludin variant 3* gene expression was unchanged between placental explants cultured under normoglycaemic conditions (5 mM D-glucose) and under a short exposure (3-hours) ($t(15) = 0.392$, $p = 0.701$) or long exposure (24-hours) ($t(15) = 1.45$, $p = 0.169$) to hyperglycaemic conditions (15 mM D-glucose).

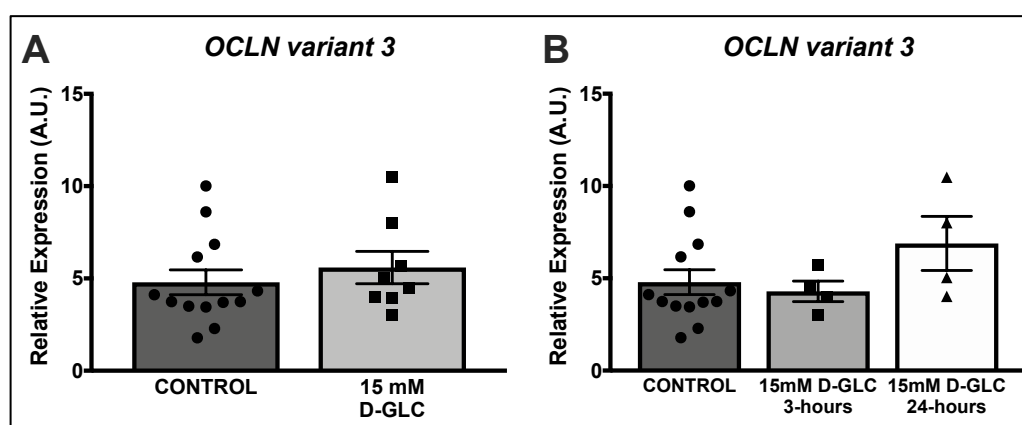


Figure 5.6. *Occludin variant 3* expression was similar in human placental chorionic villus explants subjected to normoglycaemic (5mM D-glucose) and hyperglycaemic (15 mM D-glucose) conditions. Chorionic explants were taken from normal pregnancies ($n = 8$). **(A)** *Occludin variant 3* gene expression was similar between controls (5 mM D-glucose) and hyperglycaemic conditions (15 mM D-glucose). **(B)** *Occludin variant 3* gene expression was unchanged between control and short (3-hours) or long (24-hours) exposure to hyperglycaemic (15 mM D-glucose) conditions. Mean $\pm$ SEM of eight independent experiments, each performed in replicates ($n = 4$). One-way ANOVA and post hoc Unpaired t-test. OCLN= Occludin; D-GLC= D-glucose.

Immunostaining was used to determine localisation of Occludin in placental explants (Figure 5.7). Six micrographs were taken per condition ($n = 144$ micrographs). Junctions were counted (between 100-150 junctions per condition; $n = 3100$ junctions), and junctional occupancy of Occludin was determined by the percentage of immuno-positive vessels.

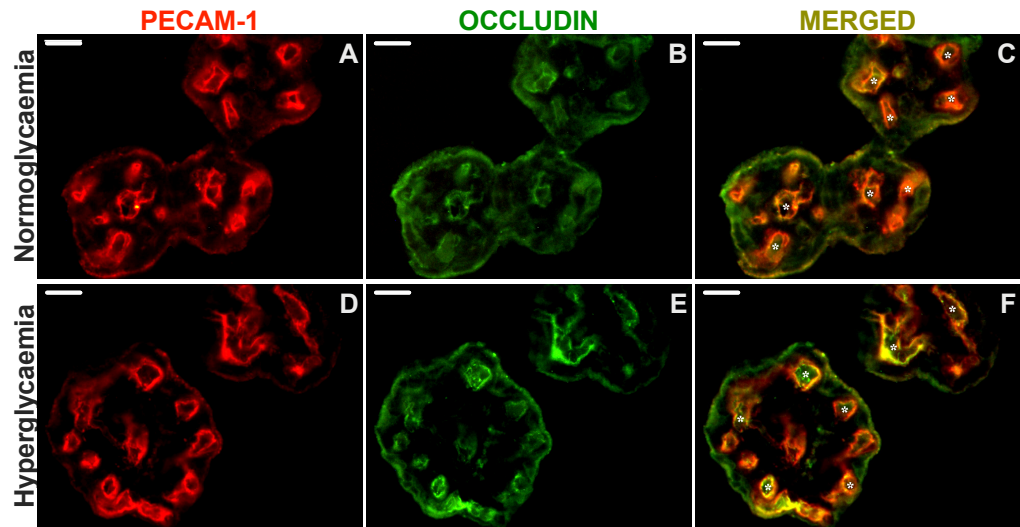


Figure 5.7. Representative fluorescent micrographs of Occludin localisation in fetoplacental vessels subjected to normoglycaemic (5mM D-glucose) and hyperglycaemic (15 mM D-glucose) conditions. Chorionic explants were taken from normal pregnancies ($n= 8$). Samples were double-stained with PECAM-1 (red; panels A, and D) and Occludin (green; panels B, and E). Panels C, and F show double-staining with PECAM-1 and Occludin (yellow; merged), stars show junctional Occludin in vessels' paracellular clefts. **(C)** Micrograph of a placental explant under normoglycaemia conditions, junctional Occludin could be visualised in all junctions (8/8). **(F)** Micrograph of a placental explant under hyperglycaemia conditions, junctional Occludin found in most of the vessels (7/9). Scale bars: 20 μm .

Junctional Occludin localisation was similar between explants under normoglycaemic (5 mM D-glucose) and hyperglycaemic (15 mM D-glucose) conditions ($t(21) = 0.342, p = 0.736$) (Figure 5.8A). Furthermore, junctional Occludin localisation was unchanged between normoglycaemic conditions and short (3-hours) or long (24-hours) of hyperglycaemic conditions ($F(2, 20) = 0.21, p = 0.813$) (Figure 5.8B).

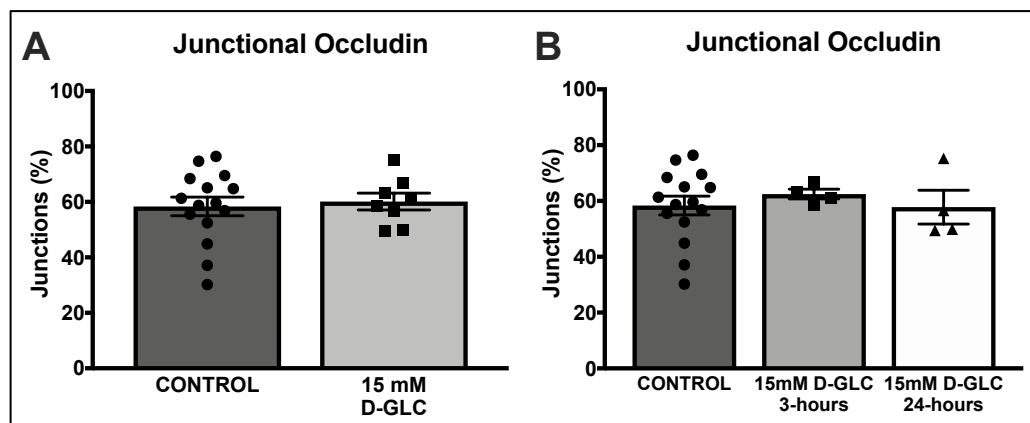


Figure 5.8. Junctional Occludin localisation was similar in fetoplacental vessels subjected to normoglycaemic (5mM D-glucose) and hyperglycaemic (15 mM D-glucose) conditions. Chorionic explants were taken from normal pregnancies ($n=8$). **(A)** Junctional occludin localisation remained similar between controls (5 mM D-glucose) and hyperglycaemic conditions (15 mM D-glucose). **(B)** Junctional occludin localisation was unchanged between control and short (3-hours) or long (24-hours) exposure to hyperglycaemic (15 mM D-glucose) conditions. Mean $\pm$ SEM of eight independent experiments, each performed in replicates ($n=4$). One-way ANOVA and post hoc Unpaired t-test. D-GLC= D-glucose.

5.4. DISCUSSION

Here we investigated the impact of hyperglycaemia on Occludin expression by using placental chorionic villus explants from non-diabetic pregnancies and a single acute hit of 15 mM D-glucose. Our results showed no change in Occludin gene expression and junctional localisation in placental explants under hyperglycaemic conditions. These results suggest that there are more factors involved in the downregulation of Occludin in a GDM environment.

Our study used the hallmark of GDM (hyperglycaemia) to determine its effect over Occludin expression and localisation in placental vasculature. The glucose concentration was chosen based on a previous report of postprandial blood glucose concentrations (15 mM) of T1DM pregnant women (Keressen *et al.*, 2004). Furthermore, a study using a CGM system on well-controlled GDM women determined daily hyperglycaemic insults (glucose levels > 8 mM) of nearly 5.5 hours per day (Jovanovic, 2000). Considering these conditions, our study exposed placental chorionic villus explants from non-diabetic pregnancies to a single acute hyperglycaemic insult (15 mM D-glucose) for either a short (3-hours) or a long (24-hours) period. This glucose concentration was 2.5-times higher than the average blood glucose concentration (HbA_{1c} results) found in the women with GDM pregnancies that agreed to be part of our study (5.7 mM blood glucose for diet-controlled GDM group, and 6.0 mM blood glucose for metformin-controlled GDM group). Nonetheless, our results showed that Occludin gene expression and junctional localisation were not affected when explants

were exposed to acute hyperglycaemic conditions. Conversely, a study using placental explants from non-diabetic pregnancies cultured under 15 mM glucose for 3-hours found downregulation of junctional VE-cadherin vessel localisation compared to controls (5 mM glucose) (Sciota, 2012). Therefore, Occludin downregulation could be a consequence of a different factor related to GDM. In this regard, studies have shown that in diabetic pregnancies there is also increased insulin secretion (Leach *et al.*, 2009), as well as increased low-density lipoprotein (LDL) (Bartels & O'Donoghue, 2011).

High insulin levels have been found in maternal blood from women with GDM pregnancies compared to normal pregnancies (Aslan *et al.*, 2012; B. Liu *et al.*, 2012). Furthermore, fetal insulin levels were higher in umbilical vein blood samples from GDM pregnancies compared to normal pregnancies (Aslan *et al.*, 2012; Westgate *et al.*, 2006). In fact, cord insulin concentrations > 120 pmol/L were found in almost 30% of offspring of mothers with GDM (Westgate *et al.*, 2006). Even when hyperinsulinemia has been reported in both maternal and fetal circulation, it is important to consider that insulin cannot cross the placental barrier in appreciable amounts. In fact, the control of insulin flow through the placenta is transferred gradually from the mother (during the first trimester, insulin receptors are localised on the syncytiotrophoblast) to the fetus (at term, insulin receptors are mostly localised on the endothelium of the placental vessels) (Desoye *et al.*, 1994). Hence, fetal hyperinsulinemia could be affecting endothelial barrier stability. In this regard, a pilot study from our group showed that perfusion of term-placentae samples from normal pregnancies with high insulin (170 pmol/L) in the fetal circulation for 20 minutes results in increased junctional disruption and vascular leakage (Lucas *et al.*, 2008). This study found that almost 50% of vascular profiles exhibited complete loss of β -catenin (an AJ protein) and almost 30% of the vessels showed dextran tracer (76-M_r) leakage when exposed to insulin (Lucas *et al.*, 2008). Hence, it could be possible that under fetal hyperinsulinemia, Occludin expression and localisation would be affected

in placental vasculature. Future work should use perfusion techniques to determine the effects of fetal hyperinsulinemia alone, as well as a combination of maternal hyperglycaemia and fetal hyperinsulinemia, over Occludin expression and localisation in the placental endothelial barrier.

Women with pregnancies complicated with GDM showed increased triglycerides, as well as reduced LDL and HDL in plasma samples compared to non-diabetic pregnant women (Qiu *et al.*, 2007). Furthermore, offspring from pregnancies complicated with GDM showed increased LDL levels in cord blood plasma samples compared to offspring from non-diabetic pregnancies (Eslamian *et al.*, 2013). Hence, dyslipidaemia can also be a feature of GDM pregnancies. Studies have shown that dyslipidaemia conditions affect the stability of the endothelial barrier. For instance, HUVEC barrier permeability was assessed when cultured under hyperglycaemic (25 mM D-glucose) (HG) and high lipopolysaccharides (1 ug/ml) (LPS) (Yan Yang *et al.*, 2017). HUVEC showed increased permeability when cultured under HG (2.0-fold), LPS (1.7-fold), and the combination of both HG and LPS (2.5-fold) compared to controls (Yan Yang *et al.*, 2017). Furthermore, a study showed that culture of human aortic endothelial cells (HAECs) under triglyceride-rich lipoproteins (TGRL) (150 mg/dl) and lipoprotein lipase (LPL) enzyme (2 U/ml) reduced the endothelial resistance (TEER), as well as reduced ZO-1 and Occludin protein expression, and discontinued ZO-1 and VE-cadherin junctional localisation in endothelial barrier compared to controls (Eiselein *et al.*, 2007). These increased barrier permeability and downregulation of Occludin protein expression were also found in brain tissue samples from mice fed with a cholesterol-rich high-fat diet to mimic hyperlipidaemia compared to controls (ElAli *et al.*, 2011). Therefore, hyperlipidaemia can affect Occludin protein expression and endothelial barrier stability. Future studies should aim to determine the effect of hyperlipidaemia, as part of a GDM environment, in placental vasculature and Occludin expression.

The stable expression and junctional localisation of Occludin found in our explant experiment can also be due to an acute exposure to hyperglycaemia. It is essential to consider that GDM is a chronic hyperglycaemic milieu. In this regard, three short periods (3-hours) of exposure within a day, for over 48-hours could replicate the hyperglycaemic conditions of a GDM as seen by Jovanovic with the CGM system (glucose levels > 8 mM for nearly 5.5 hours per day) (Jovanovic, 2000). Furthermore, the downregulation of Occludin could be the result of a change in expression of epigenetic factors in diet-controlled GDM pregnancies. Under prolonged hyperglycaemic conditions (new experiment set up), epigenetic changes will have time to occur and affect Occludin expression and localisation. Nonetheless, further work is needed to determine the levels of *Occludin* promoter DNA methylation in placental samples from normal and GDM pregnancies.

Contrary to our results, a downregulation of Occludin has been shown in cell culture studies under hyperglycaemic conditions (> 30 mM glucose for > 24-hours exposure) (M. Li *et al.*, 2017; Tien *et al.*, 2013; Ved *et al.*, 2017). However, glucose concentrations between 25 mM and 50 mM may only be seen in the clinical conditions of diabetic ketoacidosis (Fulop *et al.*, 1973) and hyperosmolar hyperglycaemic state (Arieff & Carroll, 1972; Daugirdas *et al.*, 1989). Hence, the hyperglycaemic conditions used by these cell culture studies are borderline physiological and will not be found in patients with controlled diabetes. Considering all the information discussed in this chapter, future experiments that aim to mimic a diabetic environment may need to consider a hyperglycaemic media with insulin and LDL, as well as chronic exposure.

In conclusion, we rejected our hypothesis that acute hyperglycaemic conditions alter the expression of Occludin in placental explants. Future studies that aim to mimic a diabetic pregnancy should consider glucose, insulin and LDL levels, as well as chronic exposure. Moreover, it is also important to determine whether the effects of GDM on Occludin

downregulation in placental tissue (Chapter 3) are due to epigenetic modifications (promoter methylation levels, microRNAs expression), or post-translational modifications (protein phosphorylation). These questions are addressed in Chapter 6.

CHAPTER 6

EPIGENETIC AND PHOSPHORYLATION MODIFICATIONS LINKED TO THE DOWNREGULATION OF Occludin EXPRESSION IN PLACENTAL VASCULATURE

6.1. INTRODUCTION

Our results showed lower Occludin expression and localisation in diet-controlled GDM placental samples compared to normal samples (Chapter 3). The downregulation of *Occludin* gene expression might be the result of epigenetic factors. For instance, an increase in promoter DNA methylation levels or the expression of microRNAs could be some epigenetic factors controlling *Occludin* gene expression. Furthermore, the loss of junctional Occludin from placental vasculature could be linked with post-translational modifications. For example, phosphorylation of Occludin will destabilise the protein from the junctions.

6.1.1. *Occludin* PROMOTER DNA METHYLATION

DNA methylation is an epigenetic modification that controls the levels of gene expression. DNA methylation occurs predominantly on cytosine in CpG islands and consists on the addition of a methyl group to the fifth carbon of the cytosine ring by DNA methyltransferases (DNMTs). Historically, DNA methylation was associated with repression of transcription as it occurs in promoter regions. However, recent studies have shown that when DNA methylation happens in gene-coding regions, it leads to an increase in gene expression levels (Laurent *et al.*, 2010; Schroeder *et al.*, 2013).

Our results showed that *Occludin variant 2* (which represents around the 75% of total *Occludin* gene expression) was downregulated in diet-controlled GDM placental samples (Chapter 3). Changes in the methylation levels of *Occludin* promoter could be behind this downregulation of *Occludin* gene expression. *Occludin* promoter is located around 200 bp upstream from the *Occludin* cDNA sequence (Mankertz *et al.*, 2000) (Figure 6.1). This promoter contains a CpG island that covers most of the region. Several studies have shown a downregulation of *Occludin* gene expression and an increase in the methylation levels of *Occludin* promoter in murine cancer cells (melanoma B16F10, and breast cancer AC2M2) (Osanai *et al.*, 2006), human cervical adenocarcinoma (HeLa) (J. M. Gu *et al.*, 2008), and in human melanoma cells (A-375) (Najafi *et al.*, 2017) compared to controls. However, a study using human metastatic breast tissue found lower *Occludin* gene expression and no change in *Occludin* promoter methylation levels compared to control (Martin *et al.*, 2010). To our knowledge, there are no published studies in human placental tissue quantifying *Occludin* promoter DNA methylation levels neither in normal nor in GDM placentae.

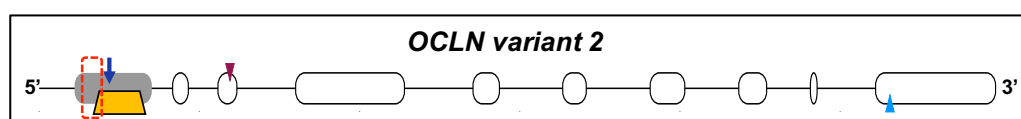


Figure 6.1. Schematic representation of *Occludin variant 2* gene, including promoter region and non-translated exons. Diagram shows: exons (white round-cornered rectangles); introns (black line); promoter region (grey round-cornered rectangle); CpG island (orange trapeze); transcriptional start site (blue arrow); translational start site (magenta triangle); stop site (light blue triangle); and the region amplified by our primers (red dotted rectangle). Promoter region reported by (Mankertz *et al.*, 2000). The intron/exon structure was displayed using the Gene Structure Display Server (GSDS 2.0) web-based bioinformatics tool (<http://gsds.cbi.pku.edu.cn>) (B. Hu *et al.*, 2015).

Several studies have compared methylation patterns from normal and GDM placental tissue and cord blood samples. Depending on the study, results have quantified either at the gene, locus, or differentially methylated regions (DMRs) levels. For instance, 3271 placenta and 3758 cord blood

genes presented differential methylation patterns between normal and GDM samples (Ruchat *et al.*, 2013). Likewise, 1708 placenta and 1485 cord blood DMRs were found between normal and GDM samples (Finer *et al.*, 2015); while 75 CpG loci were found differentially methylated in GDM cord blood samples when compared to normal pregnancies (Quilter *et al.*, 2014). These methylation patterns target different genes associated with pathways involved in metabolic diseases (Quilter *et al.*, 2014; Ruchat *et al.*, 2013), endocytosis, cancer, insulin signalling (Finer *et al.*, 2015), cell adhesion, cell-cell signalling, apoptosis (L. Liu *et al.*, 2014; Rong *et al.*, 2015), protein synthesis, lipid metabolism, among others (Petropoulos *et al.*, 2015).

6.1.2. MICRORNAS THAT TARGET *Occludin* EXPRESSION

microRNAs (miRNAs) are ~22 nucleotides-long RNA molecules that can have a profound effect in controlling gene expression by binding to the 3' UTR of the targeted mRNA. This interaction will prevent the translation of the mRNA by suppressing protein synthesis, initiating mRNA degradation, or both. Each miRNA is predicted to have multiple targets, and each mRNA may be regulated by more than one miRNA. miRNAs have been shown to be linked to cancer (Lu *et al.*, 2005), heart (Thum *et al.*, 2008) and neurological diseases (Yelamanchili *et al.*, 2010), among others.

Several miRNAs have been reported to directly or indirectly target *Occludin* expression. For instance, studies have reported that *miR-21* (Yongzhi Yang *et al.*, 2013), *miR-122* (Ye *et al.*, 2011), *miR-200c* (Elhelw *et al.*, 2017), *miR-429* (T. Yu *et al.*, 2016), and *miR-874* (Zhi *et al.*, 2014) act as direct suppressors of *Occludin* expression. Studies have also revealed indirect suppression of *Occludin* expression by *miR-18a-5p* through downregulation of Kruppel-Like Factor 4 (*KLF4*) (Y. Y. Zhao *et al.*, 2015); *miR-18a-5p* through downregulation of runt-related transcription factors (*RUNX*) (Miao *et al.*, 2015); *miR-34c* through downregulation of myc-associated zinc-finger protein (*MAZ*) (L. Zhao *et al.*, 2015); and *miR-181a-5p* through downregulation of Kruppel-Like Factor 6 (*KLF6*) (J. Ma *et al.*,

2014). These studies have used tissue samples (e.g. intestine, colon, liver) or cell culture (e.g. intestinal epithelial cells, glioma endothelial cells) to determine the effects of these miRNAs in *Occludin* gene expression. However, to our knowledge, there are no published studies in human placental tissue that quantify the expression of miRNAs that target *Occludin* gene.

Furthermore, studies have determined the miRNA profile from human placental tissue (Jiandong Li *et al.*, 2015; Muralimanoharan *et al.*, 2016; Nair *et al.*, 2018), HUVECs (Floris *et al.*, 2015; Tryggestad *et al.*, 2016) and maternal blood (C. Zhao *et al.*, 2011) from pregnancies complicated with GDM and compared it to controls. However, these studies have not determined the expression of a miRNA that target *Occludin* gene.

6.1.3. OCCLUDIN PROTEIN PHOSPHORYLATION

Protein phosphorylation is a reversible modification that consists of the addition of a phosphate group to the polar R of various amino acids through protein kinases.

Human Occludin protein is highly phosphorylated at serine (Ser) and threonine (Thr) residues in intact TJs (Sakakibara *et al.*, 1997). These phosphorylations are mainly localised at Occludin C-terminal domain; within this domain, a phosphorylation hotspot was identified from Tyr398 to Ser408 (Muller *et al.*, 2005) (Figure 6.2). This C-terminal domain is responsible for targeting Occludin to the TJs and binding to ZO-1, linking Occludin to the actin cytoskeleton (Furuse *et al.*, 1994). In this regard, junctional Occludin molecules present high serine phosphorylation, whereas non- or less-phosphorylated Occludin molecules are localised in the cytoplasm (Sakakibara *et al.*, 1997).

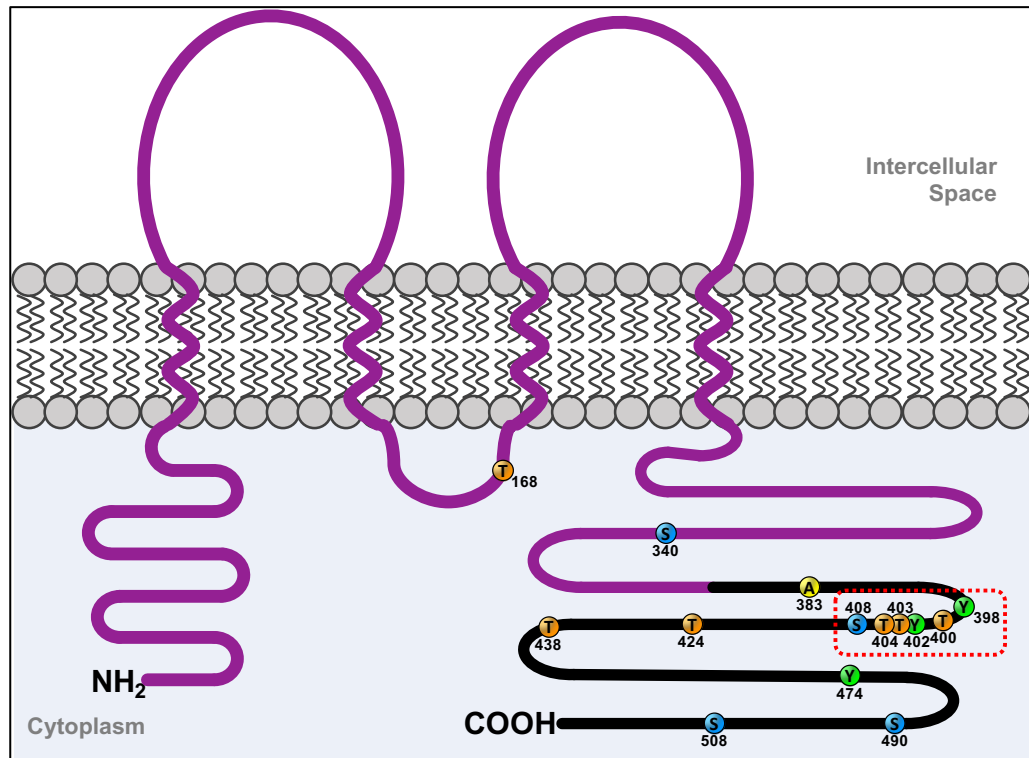


Figure 6.2. Schematic representation of human Occludin phosphorylation sites. Occludin C-terminal domain is highly phosphorylated. ZO-1 binds to amino acids 373 – 522 of the C-terminal domain (region in black). Within this region, there is a phosphorylation hotspot, a sequence of 11 amino acids from Tyr398 to Ser408 (red dotted rectangle). Threonine (T): orange circle; Serine (S): blue circle; Alanine (A): yellow circle; Tyrosine (Y): green circle.

Occludin can be phosphorylated at threonine, tyrosine and serine sites. However, different outcomes towards junctional Occludin and TJ assembly are obtained depending on the phosphorylation and dephosphorylation levels of these amino acids. For instance, Occludin threonine sites need to be phosphorylated for increased junctional Occludin and TJ assembly (Seth *et al.*, 2007; T. Suzuki *et al.*, 2009). Conversely, Occludin tyrosine sites need to be dephosphorylated to maintain junctional Occludin localisation and TJ assembly (Kale *et al.*, 2003; L. Zhu *et al.*, 2006). Phosphorylation of Occludin tyrosine (Tyr) residues (Tyr398 and Tyr402) reduces junctional Occludin and increases barrier dysfunction and membrane permeability (Elias *et al.*, 2009). In the case of serine, phosphorylation of only a handful of specific serine residues has an impact on the assembly of Occludin into the TJs. For instance, dephosphorylation of Occludin Ser408 (Raleigh *et al.*, 2011) and Ser490 (Antonetti *et al.*, 1999;

Sundstrom *et al.*, 2009), and phosphorylation of Occludin Ser338 (Andreeva *et al.*, 2001) sites are needed to enhance Occludin association with ZO-1, and stabilise Occludin in the TJs.

To our knowledge, there are no published studies in human placental tissue quantifying Occludin phosphorylation levels. However, studies have found increased number of placental vessels with total tyrosine phosphorylation in samples from GDM (Babawale *et al.*, 2000) and T1DM (Leach *et al.*, 2004) pregnancies compared to control. Additionally, studies focused on diabetes retinopathy found elevated serine/threonine phosphorylation of Occludin in retinal tissue of streptozotocin-induced diabetic rats compared to controls (Antonetti *et al.*, 1999; Harhaj & Antonetti, 2004).

6.1.4. HYPOTHESIS AND AIMS OF CHAPTER 6

Changes in Occludin expression and localisation found in GDM placental tissue are linked with epigenetic or phosphorylation modifications

To test this hypothesis, the aims of this chapter were:

- To determine the methylation levels of *Occludin variant 2* promoter in placental tissue from normal and GDM pregnancies
- To compare the expression of miRNAs known to modify the gene expression of *Occludin* in human placental tissue
- To overexpress *miR-181a-5p* (miRNA reported to target *Occludin* gene) in HUVEC isolated from normal pregnancies
- To assess *Occludin* gene expression in transfected HUVEC
- To quantify the phosphorylation levels of Occludin protein in placental tissue from normal pregnancies, diet-controlled and metformin-controlled GDM pregnancies

6.2. METHODS

For detailed methodology see Chapter 2.

6.2.1. STUDY POPULATION

Pregnant women, who were scheduled for elective C-section at term pregnancies (>37 weeks), were asked for written informed consent for placental and umbilical cord tissue collection. Placental samples were collected from normal pregnancies ($n=9$) used as a control, and pregnancies complicated with GDM where glucose levels were treated by a change of diet ($n=7$) or by medication (metformin) ($n=6$). Additionally, HUVEC isolated from normal pregnancies ($n=4$) were used for transfection experiments.

6.2.2. QUANTIFICATION OF METHYLATION LEVELS IN *Occludin* PROMOTER

DNA isolation, MeDIP and qPCR were performed as described in Sections 2.12, 2.13 and 2.14 respectively.

6.2.3. MIRNAS EXPRESSION IN HUMAN PLACENTAL TISSUE

The selection process for miRNAs that target *Occludin* expression was performed as described in Section 2.19. RNA isolation, miRNA reverse transcription and qPCR quantification were performed as described in Sections 2.6, 2.18 and 2.19 respectively. miRNA expression was analysed as described in Sections 2.11 using *U6* sn-RNA gene as a control.

6.2.4. QUANTIFICATION OF *KLF6* GENE EXPRESSION IN PLACENTAL TISSUE

RNA isolation, reverse transcription and qPCR were performed as described in Sections 2.6, 2.7 and 2.9. *KLF6* was quantified with the following primers: forward 5'-CTCTGGAGGAGTACTGGCAAC-3'; reverse 5'-CCACAGATCTTCCTGGCTGTC-3'. Briefly, qPCR reactions were run in triplicate in a final volume of 15 μ l with a final concentration of: 1X (7.5 μ l) SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma), 300 nM (0.45 μ l) primers, 1 mM (3 μ l) betaine, plus 1.5 μ l of cDNA (1:16 dilution). Detection was performed using the Rotor-Gene 6000 cyclers (Corbett Research). The thermal profile included 95°C for 15 minutes followed by 40 cycles of 95°C

for 15 seconds, annealing at 60°C for 20 seconds, and elongation at 72°C for 30 seconds.

6.2.5. OVEREXPRESSION OF *MIRNA-181A-5P* IN HUVEC

Transfection was performed as described in Section 2.20.

6.2.6. QUANTIFICATION OF *OCCUDIN* AND *KLF6* GENE EXPRESSION IN TRANSFECTED HUVEC (*MIR-181A-5P* OVEREXPRESSION)

RNA isolation and qPCR were performed as described in Sections 2.20 and 3.2.1 respectively, with minor modifications. qPCR detection was performed using the AriaMX Real-Time PCR System (Agilent Technologies). Reference genes were selected for HUVEC, as described in Section 2.10. The expression of *SDHA* and *TBP* were used as a normalisation factor for the expression of *Occludin* splice variants and *KLF6* in HUVEC.

6.2.7. PROTEIN IMMUNOPRECIPITATION

Protein isolation, immunoprecipitation and Western blot were performed as described in Sections 2.15, 2.17 and 2.16 respectively. VE-cadherin antibodies were used to standardise the IP protocol, as Occludin IP was unsuccessful (using Occludin or phosphorylated antibodies). Table 6.1 details the different protocols used to precipitate Occludin protein.

6.2.8. STATISTICAL ANALYSIS

The data was tested for normality distribution by D'Agostino & Pearson test with a confidence interval of 95%. Results from *Occludin* promoter methylation, miRNAs expression, and *Occludin* and *KLF6* gene expression in transfected HUVEC presented a normal distribution. Hence, statistical comparisons were performed by independent samples *t*-test and one-way ANOVA. Results from *KLF6* gene expression in placental tissue, and *miR-181a-5p* expression in transfected HUVEC failed to be normally distributed. Therefore, statistical comparisons were performed by Mann-Whitney test and Kruskal-Wallis test.

Table 6.1. Immunoprecipitation protocol modifications. A detailed list of the different combinations of primary antibodies, magnetic beads, buffers and incubations used during the troubleshooting of Occludin IP from placental samples.

Antibody	Beads	IP Buffer	Incubation	Elution Buffer
OCLN (71-1500)	Dynabeads	10µM Na-Phosphate pH 7.0; 140 mM NaCl; 0.05% Triton X-100	Sample + antibody + beads at 4°C for 2hs	100 µM glycine pH 2.6
			Sample + antibody 4°C overnight. Add beads and incubate at 4°C for 2hs	100 µM glycine pH 2.6
			Sample + antibody 4°C overnight. Add beads and incubate at 4°C for 2hs	100 mM glycine pH 2.5
			Sample + antibody 4°C overnight. Add beads and incubate at 4°C for 2hs	6x Laemmli buffer
		PBS, 0.1% BSA, 2mM EDTA	Sample + antibody + beads at 4°C overnight.	0.1 M citrate
	Protein A PureProt.	PBS 0.1% Tween 20 pH 7.4	Sample + antibody at 4°C overnight. Collect supernatant and add antibody; incubate 4°C overnight. Add beads and incubate at 4°C for 2hs	0.2 M glycine pH 2.5
	Protein G PureProt.	PBS 0.1% Tween 20 pH 7.4	Sample + antibody at 4°C overnight. Add beads and incubate at 4°C for 2hs	0.2 M glycine pH 2.5
	Protein G Sepharose	TBS (50mM Tris, 150mM NaCl pH 7.5)	Sample + beads at 4°C for 1h (pre-clear). Supernatant + antibody and incubate 4°C overnight. Add beads and incubate at 4°C for 1h	0.2 M glycine pH 2.5
		TBS (50mM Tris, 150mM NaCl pH 7.5) + 2M urea	Sample + denaturing buffer at 95°C for 10 minutes. Afterwards, add ice cold IP buffer + Crosslinked beads and incubate at 4°C overnight	0.2 M glycine pH 2.5

Table 6.1. Immunoprecipitation protocol modifications. Continue.

Antibody	Beads	IP Buffer	Incubation	Elution Buffer
OCLN (ab167161)	Protein G Sepharose	TBS (50mM Tris, 150mM NaCl pH 7.5)	Sample + beads at 4°C for 1h (pre-clear). Supernatant + antibody and incubate 4°C overnight. Add beads and incubate at 4°C for 1h	0.1 M glycine HCl pH 2.9 + 2 M urea
			Crosslink step, incubate beads and antibody at 4°C. Add samples and incubate 4°C overnight	0.1 M glycine HCl pH 2.9 + 2 M urea
OCLN (ab216327)	Protein G Sepharose	TBS (50mM Tris, 150mM NaCl pH 7.5)	Sample + beads at 4°C for 1h (pre-clear). Supernatant + antibody and incubate 4°C overnight. Add beads and incubate at 4°C for 1h	0.2 M glycine pH 2.5
OCLN (sc-133256)	Protein G Sepharose	TBS (50mM Tris, 150mM NaCl pH 7.5)	Sample + beads at 4°C for 1h (pre-clear). Supernatant + antibody and incubate 4°C overnight. Add beads and incubate at 4°C for 1h	0.1 M glycine HCl pH 2.9 + 2 M urea
		TBS (50mM Tris, 150mM NaCl pH 7.5) + 2M urea	Sample and denaturing buffer at 95°C for 10 minutes. Add ice cold IP buffer and Crosslinked beads; incubate at 4°C overnight	0.2 M glycine pH 2.5
Phospho- Serine (9606)	Protein G Sepharose	TBS (50mM Tris, 150mM NaCl pH 7.5)	Sample + beads at 4°C for 1h (pre-clear). Supernatant + antibody and incubate 4°C overnight. Add beads and incubate at 4°C for 1h	0.2 M glycine pH 2.5
Phospho- Tyrosine (9411)	Protein G Sepharose	TBS (50mM Tris, 150mM NaCl pH 7.5)	Sample + beads at 4°C for 1h (pre-clear). Supernatant + antibody and incubate 4°C overnight. Add beads and incubate at 4°C for 1h	0.2 M glycine pH 2.5

6.3. RESULTS

6.3.1. STABLE LEVELS OF DNA METHYLATION OF *OCLUDIN* PROMOTER IN PLACENTAL SAMPLES BETWEEN NORMAL AND GDM PREGNANCIES

Occludin promoter DNA methylation levels were similar between normal and GDM placental samples ($t(17) = .51, p = .620$) (Figure 6.3A). Likewise, no statistical difference was found when comparing the DNA methylation levels of *Occludin* promoter between placental samples from normal, diet-controlled and metformin-controlled GDM pregnancies ($F(2, 16) = 1.96, p = .173$) (Figure 6.3B). However, a Post hoc comparison indicated that the methylation levels for diet-controlled GDM placental samples tended to be higher than metformin-controlled GDM ($t(9) = 2.11, p = .065$). No statistical difference was found in the DNA methylation levels of *Occludin* promoter between placental samples from normal pregnancies and diet-controlled GDM pregnancies ($t(12) = 1.29, p = .222$) or metformin-controlled GDM samples ($t(11) = .705, p = .495$). Additionally, our results showed two distinct subgroups within the diet-controlled GDM samples. Same was found for the placental samples from normal pregnancies. These subgroups cannot be due to altered maternal clinical characteristics as women from both groups (normal and GDM) have similar maternal BMI, maternal age, and gestational age at delivery.

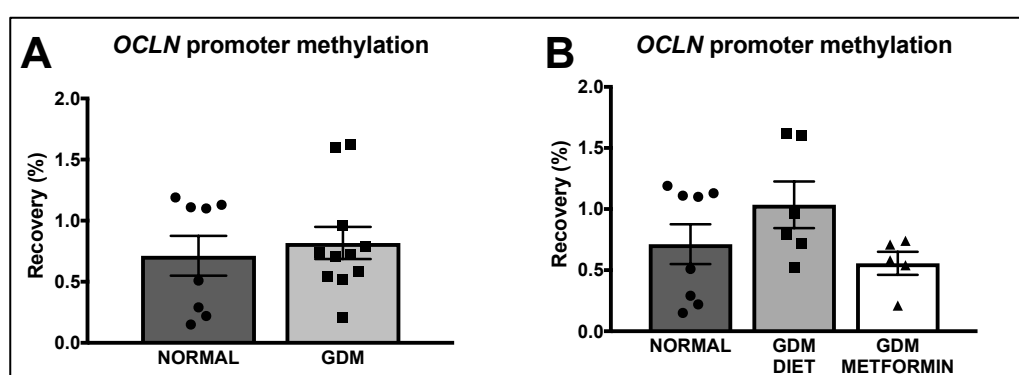


Figure 6.3. *Occludin* promoter DNA methylation was similar between normal and GDM placental samples. Placental samples were taken from normal pregnancies ($n = 9$), and pregnancies complicated with GDM where glucose levels were managed with diet ($n = 7$) or metformin ($n = 6$). **(A)** *Occludin* promoter methylation levels between normal and GDM placental samples. **(B)** *Occludin* promoter methylation levels between normal, diet-controlled and metformin-

controlled GDM placental samples. Mean $\pm$ SEM. One-way ANOVA and post hoc Unpaired t-test.

6.3.2. FIVE POTENTIAL miRNAs THAT TARGET *Occludin* GENE EXPRESSION

miRNAs with the highest score were selected for quantification in human placental samples from normal and GDM pregnancies. A positive (1) or negative (0) score was given to potential miRNAs according to:

- miRbase database information of annotation confidence
- miRTarBase database information of gene target and validation method
- DIANA database information of gene target probability (miTG score)
- Literature reports of the miRNA interacting with Occludin
- Literature reports of the miRNA expression in a diabetic environment
- Literature reports of the miRNA expression in human placental tissue

Five miRNAs were selected. Table 6.2 describes the bioinformatics score and the facts found in literature linking *miR-18a-5p*, *miR-21-3p*, *miR-122-5p*, *miR181a-5p*, and *miR-340-5p* to *Occludin* expression, diabetes, and human placenta.

Table 6.2. Five microRNAs, with potential regulatory effect on *Occludin* expression, were selected for this study based on bioinformatics and literature information. DIANA database gives a miTG score that determines the probability of the miRNA to target the gene of interest (higher miTG Score, higher probability of targeting)

microRNA	miTG Score	Literature Occludin	Literature Diabetes	Literature Placenta
<i>miR-18a-5p</i>	0.492	- Regulates <i>Occludin</i>, <i>ZO-1</i> and <i>claudin-5</i> expression by downregulation of <i>RUNX1</i> (Miao <i>et al.</i>, 2015) - Regulates <i>Occludin</i>, <i>ZO-1</i> and <i>claudin-5</i> expression by downregulation of <i>KLF4</i> (Y. Y. Zhao <i>et al.</i>, 2015)	Upregulated in GDM (Tagoma <i>et al.</i>, 2018)	Expression reported in human placental samples (Y. Gu <i>et al.</i>, 2013; Jing Li <i>et al.</i>, 2015; X. Zhu <i>et al.</i>, 2009)
<i>miR-21-3p</i>	0.791	<i>miR-21</i> downregulates <i>Occludin</i> and <i>ZO-1</i> expression (Yongzhi Yang <i>et al.</i>, 2013)	- Lower levels of <i>miR-21</i> in T2DM (C. Zhao <i>et al.</i>, 2011) - Increased <i>miR-21</i> levels in diabetic retinopathy, and glomerular tissue (Prattichizzo <i>et al.</i>, 2015)	Expression reported in human placental samples (Y. Gu <i>et al.</i>, 2013; Luo <i>et al.</i>, 2009; Maccani <i>et al.</i>, 2011)
<i>miR-122-5p</i>	0.972	<i>miR-122</i> downregulates <i>Occludin</i> expression in liver, helping HCV replication (Elhelw <i>et al.</i>, 2017) <i>miR-122</i> downregulates <i>Occludin</i> expression, leading to an increase in intestinal permeability (Ye <i>et al.</i>, 2011)	Downregulated in T2DM (Z. Zhu <i>et al.</i>, 2017)	Expression reported in exosomes of human placental explants (Nair <i>et al.</i>, 2018)

Table 6.2. Five microRNAs, with potential regulatory effect on *Occludin* expression, were selected for this study based on bioinformatics and literature information. Continue

microRNA	miTG Score	Literature Occludin	Literature Diabetes	Literature Placenta
<i>miR-181a-5p</i>	N/A	<i>miR-181a-5p</i> regulates <i>Occludin</i>, <i>ZO-1</i> and <i>claudin-5</i> expression by downregulation of <i>KLF6</i> (J. Ma <i>et al.</i>, 2014).	Upregulated in T2DM (B. Zhou <i>et al.</i>, 2012)	Expression reported in human placental samples (Y. Hu <i>et al.</i>, 2009; Kane <i>et al.</i>, 2012; Mayor-Lynn <i>et al.</i>, 2011)
<i>miR-340-5p</i>	0.984	N/A	Upregulated in GDM (Stirm <i>et al.</i>, 2018)	Expression reported in exosomes of human placental explants (Nair <i>et al.</i>, 2018)

N/A: Not applicable

6.3.3. INCREASED *MIR-181A-5P* EXPRESSION IN GDM PLACENTAL SAMPLES

qPCR results for *miR-122-5p*, and *miR-340-5p* could not be analysed as these miRNAs presented very low expression, making it difficult to obtain accurate measurements of expression. Thus, we focused on the expression of *miR-18a-5p*, *miR-21-3p*, and *miR181a-5p* whose expression was high enough to enable reliable quantification.

miR-18a-5p expression was similar in placental samples between normal and GDM pregnancies ($t(17) = 1.08$, $p = .296$) (Figure 6.4A). Likewise, no statistical difference was found when comparing *miR-18a-5p* expression between placental samples from normal, diet-controlled and metformin-controlled GDM pregnancies ($F(2, 16) = 1.32$, $p = .293$) (Figure 6.4B). Furthermore, a Post hoc comparison found no statistical difference in *miR-18a-5p* expression between placental samples from normal pregnancies and diet-controlled GDM samples ($t(12) = 1.51$, $p = .156$) or metformin-controlled GDM samples ($t(11) = .224$, $p = .827$). Additionally, our results showed two distinct subgroups within the diet-controlled GDM samples. These subgroups cannot be due to altered maternal clinical characteristics as women from the diet-controlled GDM group have similar maternal BMI, maternal age, and gestational age at delivery. Furthermore, in normal and metformin-controlled GDM placental groups, outliers resemble the expression found in the upper subgroup of the diet-controlled GDM samples. As for diet-controlled GDM, the outliers were independent from maternal clinical characteristics.

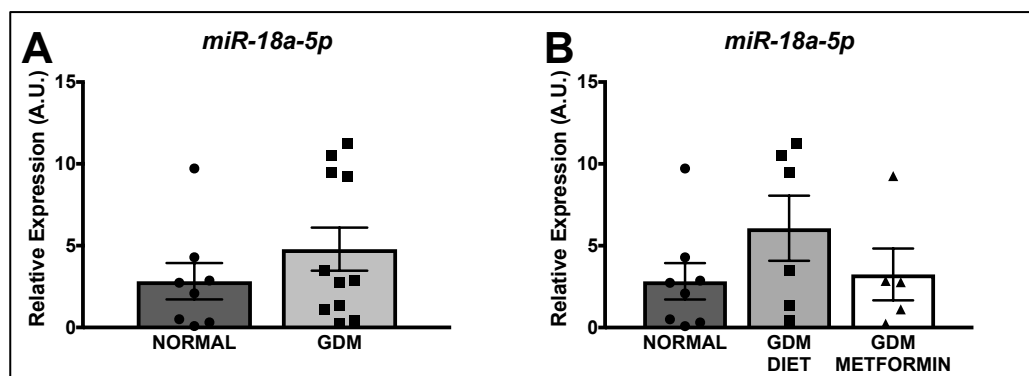


Figure 6.4. *miR-18a-5p* expression was similar between normal and GDM placental samples. Placental samples were taken from normal pregnancies (n= 9), and pregnancies complicated with GDM where glucose levels were managed with diet (n= 7) or metformin (n= 6). **(A)** *miR-18a-5p* expression between normal and GDM placental samples. **(B)** *miR-18a-5p* expression between normal, diet-controlled and metformin-controlled GDM placental samples. Mean $\pm$ SEM. One-way ANOVA and post hoc Unpaired t-test.

miR-21-3p expression was similar in placental samples between normal and GDM pregnancies ($t(19) = .04, p = .971$) (Figure 6.5A). Likewise, no statistical difference was found when comparing *miR-21-3p* expression between placental samples from normal, diet-controlled and metformin-controlled GDM pregnancies ($F(2, 18) = .25, p = .780$) (Figure 6.5B). Furthermore, a Post hoc comparison indicated that *miR-21-3p* expression of normal placental samples did not differ from diet-controlled GDM samples ($t(13) = .35, p = .730$), or metformin-controlled GDM samples ($t(13) = .37, p = .717$). Additionally, our results showed two distinct subgroups within the metformin-controlled GDM samples. Same was found for the placental samples from normal pregnancies. These subgroups cannot be due to altered maternal clinical characteristics as women from both groups (normal and GDM) have similar maternal BMI, maternal age, and gestational age at delivery.

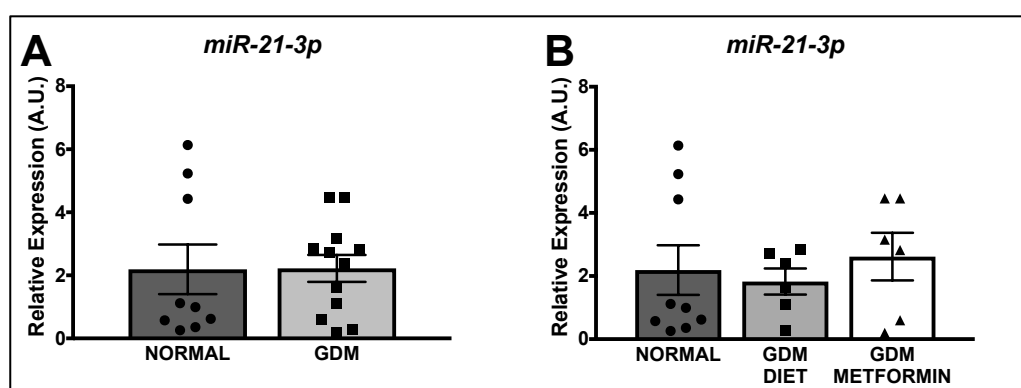


Figure 6.5. *miR-21-3p* expression was similar between normal and GDM placental samples. Placental samples were taken from normal pregnancies (n= 9), and pregnancies complicated with GDM where glucose levels were managed with diet (n= 7) or metformin (n= 6). **(A)** *miR-21-3p* expression between normal and GDM placental samples. **(B)** *miR-21-3p* expression between normal, diet-controlled and metformin-controlled GDM placental samples. Mean $\pm$ SEM. One-way ANOVA and post hoc Unpaired t-test.

miR-181a-5p expression in placental samples was higher in GDM samples compared to normal samples ($t(19) = 2.27, p = .035$) (Figure 6.6A). However, no statistical difference was found when comparing *miR-181a-5p* expression between placental samples from normal, diet-controlled and metformin-controlled GDM pregnancies ($F(2, 18) = 2.53, p = .108$) (Figure 6.6B). Nonetheless, a Post hoc comparison indicated that *miR-181a-5p* expression for diet-controlled GDM placental samples was significantly higher than normal samples ($t(13) = 2.25, p = .043$). No statistical differences were found in *miR-181a-5p* expression between normal placental samples and metformin-controlled GDM placental samples ($t(13) = 1.74, p = .105$). Additionally, our results showed at least two distinct subgroups within the GDM samples. Same was found for the placental samples from normal pregnancies. These subgroups cannot be due to altered maternal clinical characteristics as women from both groups (normal and GDM) have similar maternal BMI, maternal age, and gestational age at delivery.

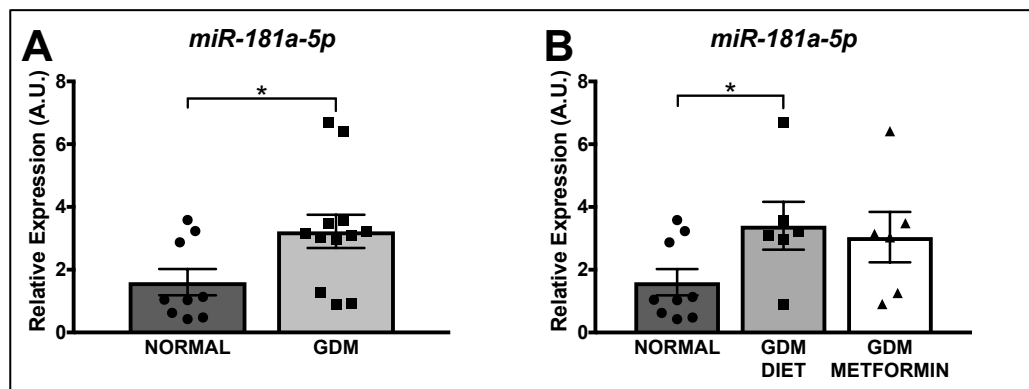


Figure 6.6. *miR-181a-5p* expression was higher in GDM placental samples compared to normal samples. Placental samples were taken from normal pregnancies ($n = 9$), and pregnancies complicated with GDM where glucose levels were managed with diet ($n = 7$) or metformin ($n = 6$). **(A)** *miR-181a-5p* expression between normal and GDM placental samples. **(B)** *miR-181a-5p* expression between normal, diet-controlled and metformin-controlled GDM placental samples. Mean $\pm$ SEM. One-way ANOVA and post hoc Unpaired t-test ($*p < 0.05$).

6.3.4. SIMILAR *KLF6* GENE EXPRESSION IN PLACENTAL SAMPLES BETWEEN NORMAL AND GDM PREGNANCIES

Previous studies have found that *miR-181a-5p* regulates *Occludin* expression by altering *KLF6* gene expression (J. Ma *et al.*, 2014). As higher expression of *miR-181a-5p* was found in GDM placental samples, we thus determined *KLF6* expression in placental samples.

KLF6 gene expression was similar in placental samples between normal and GDM pregnancies ($U = 54.0$, $p = .794$) (Figure 6.7A). Likewise, no statistical difference was found when comparing *KLF6* gene expression in placental samples from normal, diet-controlled and metformin-controlled GDM pregnancies ($H = .16$, $p = .926$) (Figure 6.7B).

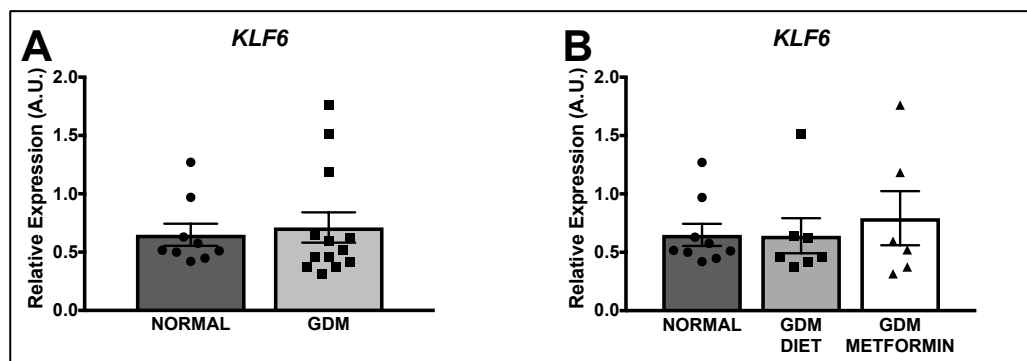


Figure 6.7. *KLF6* expression was similar between normal and GDM placental samples. Placental samples were taken from normal pregnancies (n= 9), and pregnancies complicated with GDM where glucose levels were managed with diet (n= 7) or metformin (n= 6). **(A)** *KLF6* expression between normal and GDM placental samples. **(B)** *KLF6* expression between normal, diet-controlled and metformin-controlled GDM placental samples. Mean $\pm$ SEM. Kruskal Wallis test and Mann-Whitney post hoc test.

6.3.5. OVEREXPRESSION OF *MIR-181A-5P* IN TRANSFECTED HUVEC

Based on our results, we wanted to confirm the effect of *miR-181a-5p* on *Occludin* gene expression. Hence, a transfection experiment was set up with HUVEC isolated from normal pregnancies. We confirmed the efficacy of the transfection by comparing *miR-181a-5p* expression in HUVEC under untreated control, transfection control, negative control and HUVEC under *miR-181a-5p* transfection conditions ($H = 9.62$, $p = .007$) (Figure 6.8).

miR-181a-5p expression was unchanged in HUVEC between untreated control and transfection control ($U = 3.0, p = .200$), or negative control ($U = 7.0, p = .886$) conditions. *miR-181a-5p* expression was higher in transfected HUVEC compared to untreated controls ($U = 0.0, p = .029$), transfection control ($U = 0.0, p = .029$), and negative control ($U = 0.0, p = .029$).

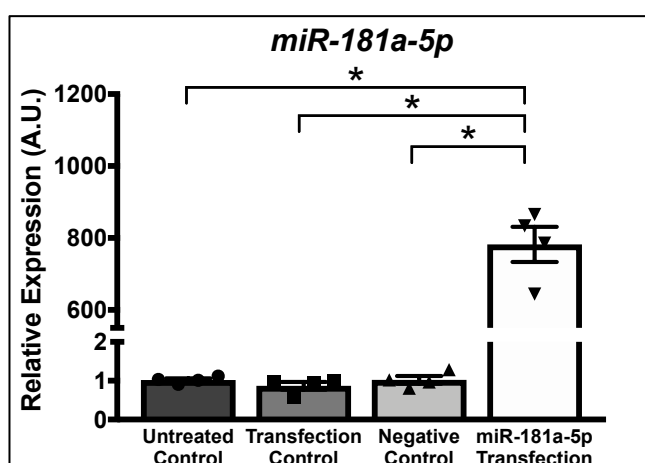


Figure 6.8. Higher expression of *miR-181a-5p* in transfected HUVEC. HUVEC isolated from normal pregnancies ($n = 4$) were subject to transfection conditions for 48-hours. qPCR results of *miR-181a-5p* expression between HUVEC subjected to untreated control, transfection control (no mimic), transfection of negative control (15 η M negative control mimic), and *miR-181a-5p* transfection (15 η M *miR-181a-5p* mimic). Mean $\pm$ SEM of four independent experiments, each performed in triplicates. Kruskal Wallis test and Mann-Whitney post hoc test. (* $p < 0.05$).

6.3.6. OVEREXPRESSION OF *MIR-181A-5P* DOWNREGULATES THE GENE EXPRESSION OF *OCLUDIN VARIANTS 1 AND 2* IN TRANSFECTED HUVEC

A statistical difference was found in *Occludin variant 1* gene expression between HUVEC under untreated control, transfection control, negative control and *miR-181a-5p* transfection conditions ($F(3, 12) = 8.01, p = .003$) (Figure 6.9). Overexpression of *miR-181a-5p* significantly decreased *Occludin variant 1* gene expression in HUVEC compared to untreated control ($t(6) = 3.74, p = .009$), transfection control ($t(6) = 4.52, p = .004$), and negative control ($t(6) = 9.19, p < .0001$). No statistical differences were found between controls.

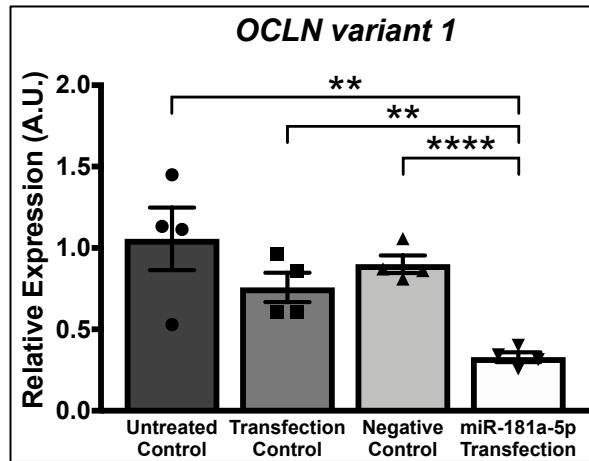


Figure 6.9. Occludin variant 1 gene expression was downregulated in HUVEC overexpressing *miR-181a-5p*. HUVEC isolated from normal pregnancies ($n= 4$) were subject to transfection conditions for 48-hours. qPCR results of *Occludin variant 1* expression between HUVEC subjected to untreated control, transfection control (no mimic), transfection of negative control (15 η M negative control mimic), and *miR-181a-5p* transfection (15 η M *miR-181a-5p* mimic). Mean $\pm$ SEM of four independent experiments, each performed in triplicates. One-way ANOVA and post hoc Unpaired t-test (** $p < 0.01$; **** $p < 0.0001$).

An analysis of variance showed that the expression of *Occludin variant 2* was statistically different between HUVEC under untreated control, transfection control, negative control and *miR-181a-5p* transfection conditions ($F(3, 12) = 4.754, p = .021$) (Figure 6.10). Overexpression of *miR-181a-5p* significantly decreased *Occludin variant 2* gene expression in HUVEC compared to untreated control ($t(6) = 3.85, p = .009$), and transfection control ($t(6) = 3.21, p = .018$). *Occludin variant 2* gene expression in HUVEC overexpressing *miR-181a-5p* did not differ from HUVEC cultured under negative control conditions ($t(6) = 1.29, p = .245$). No statistical differences were found between controls.

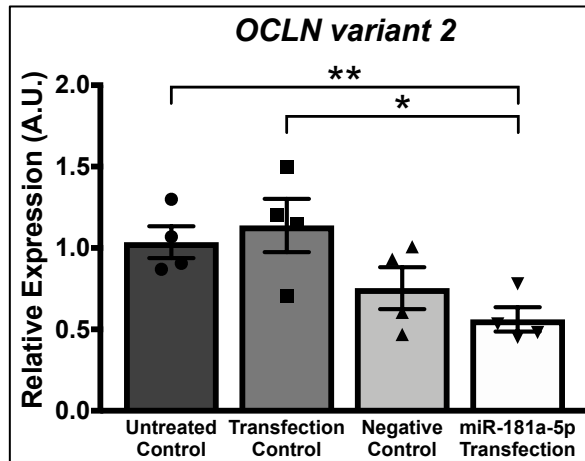


Figure 6.10. Occludin variant 2 gene expression was downregulated in HUVEC overexpressing *miR-181a-5p*. HUVEC isolated from normal pregnancies ($n= 4$) were subject to transfection conditions for 48-hours. qPCR results of *Occludin variant 2* expression between HUVEC subjected to untreated control, transfection control (no mimic), transfection of negative control (15 η M negative control mimic), and *miR-181a-5p* transfection (15 η M *miR-181a-5p* mimic). Mean $\pm$ SEM of four independent experiments, each performed in triplicates. One-way ANOVA and post hoc Unpaired t-test (* $p < 0.05$; ** $p < 0.01$).

There were no statistical differences in *Occludin variant 3* gene expression in HUVEC under untreated control, transfection control, negative control and *miR-181a-5p* transfection conditions ($F(3, 12) = .817$, $p = .509$) (Figure 6.11). Overexpression of *miR-181a-5p* did not affect *Occludin variant 3* gene expression in HUVEC compared to untreated control ($t(6) = 1.02$, $p = .347$), transfection control ($t(6) = 2.02$, $p = .089$), and negative control ($t(6) = .343$, $p = .743$). No statistical differences were found between controls.

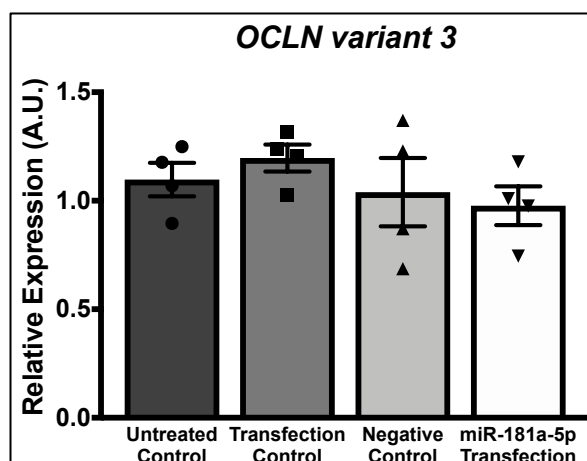


Figure 6.11. *Occludin variant 3* gene expression was unchanged in HUVEC overexpressing *miR-181a-5p*. HUVEC isolated from normal pregnancies ($n= 4$) were subject to transfection conditions for 48-hours. qPCR results of *Occludin variant 3* expression between HUVEC subjected to untreated control, transfection control (no mimic), transfection of negative control (15 η M negative control mimic), and *miR-181a-5p* transfection (15 η M *miR-181a-5p* mimic). Mean $\pm$ SEM of four independent experiments, each performed in triplicates. One-way ANOVA and post hoc Unpaired t-test

6.3.7. OVEREXPRESSION OF *MIR-181A-5P* DOES NOT AFFECT *KLF6* GENE EXPRESSION IN TRANSFECTED HUVEC

An analysis of variance showed that the expression of *KLF6* was unchanged between HUVEC under untreated control, transfection control, negative control and *miR-181a-5p* transfection conditions ($F(3, 12) = 1.02$, $p = .419$) (Figure 6.12). However, there was a tendency for lower *KLF6* gene expression between HUVEC overexpressing *miR-181a-5p* and untreated control conditions ($t(6) = 2.43$, $p = .051$). Furthermore, overexpression of *miR-181a-5p* did not affect the gene expression of *KLF6* in HUVEC compared to transfection control ($t(6) = 1.23$, $p = .265$), and negative control ($t(6) = 1.59$, $p = .164$). No statistical differences were found between controls.

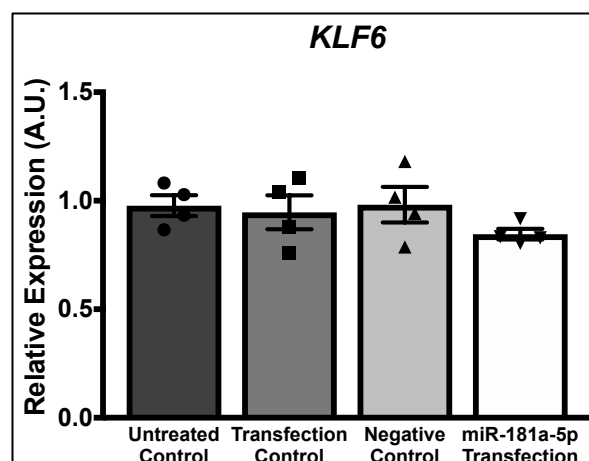


Figure 6.12. *KLF6* gene expression was unchanged in HUVEC overexpressing *miR-181a-5p*. HUVEC isolated from normal pregnancies ($n= 4$) were subject to transfection conditions for 48-hours. qPCR results of *KLF6* expression between HUVEC subjected to untreated control, transfection control (no mimic), transfection of negative control (15 η M negative control mimic), and *miR-181a-5p* transfection (15 η M *miR-181a-5p* mimic). Mean $\pm$ SEM of four independent experiments, each performed in triplicates. One-way ANOVA and post hoc Unpaired t-test

6.3.8. TECHNICAL CHALLENGES IN THE IMMUNOPRECIPITATION OF OCCLUDIN FROM PLACENTAL PROTEIN SAMPLES

Using different protocols, several attempts were made to precipitate Occludin protein from placental samples (Figure 6.13). Occludin IP was unsuccessful, even after the use of different Occludin primary antibodies, magnetic beads, and modifications to the protocol (Table 6.1). Additionally, samples presented a residual from the antibody used for IP, hence a negative control for rabbit IgG was used to differentiate between Occludin isoform A band (~60 kDa) and the IgG heavy chain band (~55 kDa). This IgG heavy chain presented a strong band with most of the antibodies. This band was stronger than the Occludin band present in the control samples. Due to the unsuccessful precipitation of Occludin with its antibodies, protein immunoprecipitation was attempted with phosphorylation antibodies: Phospho-Tyrosine (Figure 6.13G) and Phospho-Serine (Figure 6.13H). Like before, Occludin protein precipitation was unsuccessful.

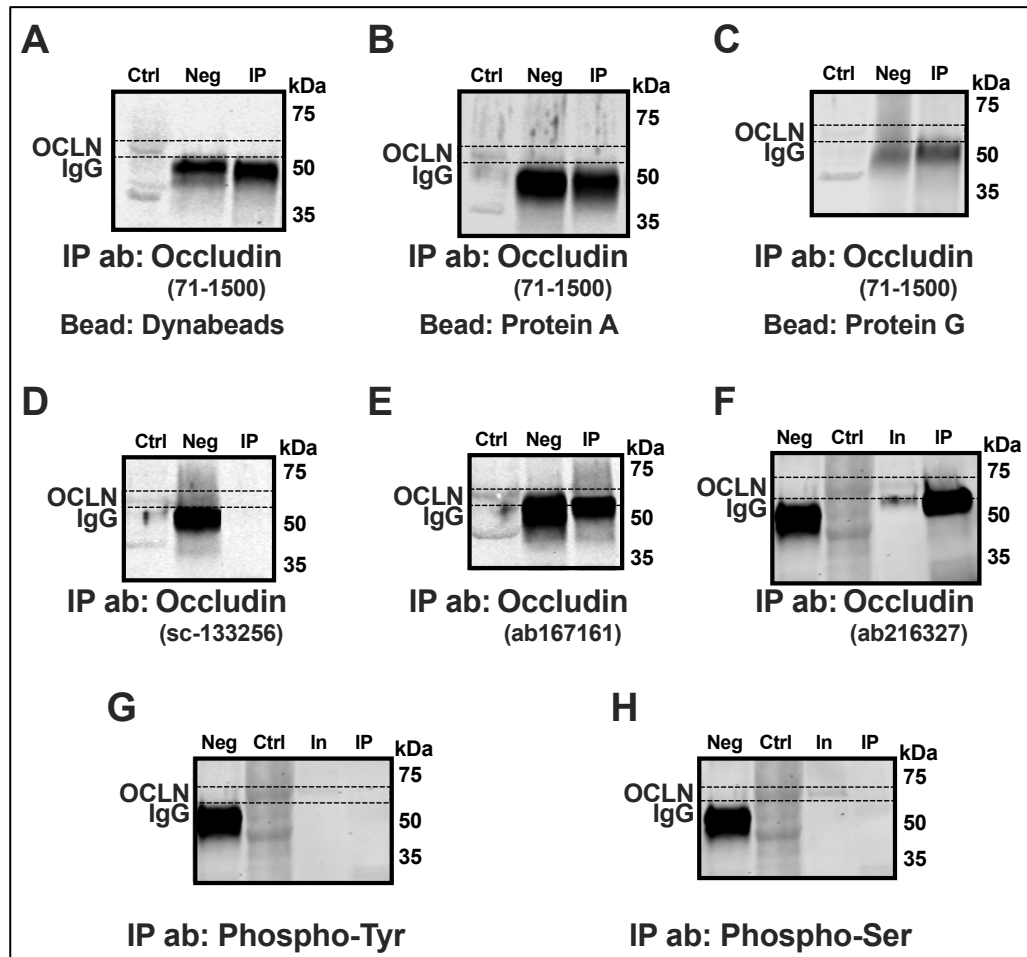


Figure 6.13. Representation of Occludin protein immunoprecipitation attempts. Placental total protein samples from normal pregnancies were used to optimise Occludin immunoprecipitation by various methods. Western blot membranes were incubated with polyclonal rabbit anti-Occludin (71-1500). Occludin isoform A band (~60 kDa) and the IgG heavy chain band (~55 kDa). **(A)** Occludin rabbit polyclonal (71-1500) antibody with Dynabeads magnetic beads were used for IP. **(B)** Occludin rabbit polyclonal (71-1500) antibody with Protein A magnetic beads were used for IP. **(C)** Occludin rabbit polyclonal (71-1500) antibody with Protein G magnetic beads were used for IP. **(D)** Occludin mouse monoclonal (sc-133256) antibody with Protein G magnetic beads were used for IP. **(E)** Occludin rabbit monoclonal (ab167161) antibody with Protein G magnetic beads were used for IP. **(F)** Occludin rabbit monoclonal (ab216327) antibody with Protein G magnetic beads were used for IP. **(G)** Phospho-Tyrosine mouse monoclonal (9411) antibody with Protein G magnetic beads were used for IP. **(H)** Phospho-Serine mouse monoclonal (9606) antibody with Protein G magnetic beads were used for IP. Ctrl: control (sample without immunoprecipitation); Neg: negative control (IgG); In: supernatant after magnetic separation; IP: sample after immunoprecipitation.

Furthermore, we determined whether the problem with the precipitation of Occludin was due to a faulty protocol. We used VE-cadherin

primary antibody to test the IP protocol (Figure 6.14). The IP sample showed a lighter band of VE-cadherin isoform-1 (~125 kDa). Both negative control and IP sample presented the IgG heavy chain (~55 kDa). This result showed that the placental samples and the IP protocol can be used for protein precipitation. However, the commercially available Occludin antibodies are not good enough for precipitation of the protein. It could be that a modification in the protocol will enable the precipitation of Occludin with the current antibodies. Due to time constraints, no further attempts were made to precipitate Occludin from placental samples. Consequently, we could not quantify the phosphorylation levels of Occludin in placental samples.

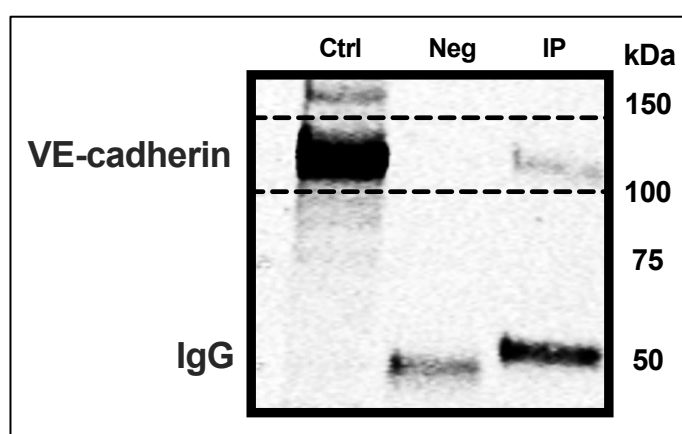


Figure 6.14. VE-cadherin protein immunoprecipitation from a placental sample from normal pregnancy. Placental total protein samples from normal pregnancies were used to precipitate VE-cadherin isoform-1 (~125 kDa). The IgG heavy chain band (~55 kDa) also present in negative control and IP sample. A dull band can be seen in the IP sample corresponding to VE-cadherin isoform-1 (~125 kDa). Ctrl: control (sample without immunoprecipitation); Neg: negative control (IgG); IP: sample after immunoprecipitation.

6.4. DISCUSSION

In this chapter, we explored whether the changes in Occludin expression and localisation found in placental samples from GDM pregnancies were linked to epigenetic modifications or protein phosphorylation. Higher expression of *miR-181a-5p* was found in diet-controlled GDM placental samples compared to controls. Additionally, overexpression of *miR-181a-5p* in HUVEC resulted in downregulation of

Occludin gene expression. There were no differences either in DNA methylation levels of *Occludin* promoter or in miRNA expression between metformin-controlled GDM placental samples and controls. Quantification of *Occludin* phosphorylation levels was unsuccessful due to technical complications in the immunoprecipitation of *Occludin* from placental samples.

Our study found a downregulation of *Occludin* gene expression in placental samples from diet-controlled GDM pregnancies. Previous studies have demonstrated that a downregulation of *Occludin* expression is caused by higher methylation levels of its promoter (J. M. Gu *et al.*, 2008; Osanai *et al.*, 2006). However, our results showed that the methylation levels of *Occludin* promoter were stable between normal and GDM placental samples. It is important to highlight that placental samples from metformin-controlled GDM pregnancies presented the lowest levels of DNA methylation, compared to normal (1.3-fold decrease) and diet-controlled GDM (1.9-fold decrease) samples. This could suggest that metformin may be downregulating DNA methylation levels of *Occludin* promoter. Further work is needed to determine if other epigenetic factors (e.g. histone modifications, non-coding RNAs) might be controlling *Occludin* gene expression. Additionally, further work is needed to confirm the effect of metformin over DNA methylation levels of *Occludin* promoter. Furthermore, our results presented two subgroups with different DNA methylation levels within the normal and diet-controlled GDM samples. We considered if these subgroups were linked to maternal clinical characteristics, but no correlations were found. This suggests that there could be a different factor controlling the levels of DNA methylation of *Occludin* promoter. A limitation of our study was that we did not include positive (fully methylated) and negative (non-methylated) controls to verify the specificity of the MeDIP. However, the MeDIP antibody (anti-5-methylcytosine) was previously validated in our laboratory (Jessop & Toledo-Rodriguez, 2018). In human placental studies, validation of MeDIP can be done by amplification of the methylated *testis-specific histone 2B*

(*HIST1H2BA*) as a positive control (Mitsuya *et al.*, 2017), and the unmethylated *ubiquitin-conjugating enzyme E2 B (UBE2B)* promoter as a negative control (Monk *et al.*, 2011). Future studies that aim to determine DNA methylation levels using MeDIP should also include controls to confirm the specificity of the protocol.

Furthermore, our study determined if a change in miRNAs expression was behind the downregulation of *Occludin* gene expression found in placental samples from diet-controlled GDM pregnancies. We selected five miRNAs that target *Occludin* expression: *miR-18a-5p*, *miR-21-3p*, *miR-122-5p*, *miR-181a-5p*, and *miR-340-5p*. Additionally, we confirmed that these miRNAs have been reported to be expressed in human placental samples. Studies have found *miR-18a-5p* (Y. Gu *et al.*, 2013; Jing Li *et al.*, 2015; X. Zhu *et al.*, 2009), *miR-21-3p* (Y. Gu *et al.*, 2013; Luo *et al.*, 2009; Maccani *et al.*, 2011), and *miR-181a-5p* (Y. Hu *et al.*, 2009; Kane *et al.*, 2012; Mayor-Lynn *et al.*, 2011) expressed in human placental samples. Moreover, *miR-122-5p* and *miR-340-5p* were found in exosomes of human placental explants of GDM pregnancies under hypoxic conditions (Nair *et al.*, 2018). Our study was not able to compare the expression of *miR-122-5p* and *miR-340-5p* in placental samples between normal and GDM pregnancies due to the low expression. As previously mentioned, the expression of these two miRNAs was previously quantified in exosomes of human placental explants (Nair *et al.*, 2018). Hence, it is possible that *miR-122-5p*, and *miR-340-5p* were lost during sample collection.

Our results showed unchanged expression of *miR-18a* in human placental samples between normal and GDM pregnancies. To our knowledge, no information regarding *miR-18a* expression in samples from GDM pregnancies have been published to date. Nonetheless, contradictory results have been reported for *miR-18a* expression in diabetic environments. For instance, a study found higher expression of *miR-18a* in retinal tissue of streptozotocin-induced diabetic rats compared to controls (Kovacs *et al.*, 2011); whereas lower expression of *miR-18a* was found in

human blood samples of T2DM patients compared to controls (Mao & Liu, 2014). Hence, a clear effect of diabetes over the expression of *miR-18a* cannot be established. Additionally, our results presented two subgroups with different *miR-18a* expression levels within the diet-controlled GDM samples. No correlations were found between maternal clinical characteristics and these subgroups. Therefore, there is a possibility that a different factor is altering the expression of these miRNA.

Our results showed stable expression of *miR-21-3p* in human placental samples between normal and GDM pregnancies. Contrary to our results, studies have found higher expression of *miR-21* in kidney biopsies from diabetic nephropathy patients (Fiorentino *et al.*, 2013), and serum samples from diabetic retinopathy patients (Qing *et al.*, 2014) compared to controls. These suggest that chronic diabetic conditions (e.g. diabetic nephropathy, retinopathy) might alter the expression of *miR-21-3p*. A GDM pregnancy might not provide the environment for a higher expression of this miRNA.

Human *miR-181a-5p* is located in chromosome 9, its expression was first predicted by computational methods with mouse sequences (L. P. Lim, 2003), and then verified in the human genome (Landgraf *et al.*, 2007; Lui *et al.*, 2007). Bioinformatic analyses have shown that *miR-181a-5p* targets different mRNAs codifying proteins related to apoptosis, cell cycle progression, cell cycle inhibition, proliferation and differentiation, cell survival, as well as insulin signal and resistance (Assmann *et al.*, 2017; He *et al.*, 2015). These pathways will affect the pathogenesis of cancer and diabetes. Regarding diabetes, there is no clear evidence that links the expression of *miR-181a-5p* to a diabetic environment. However, *miR-181a-5p* is upregulated in diabetes. For instance, studies have reported upregulation of *miR-181a-5p* expression in serum samples (B. Zhou *et al.*, 2012), skeletal muscle samples (Gallagher *et al.*, 2010), as well as adipose tissue samples (Klötting *et al.*, 2009) from T2DM patients compared to controls. Likewise, higher expression of *miR-181a* has been reported in

blood samples from T1DM patients (Nabih & Andrawes, 2016), and in human liver cells (HepG2) cultured under hyperglycaemic conditions (B. Zhou *et al.*, 2012) compared to controls. To our knowledge, there is no published information regarding *miR-181a-5p* expression in samples from pregnancies complicated with GDM. Hence, our study is the first one to report a 2.0-fold increase in expression of *miR-181a-5p* in placental samples from GDM pregnancies compared to control. When comparing between treatments, diet-controlled GDM placental samples presented 2.1-fold increase expression of this miRNA compared to normal samples. Hence, our results agree with the studies previously mentioned which used samples from T1DM and T2DM patients. Furthermore, a previous study demonstrated that *miR-181a-5p* downregulates *ZO-1*, *Occludin* and *claudin-5* gene expression by downregulation of *KLF6* (J. Ma *et al.*, 2014). Hence, we compared *KLF6* gene expression in placental samples from normal and GDM pregnancies. Our results showed that *KLF6* expression was similar between normal and GDM placental samples. Thus, further work was needed to determine if *miR-181a* was affecting the expression of *Occludin* in GDM placental samples. Additionally, *miR-181a-5p* expression was similar between placental samples from normal and metformin-controlled GDM pregnancies. Therefore, further work will be needed to determine if metformin affects the expression of *miR-181a-5p* in a diabetic environment. Moreover, as a previous study reported that *miR-181a-5p* downregulates *ZO-1* and *claudin-5* gene expression, future studies should compare the gene expression of *ZO-1* and *claudin-5* in placental samples from GDM pregnancies.

Our study set up a pilot experiment to overexpress *miR-181a-5p* in HUVEC from normal pregnancies. Transfection results showed that overexpression of *miR-181a-5p* downregulates *Occludin variant 1* (3.2-fold decrease) and *variant 2* (1.8-fold decrease) gene expression in HUVEC. Our results also showed that overexpression of *miR-181a* did not affect the expression of *KLF6* in HUVEC. These results open the possibility that *miR-181a* is a direct regulator of *Occludin* gene. Further work is needed to

determine the relation between *miR-181a-5p* and *Occludin* gene. Additionally, our study found that HUVEC present Occludin protein in the cytoplasm (Appendix B). Hence, we could not determine if overexpression of *miR-181-5p* will also affect the localisation of Occludin in the TJs. Future studies should determine the effects of *miR-181-5p* in Occludin protein expression and junctional localisation.

Our study aimed to compare Occludin phosphorylation levels in placental samples between normal and GDM pregnancies. To our knowledge, there is no published information regarding Occludin phosphorylation levels in human placental vasculature. However, despite having used tried and tested protocols and different Occludin antibodies, immunoprecipitation of Occludin was unsuccessful. Studies have shown that higher Occludin tyrosine phosphorylation will generate disassembly of the TJ and remove Occludin from the junctions (Kale *et al.*, 2003; L. Zhu *et al.*, 2006). Furthermore, studies in diabetic retinopathy have shown that phosphorylation of Occludin, by VEGF, generates a reduction in junctional localisation and an increase in barrier permeability (Antonetti *et al.*, 1999; Harhaj & Antonetti, 2004). Additionally, a study demonstrated that placental vessels from GDM pregnancies present higher tyrosine phosphorylation compared to controls (Babawale *et al.*, 2000). Based on these findings, we can speculate the loss of junctional Occludin from placental vessels found diet-controlled GDM samples was due to an increase phosphorylation on Occludin tyrosine residues. Further work is needed to determine Occludin phosphorylation levels in placental samples from GDM pregnancies.

In conclusion, we confirmed our hypothesis that *Occludin* gene expression could be altered by epigenetic factors. Methylation levels of *Occludin* promoter were not affected in GDM placental samples. However, increased expression of *miR-181a-5p* was reported in diet-controlled GDM samples. Overexpression of this miRNA in HUVEC showed downregulation of *Occludin* gene expression. These results confirm the effect of *miR-181a-*

5p over *Occludin* expression in human placental endothelial cells. Future studies should determine if *miR-181a-5p* overexpression affects the protein localisation of Occludin in TJs of endothelial cells. Additionally, there was no change in the expression of any miRNA in placental samples from metformin-controlled GDM pregnancies compared to controls. This suggests that metformin might have an effect through the normalisation of epigenetic changes resulting from increased glucose levels.

CHAPTER 7

GENERAL DISCUSSION

7.1. SUMMARY OF RESULTS

The main purpose of this study was to examine the effect of gestational diabetes (GDM) in the expression of junctional molecules in the human placental endothelial barrier. Furthermore, as a secondary goal, our study aimed to determine whether the treatment to control maternal glucose levels during a GDM pregnancy had an impact on the expression of junctional molecules.

Our study showed that GDM affected the gene and protein expression of Occludin, as well as its localisation in the junctions in placental vasculature (Figure 7.1). Additionally, total VEGF-A protein expression was downregulated in GDM placental samples compared to controls. The gene expression of *VEGF-A*, *Ang-1*, *Ang-2*, *VEGFR-2* and *Tie-2* remained unchanged between normal and GDM placental samples. Furthermore, placental explants exposed to an acute hyperglycaemic insult (15 mM D-glucose) were used as an *ex vivo* model of hyperglycaemia. Our study demonstrated that neither a short (3-hours) nor a long (24-hours) duration of exposure had an impact on Occludin gene expression and junctional localisation in placental vasculature. Moreover, GDM placental samples presented an upregulation of *miR-181a-5p* expression compared to controls. Overexpression of *miR-181a-5p* in HUVEC resulted in downregulation of *Occludin* gene expression. Thus, *miR-181a-5p* may be, in part, responsible for the Occludin downregulation found in GDM placental samples.

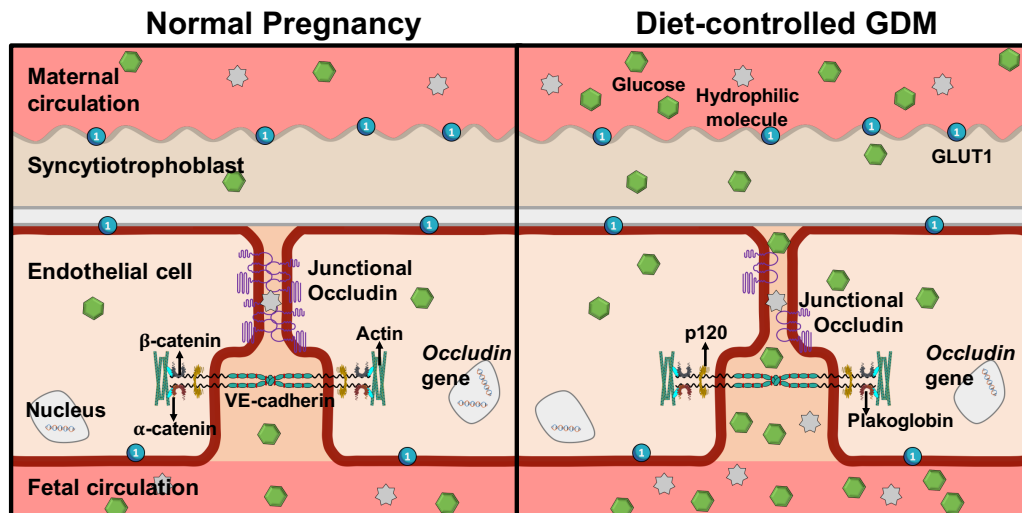


Figure 7.1. Summary of the results from this study. In normal pregnancies (left panel) Occludin (purple) expression and junctional localisation maintain the endothelial paracellular barrier integrity. Glucose (green) moves across the placental barriers through its transporter (GLUT1, blue) or the paracellular clefts. Hydrophilic molecules (grey) move across the placenta through the paracellular transport. In diet-controlled GDM pregnancies (right panel) Occludin gene and protein expression are downregulated. Paracellular barrier integrity is compromised, paracellular transport of hydrophilic molecules (including glucose) is increased to the fetal circulation.

Interestingly, these changes in expression were present only in placental samples from GDM pregnancies where glucose levels were controlled by a change of diet. When metformin was used to control maternal glucose levels, there was no significant change in the expression of the different molecules analysed in this study.

7.2. SIGNIFICANCE OF THE ENDOTHELIAL BARRIER IN THE PLACENTA

The placenta presents two cell barriers (syncytiotrophoblast and endothelium) that are important for nutrient and gas exchange, and waste removal between the maternal and the fetal circulation. The placental endothelium restricts the transport of hydrophilic molecules larger than 65 kDa across the paracellular barrier (Eaton *et al.*, 1993). Thus, alterations of this endothelial barrier will affect the overall trans-placental transport of solutes to the fetus. For instance, glucose transport across the endothelium can be either transcellular (i.e. through transporters GLUTs) or paracellular (Leach *et al.*, 1995; Leach & Firth, 1997; K. Takata & Hirano, 1997). A

disruption of the endothelial barrier will influence the rate of glucose transfer. In this regard, higher glucose uptake was reported for placental explants from GDM pregnancies compared to controls (Visiedo *et al.*, 2015).

In a GDM pregnancy, a disruption to this endothelial paracellular barrier could be, in part, responsible for some fetal complications related to this condition (e.g. hyperglycaemia, hyperinsulinemia, overgrowth, among others). In this regard, the 'Barker hypothesis', also known as the theory of developmental origins of health and disease (DOHAD), states that antenatal environment factors lead to alterations in fetal programming that can induce permanent biological and physiologic changes later in life (Barker *et al.*, 1989; Gluckman *et al.*, 2005). Hence, offspring from GDM pregnancies have a higher risk of developing diabetes later in life.

7.3. OCCLUDIN'S CONTRIBUTION TO THE INTEGRITY OF THE BARRIER

Occludin is a TJ molecule that regulates the permeability of the barrier. Our study found downregulation of Occludin expression (gene and protein), as well as reduced localisation of Occludin in the junctions of placental vasculature in samples from diet-controlled GDM pregnancies compared to controls. Our results are consistent with previous findings of reduced junctional Occludin levels in placental vasculature from insulin-controlled GDM pregnancies compared to controls (Babawale *et al.*, 2000). Moreover, our results increase the knowledge of the effect of GDM in Occludin gene and protein expression in placental vessels and confirm the expression of predicted *Occludin* gene splice variants in human placenta.

Furthermore, our results agree with the findings of previous studies investigating Occludin expression and localisation in non-placental tissues. For instance, downregulation of Occludin protein expression and junctional localisation was found in the endothelial membranes of retinal (Antonetti *et al.*, 1998; Barber & Antonetti, 2003), and kidney (Molina-Jijón *et al.*, 2014) samples from streptozotocin-induced diabetic rats compared to controls. Additionally, studies have shown lower Occludin protein levels

and increased BBB permeability in cerebral tissue from streptozotocin-induced diabetic rats compared to controls (Chehade *et al.*, 2002; Yoo *et al.*, 2016). These results emphasise the effects of diabetes over Occludin expression, and the importance of junctional Occludin for the maintenance of the barrier integrity.

It is important to highlight that the human placental vascular system is continuous with the vasculature of the developing fetus (Leach, 2011). Therefore, if Occludin expression is being affected in the placental vasculature, there might be a downregulation of Occludin expression in fetal microvasculature during development or later in life. Indeed, an Occludin-knockout study showed that the absence of Occludin expression during development resulted in complications as growth retardation and mineral deposits in the brain (Saitou *et al.*, 2000). Downregulation of Occludin during a GDM pregnancy will not have as drastic effects as knockdown. However, the offspring of GDM pregnancies presents an increased risk of metabolic and cardiovascular diseases. Hence, we could speculate that offspring from GDM pregnancies could also be prone to downregulation of Occludin from the endothelial barrier leading to complications as diabetic retinopathy or nephropathy.

Maternal vasculature could also be compromised during a GDM pregnancy. For instance, women with GDM pregnancies present retinal vasculature abnormalities compared to controls (L. J. Li, Kramer, *et al.*, 2017). Even if these abnormalities were not categorised as diabetic retinopathy, the authors suggest that GDM may cause small-vessel dysfunction in maternal vasculature. Additionally, women who had a pregnancy complicated with GDM present increased incidence of: metabolic syndrome (L. J. Li *et al.*, 2018), developing GDM in subsequent pregnancies, and developing T2DM (L. J. Li, Tan, *et al.*, 2017) within 5 years postpartum.

7.4. METFORMIN EFFECT ON THE STABILITY OF THE ENDOTHELIAL BARRIER

Metformin is a biguanide compound that works as an oral hypoglycaemic agent by increasing insulin sensitivity, reducing hepatic gluconeogenesis, and enhancing peripheral glucose uptake (Rena *et al.*, 2017). There are two mechanisms by which metformin acts: activation of AMP-activated protein kinase (AMPK) (G. Zhou *et al.*, 2001), and reduction of cyclic AMP (cAMP) levels and phosphorylation levels of PKA (cAMP-dependent protein kinase) (Miller *et al.*, 2013). The activation of AMPK will inhibit gluconeogenesis, promote fatty acid oxidation, and decrease lipid synthesis (Y. Li *et al.*, 2011). During pregnancy, metformin can cross the placenta, thus the drug is detected at similar concentrations in fetal and maternal circulating plasma (Charles *et al.*, 2006; Vanky *et al.*, 2005). Nevertheless, there is no accumulation of metformin in the placental tissue (Kovo *et al.*, 2008). The transport of metformin across the placenta is regulated by P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), multidrug resistance-associated protein (MRP1), and organic cation transporter (OCT3) (Hemauer *et al.*, 2010; Tertti *et al.*, 2010).

Metformin and AMPK are also involved in the maintenance of the endothelial paracellular barrier. For instance, rat brain capillary endothelial cells (RBECs) cultured with metformin (1 mM) showed up-regulation of BBB functions (resistance and permeability) via AMPK activation (F. Takata *et al.*, 2013). Furthermore, cell culture studies also determined the impact of a hyperglycaemic environment and the activation of AMPK on the permeability of the endothelial barrier as well as the expression of junctional molecules. Increased endothelial permeability and reduced protein expression of Occludin, ZO-1 and claudin-5 were also found when human brain microvascular endothelial cell (HBMEC) were cultured under hyperglycaemic (25 mM D-glucose) conditions; activation of AMPK upregulated the expression of TJ molecules and recovered endothelial integrity (C. Liu *et al.*, 2012). Similarly, HUVEC cultured under hyperglycaemic (25 mM D-glucose) (HG), lipopolysaccharides (1 ug/ml) (LPS), or both conditions (HG + LPS) showed downregulation of AMPK

phosphorylation levels, increase paracellular permeability, and increased VE-cadherin phosphorylation levels; upregulation of AMPK abolished these effects (Yan Yang *et al.*, 2017). Hence, considering the results from our study, the placental vasculature from metformin-controlled pregnancies could present higher levels of activated AMPK which could have regulated the expression and localisation of Occludin during gestation. Nonetheless, further work is needed to quantify AMPK phosphorylation levels in placental vasculature from normal and GDM pregnancies.

7.5. LIMITATIONS AND FUTURE WORK

We used the results obtained by Babawale and collaborators as a baseline to calculate the number of samples needed for our study (Babawale *et al.*, 2000). These power calculations estimated that, in order to obtain significant results, a minimum of seven samples per condition were needed. In this regard, we acknowledge that the metformin-controlled GDM group was under-powered ($n=6$). However, no statistical differences or tendencies were found between this group and the control group (normal pregnancies). Most of our results showed a tendency of change in the diet-controlled GDM group. This lack of statistical significance could be due to a large variability in our samples. The high variation within our samples could be due to factors not accounted for in our study (e.g. ethnicity, previous history of pregnancy complications, hypoxia, among others). Future studies should consider increasing the number of samples, as well as collecting maternal background information when comparing between normal and GDM pregnancies.

Our study broadened the knowledge of Occludin downregulation in a diabetic milieu. However, there are still unknowns that should be addressed:

- Future work should determine the role of metformin in Occludin expression and localisation. This could be done by using retinal pigmented epithelial (RPE) cells as an *in vitro* model of hyperglycaemia. RPE cells cultured under hyperglycaemic conditions (35 mM glucose)

presented a reduced Occludin protein expression and junctional localisation (Ved *et al.*, 2017). Hence, a study could determine if metformin can rescue Occludin expression and localisation from the effects of a hyperglycaemic milieu.

- Future work should compare MMP-9 proteolytic activity in placental samples between normal and GDM pregnancies. The use of gelatine zymography gel could determine the enzymatic levels of MMP-9. Elevated MMP-9 proteolytic activity could be responsible for the downregulation of Occludin protein expression found in our study.
- Future work should quantify the tyrosine phosphorylation levels of Occludin protein in placental samples between normal and GDM pregnancies. As specific antibodies that target Occludin-tyrosine phosphorylation are not commercially available, quantification of Occludin phosphorylation levels will be dependent on an efficient immunoprecipitation protocol. Increased Occludin-tyrosine phosphorylation levels could be responsible for the reduced junctional Occludin localisation in placental vasculature found in our study.
- Further work is needed to determine the localisation of Occludin isoform-B in endothelial cells. Occludin isoform-A and isoform-B present a 100% identity, as both isoforms include the last two domains (transmembrane and C-terminal domains) of Occludin protein. Hence, the use of antibodies for an immuno-location experiments will not differentiate between both isoforms. Therefore, transfection of *Occludin variant 3* gene with a GFP tag could help follow the translation of Occludin isoform-B and trace its localisation in cells.
- Future work should compare VE-cadherin phosphorylation levels in placental vessels from normal and GDM pregnancies. VE-cadherin phosphorylation levels can be determined using commercially available antibodies by Western blot. Increased phosphorylation of VE-cadherin could be responsible for the reduced localisation of VE-cadherin in placental vasculature from T1DM, T2DM and insulin-controlled GDM pregnancies compared to controls (Babawale *et al.*, 2000; Baumüller *et al.*, 2015; Leach *et al.*, 2004).

- Further work is needed to determine Plakoglobin protein expression and localisation in placental samples. Additionally, it is important to determine Plakoglobin participation in AJs or desmosomes. Co-immunoprecipitation experiments could help elucidate how much Plakoglobin is binding to VE-cadherin and Desmoglein in placentae.
- Future work should determine if inhibition of *miR-181a-5p* expression will restore *Occludin* gene expression in endothelial cells. Transfection of *miR-181a-5p* inhibitor on HUVEC could be used to block the expression of the microRNA. Afterwards, Occludin expression can be quantified by qPCR and Western blot.
- Future work is needed to confirm whether metformin affects *miR-181a-5p* expression in endothelial cells (HUVEC), as metformin-controlled placental samples did not show overexpression of *miR-181a-5p*.

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APPENDIX A

CONFIRMATION OF PRIMERS SPECIFICITY – SEQUENCING RESULTS

A.1. Occludin variant 1

Primers:

F: 5'- TGAATTGGTCACCGAGGGAG-3'

R: 5'- TAAACCAATCTGCTGCGTCCTA-3'

Consensus sequence (157 bp):

5'- TTGAATTGGTCACCGAGGGAGGAGGCCGAAYWYWCCMCMCSTAC
ACTYCCGCGTCCACCTCTCCCTCCCTGCTTCCTCTGGCGGAGGCGGCAG
GAACCGAGAGCCAGGTCYATTRATGYRCCGAGSATKCCGGTCTAGGAC
GCAGCAGATTGGTTTAA -3'

BLAST results:

Occludin variant 1 was the only confirmed splice variant that was found, which ratifies the specificity of the primers (Table A.1). Alignment to *Occludin variant 1* covered most of the consensus sequence (156 of 157 bp) (Figure A.1.A). Alignment to the predicted *Occludin variant X2* showed a shorter coverage of the consensus sequence (116 of 157 bp) (Figure A.1.B).

Table A.1. BLAST results for *Occludin (OCLN) variant 1* qPCR primers. Detailed information of Transcript, Total score, Percentage of query cover, Expect value, Percentage of identity, Number of matches (Mat.), and Accession number. *Occludin* X splice variants are predicted using gene prediction method: Gnomon.

Transcript	Total Score	Query Cover	Expect Value	Identity	Mat.	Accession
<i>OCLN var 1</i>	196	98%	4.0E-48	87.82%	1	NM_002538.3
<i>OCLN X2</i>	160	77%	3.0E-37	88.62%	1	XM_017008914.2

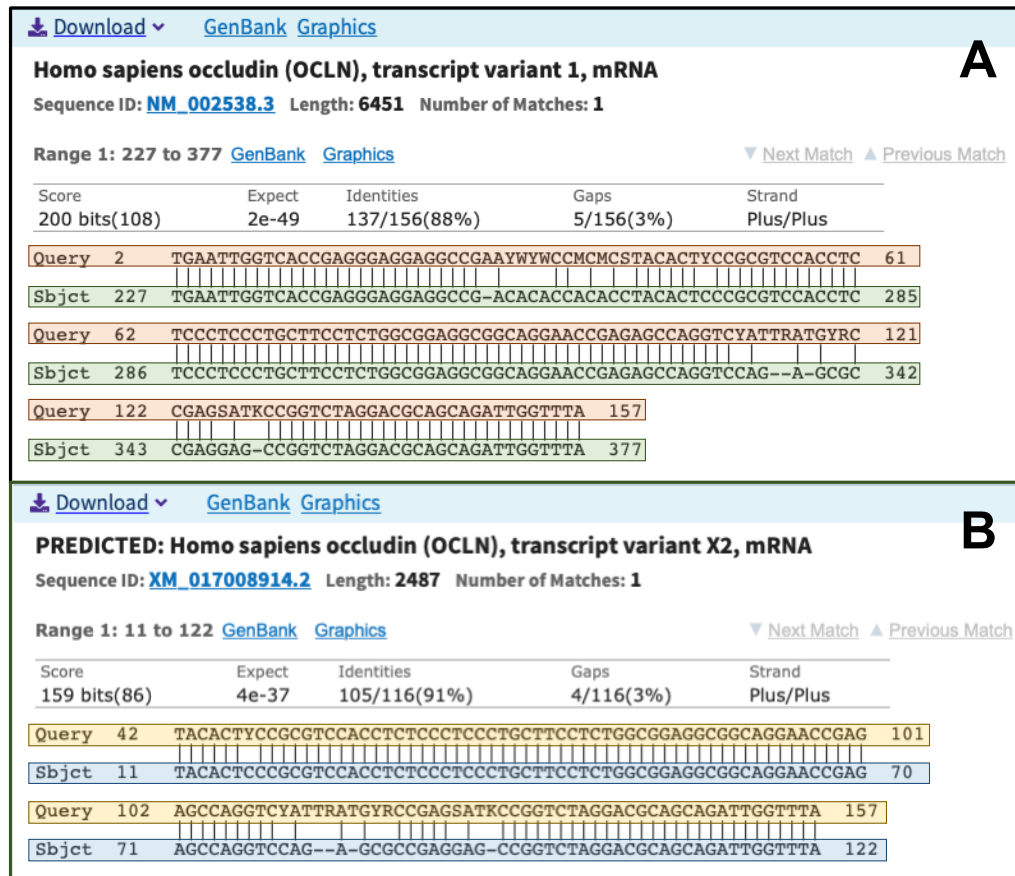


Figure A.1. BLAST alignment for Occludin variant 1 qPCR primers. (A) Alignment of the sequencing consensus results (Query; orange) extends from 2 to 157 bp (98% cover) and matches *Occludin variant 1* (Sbjct; green) with an 88% identity and 3% gaps between sequences. **(B)** Alignment of the sequencing consensus results (Query; yellow) extends from 42 to 157 bp (77% cover) and matches the predicted *Occludin variant X2* (Sbjct; blue) with an 91% identity and 3% gaps between sequences.

A.2. OCCLUDIN VARIANT 2

Primers:

F: 5'- GGCGAGCGGATTGGTTTAT-3'

R: 5'- AAGGAGGTGGACTTTCAAGAGG-3'

Consensus sequence (121 bp):

5'- CCCAGTTGCGGCGAGCGGATTGGTTTATCTTGGAAGCTAAAGGGCA
TTGCTCATCMTGWAGATCAGCTGACCATTGACRAKSAGCCATGTCAT
CCAGGCCTCTTGAAAGTCCACCTCCTTA -3'

BLAST results:

Three confirmed *Occludin* splice variants were identified (Table A.2). From these, only *Occludin variant 2* showed 100% query cover (all 121 bp), corroborating the specificity of the primers (Figure A.2).

Table A.2. BLAST results for *Occludin (OCLN) variant 2* qPCR primers. Detailed information of Transcript, Total score, Percentage of query cover, Expect value, Percentage of identity, Number of matches (Mat.), and Accession number. *Occludin* X splice variants are predicted using gene prediction method: Gnomon.

Transcript	Total Score	Query Cover	Expect Value	Identity	Mat.	Accession
<i>OCLN var 2</i>	206	100%	3.0E-51	95.87%	1	NM_001205254.1
<i>OCLN X1</i>	206	100%	3.0E-51	95.87%	1	XM_017008913.2
<i>OCLN var 1</i>	176	89%	3.0E-42	94.44%	1	NM_002538.3
<i>OCLN var 3</i>	172	85%	3.0E-41	95.15%	1	NM_001205255.1
<i>OCLN X2</i>	176	89%	3.0E-42	94.44%	1	XM_017008914.2

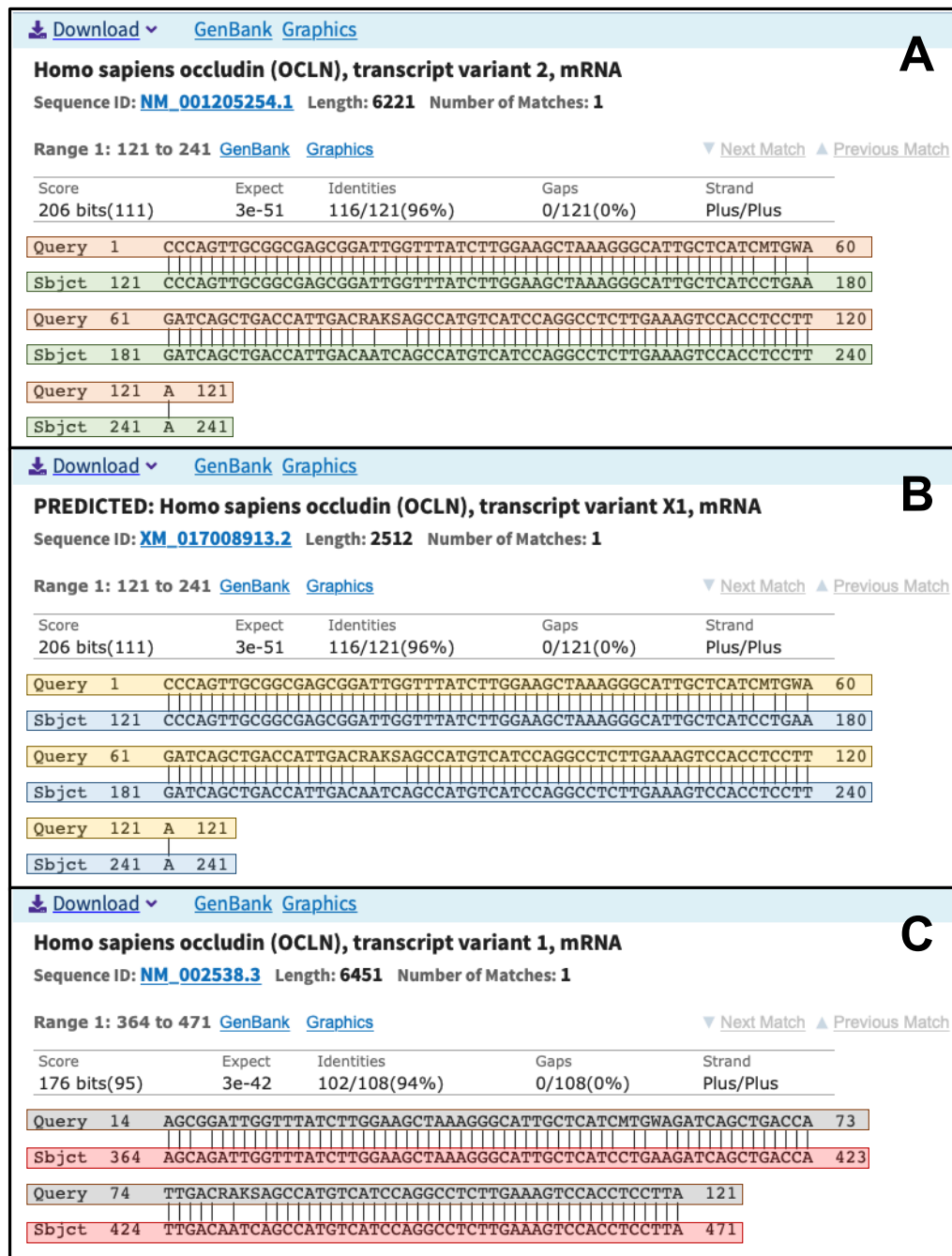


Figure A.2. BLAST alignment for Occludin variant 2 qPCR primers. (A) Alignment of the sequencing consensus results (Query; orange) extends from 1 to 121 bp (100% cover) and matches *Occludin variant 2* (Sbjct; green) with an 96% identity and 0% gaps between sequences. (B) Alignment of the sequencing consensus results (Query; yellow) extends from 1 to 121 bp and matches the predicted *Occludin variant X1* (Sbjct; blue) with an 96% identity and 0% gaps between sequences. (C) Alignment of the sequencing consensus results (Query; grey) extends from 14 to 121 bp (89% cover) and matches *Occludin variant 1* (Sbjct; red) with an 94% identity and 0% gaps between sequences.

A.3. OCCLUDIN VARIANT 3

Primers:

F: 5'- CCTTACAGGCCTGATGAATTGC-3'

R: 5'- TGTCCATCTTTCTTCGAGTTTTC-3'

Consensus sequence (108 bp):

5'- CCTTACAGGCCTGATGAATTGCCATTGCCATTGTACTGGGGTTCAT
GATTATTGTGGCTTTTGCTTTAATAATTTTCTTTGCTGTGAAAACG
AAGAAAGATGGACA -3'

BLAST results:

Three confirmed *Occludin* splice variants presented 100% query cover and identity (Table A.3). From these, only *Occludin variant 3* showed an only match confirming the specificity of the primers (Figure A.3).

Table A.3. BLAST results for *Occludin (OCLN) variant 3* qPCR primers. Detailed information of Transcript, Total score, Percentage of query cover, Expect value, Percentage of identity, Number of matches, and Accession number. *Occludin X* splice variants are predicted using gene prediction method: Gnomon.

Transcript	Total Score	Query Cover	Expect Value	Identity	Mat.	Accession
<i>OCLN var 3</i>	214	100%	8.0E-54	100%	1	NM_001205255.1
<i>OCLN var 2</i>	215	100%	7.0E-42	100%	2	NM_001205254.1
<i>OCLN var 1</i>	215	100%	7.0E-42	100%	2	NM_002538.3
<i>OCLN X2</i>	40.1	18%	2.8E-01	100%	1	XM_017008914.2
<i>OCLN X1</i>	40.1	18%	2.8E-01	100%	1	XM_017008913.2

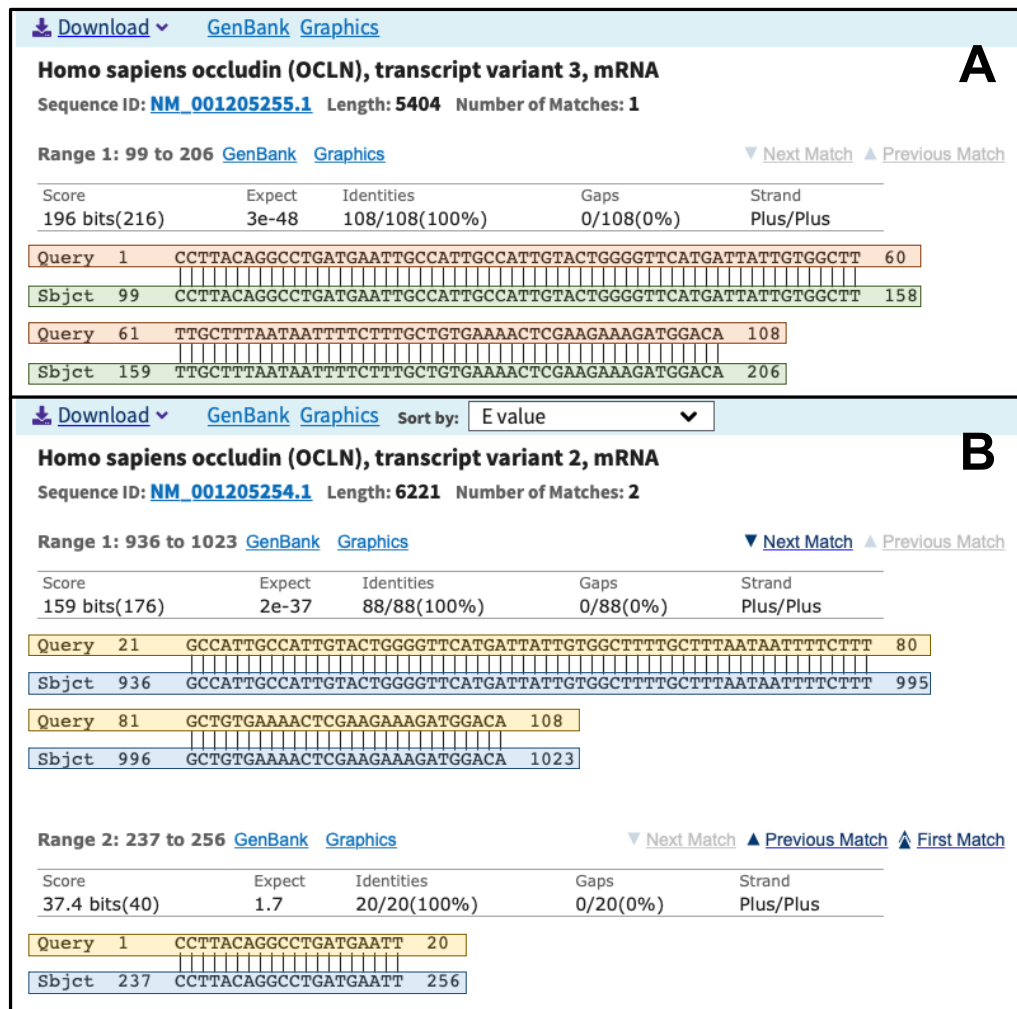


Figure A.3. BLAST alignment for Occludin variant 3 qPCR primers. (A) Alignment of the sequencing consensus results (Query; orange) extends from 1 to 108 bp (100% cover) and matches *Occludin variant 3* (Sbjct; green) with an 100% identity and 0% gaps between sequences. **(B)** Alignment of the sequencing consensus results (Query; yellow) presents two matches: the first one extends from 21 to 108 bp and the second one extends from 1 to 20 bp; these matches *Occludin variant 2* (Sbjct; blue) with an 100% identity and 0% gaps between sequences. The gap between these two matches is equivalent to the missing exon 3 (680 bp) in *Occludin variant 3*.

A.4. VE-CADHERIN VARIANTS 1, 2, 3

Primers:

F: 5'- TGGTAGCCATCTTACTCTGCAT-3'

R: 5'- GAGTTGAGCACCGACACATC-3'

Consensus sequence (201 bp):

5'- GGCAGTGGTAGCCATCTTACTCTGCATCCTCACATCACAGTGATC
ACCCTGCTCATCTKMCTGCGGCGGCGGCTMCGGAAGCAGGCCWGGCGC
GCASGGCAAGAGCGTGMCKGAGATCCACKAGCAGCTGGTCACSTACGA
CGAGKAGGGCGGMGGCGAGATGGACACCACCAGCTACGATGTGTCGG
TGCTCAACTCGGT -3'

BLAST results:

Three confirmed *VE-cadherin* splice variants presented 100% query cover and 94% identity (Table A.4; Figure A.4). *VE-cadherin variant 4* was not detected with these primers, which ratifies the specificity of the designed primers.

Table A.4. BLAST results for *VE-cadherin (VE-cad)* variants 1, 2 and 3 qPCR primers. Detailed information of Transcript, Total score, Percentage of query cover, Expect value, Percentage of identity, Number of matches, and Accession number. *VE-cadherin* X splice variants are predicted using gene prediction method: Gnomon.

Transcript	Total Score	Query Cover	Expect Value	Identity	Mat.	Accession
<i>VE-cad var 3</i>	326	100%	2.0E-92	94.03%	1	ENST00000539168.1
<i>VE-cad var 2</i>	326	100%	2.0E-92	94.03%	1	ENST00000614547.4
<i>VE-cad var 1</i>	326	100%	2.0E-92	94.03%	1	NM_001795.4
<i>VE-cad var X1</i>	326	100%	2.0E-92	94.03%	1	XM_011522801.2
<i>VE-cad var X2</i>	326	100%	2.0E-92	94.03%	1	XM_024450133.1

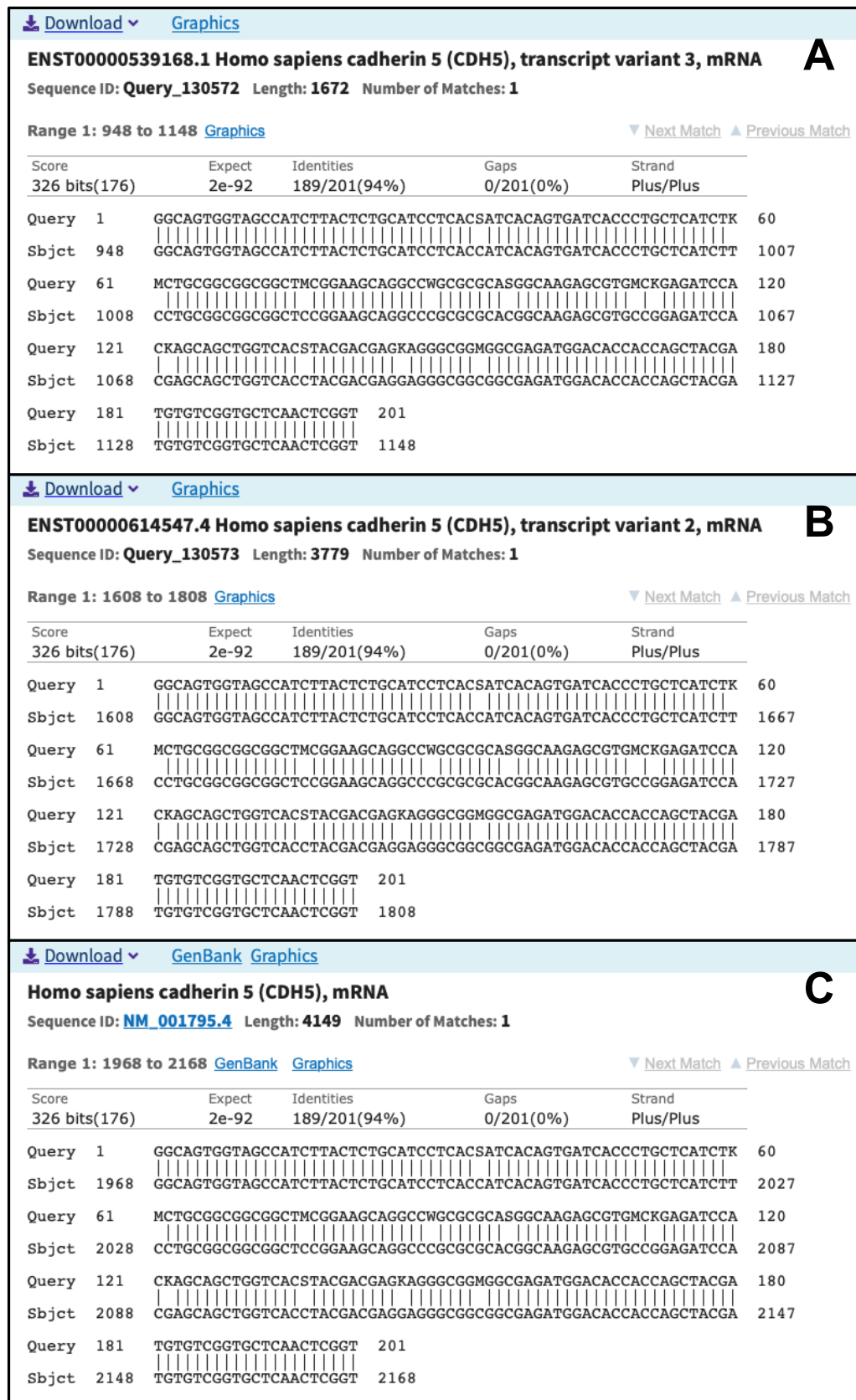


Figure A.4. BLAST alignment for *VE-cadherin* variants 1, 2 and 3 qPCR primers. Alignment of the sequencing consensus results (Query) extends from 1 to 201 bp (100% cover) and matches *VE-cadherin* variant 3 **(A)**, *VE-cadherin* variant 2 **(B)**, and *VE-cadherin* variant 1 **(C)**, with an 94% identity and 0% gaps between sequences.

A.5. VE-CADHERIN VARIANTS 1, 2, 4

Primers:

F: 5'- CCAGCTTCACCATCAAAGTTCA-3'

R: 5'- GGCAAATACTCTTTCCCCTTC-3'

Consensus sequence (227 bp):

5'- GAGACTCCTTCCAGCTTCACCATCAAAGTTCATGWCCTGAACGAC
ATACTGGCCTCGTGTTACGCATCGRTTGTTTCMATGCGTCCGTGCTCT
AGAGTCGTCGGCTGTCGGGGACCTCRGTCATCTGTGATCAGYAGTG
GATGCAGACGMCCCCACGTGTGGGAGACCACGCCCKCTGTCATGTACCA
AATCCTGAAGGGGAAAGAGTATTTTGCCATCGATAATT -3'

BLAST results:

Three confirmed *VE-cadherin* splice variants presented 100% query cover and 94% identity (Table A.5; Figure A.5). *VE-cadherin variant 3* was not detected with these primers, which ratifies the specificity of the designed primers.

Table A.5. BLAST results for *VE-cadherin (VE-cad)* variants 1, 2 and 4 qPCR primers. Detailed information of Transcript, Total score, Percentage of query cover, Expect value, Percentage of identity, Number of matches, and Accession number. *VE-cadherin* X splice variants are predicted using gene prediction method: Gnomon.

Transcript	Total Score	Query Cover	Expect Value	Identity	Mat.	Accession
<i>VE-cad var 4</i>	348	100%	6.0E-99	93.83%	1	ENST00000563425.2
<i>VE-cad var 2</i>	348	100%	6.0E-99	93.83%	1	ENST00000614547.4
<i>VE-cad var 1</i>	348	100%	6.0E-99	93.83%	1	NM_001795.4
<i>VE-cad var X1</i>	348	100%	6.0E-99	93.83%	1	XM_011522801.2
<i>VE-cad var X2</i>	348	100%	6.0E-99	93.83%	1	XM_024450133.1

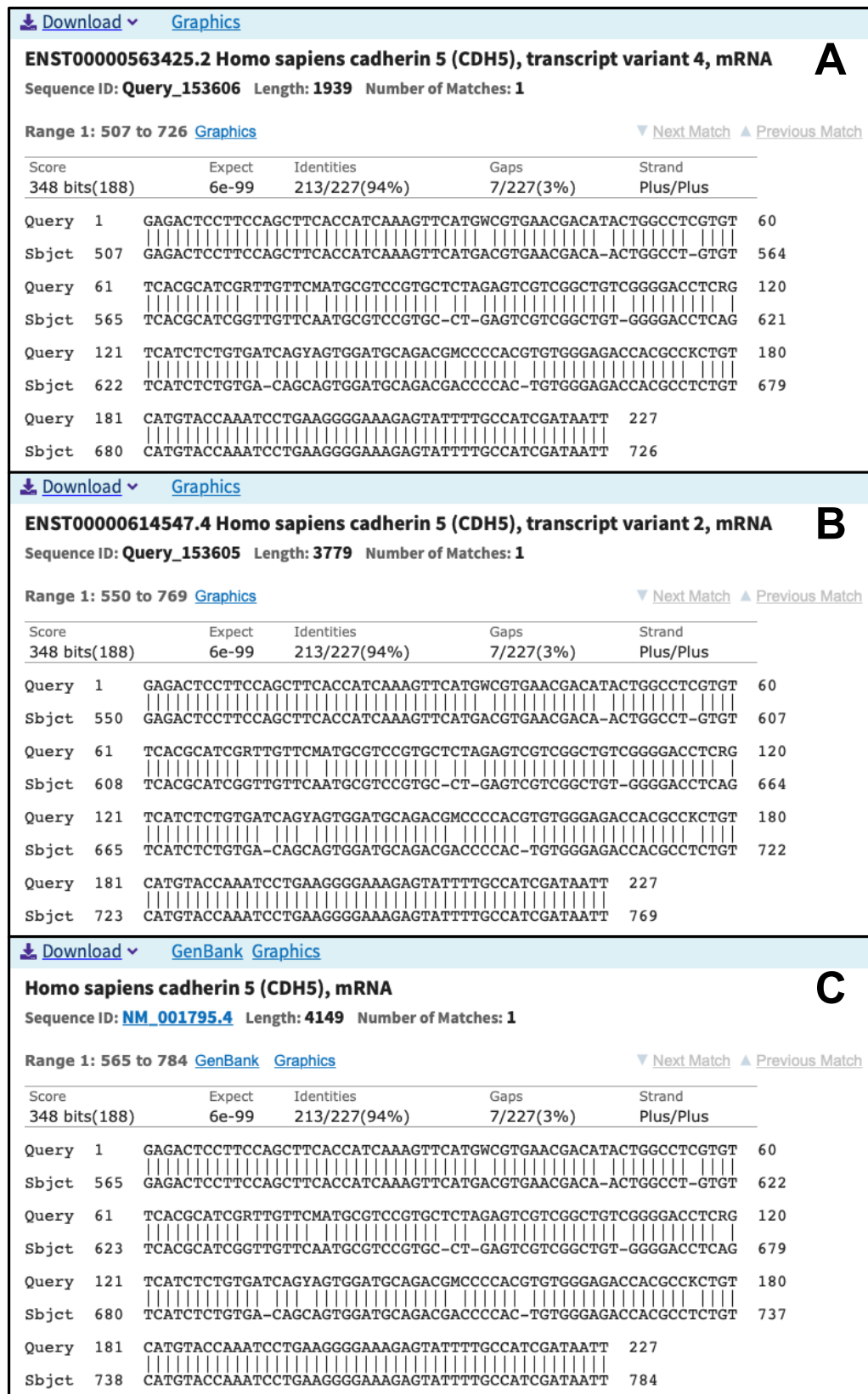


Figure A.5. BLAST alignment for *VE-cadherin* variants 1, 2 and 4 qPCR primers. Alignment of the sequencing consensus results (Query) extends from 1 to 227 bp (100% cover) and matches *VE-cadherin* variant 4 (**A**), *VE-cadherin* variant 2 (**B**), and *VE-cadherin* variant 1 (**C**), with an 94% identity and 3% gaps between sequences.

A.6. PLAKOGLOBIN

Primers:

F: 5'- AGTACACGCTCAAGAAAACCAC-3'

R: 5'- CTGACACACCAGGGCACAT-3'

Consensus sequence (130 bp):

5'- AGTACACGCTCAAGAAAACCACCACTTACACCCAGGGGGTGCCCCC
CAGCCAAGGTGACCTGGAGTACCAGATGTCCACAACAGCCAGGGCCAA
ACGGGTGCGGGAGGCCATGTGCCCTGGTGTGTCAGA -3'

BLAST results:

Seven confirmed *Plakoglobin* splice variants presented 99% query cover and identity, which corroborates the specificity of the primers (Table A.6; Figure A.6).

Table A.6. BLAST results for *Plakoglobin (JUP)* splice variants qPCR primers. Detailed information of Transcript, Total score, Percentage of query cover, Expect value, Percentage of identity, Number of matches, and Accession number. *Plakoglobin* X splice variants are predicted using gene prediction method: Gnomon.

Transcript	Total Score	Query Cover	Expect Value	Identity	Mat.	Accession
<i>JUP var 3</i>	233	99%	2.0E-59	99.22%	1	NM_001352773.1
<i>JUP var 6</i>	233	99%	2.0E-59	99.22%	1	NM_001352776.1
<i>JUP var 5</i>	233	99%	2.0E-59	99.22%	1	NM_001352775.1
<i>JUP var 4</i>	233	99%	2.0E-59	99.22%	1	NM_001352774.1
<i>JUP var 1</i>	233	99%	2.0E-59	99.22%	1	NM_002230.3
<i>JUP var 7</i>	233	99%	2.0E-59	99.22%	1	NM_001352777.1
<i>JUP var 2</i>	233	99%	2.0E-59	99.22%	1	NM_021991.3
<i>JUP var X2</i>	233	99%	2.0E-59	99.22%	1	XM_006721874.3
<i>JUP var X1</i>	233	99%	2.0E-59	99.22%	1	XM_017024588.2
<i>JUP var X3</i>	233	99%	2.0E-59	99.22%	1	XM_011524753.2

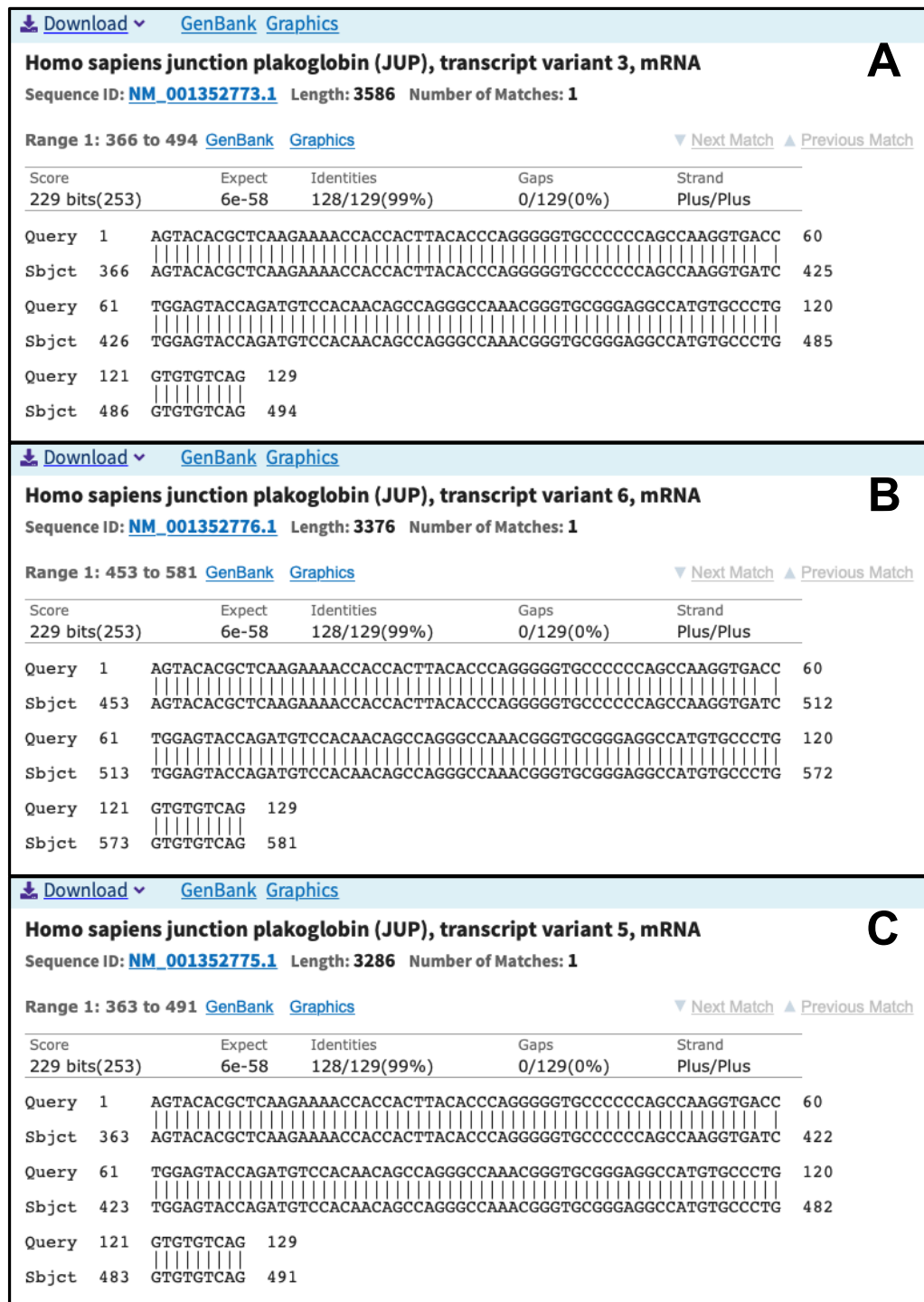


Figure A.6. Example of BLAST alignment for *Plakoglobin* variants 3, 5 and 6 qPCR primers. Alignment of the sequencing consensus results (Query) extends from 1 to 129 bp (99% cover) and matches *Plakoglobin* variant 3 (A), *Plakoglobin* variant 6 (B), and *Plakoglobin* variant 5 (C), with an 99% identity and 0% gaps between sequences.

A.7. VEGF-A

Primers:

F: 5'- ATCTTCAAGCCATCCTGTGTGC -3'

R: 5'- TATGTGCTGGCCTTGGTGAG -3'

Consensus sequence (140 bp):

5'- TATCTTCAAGCCATCCTGTGTGCCCTGATGCGATGCGGGGGCTGC
TGCAATGACGAGGGCCTGGAGTGTGTGCCCCACTGAGGAGTCCAACATC
ACCATGCAGATTATGCGGATCAAACCTCACCAAGGCCAGCACATAA -3'

BLAST results:

Eight confirmed *VEGF-A* splice variants presented 98% query cover and 100% identity, which corroborates the specificity of the primers (Table A.7; Figure A.7). Within the detected variants, *VEGF-A_{165b}* (anti-angiogenic variant) is also found by the primers.

Table A.7. BLAST results for *VEGF-A* splice variants qPCR primers. Detailed information of Transcript, Total score, Percentage of query cover, Expect value, Percentage of identity, Number of matches, and Accession number. *Occludin X* splice variants are predicted using gene prediction method: Gnomon.

Transcript	Total Score	Query Cover	Expect Value	Identity	Mat.	Accession
<i>VEGF-A₁₂₁</i>	255	98%	4.0E-66	100%	1	NM_001171628.1
<i>VEGF-A₁₄₅</i>	255	98%	4.0E-66	100%	1	NM_001204384.1
<i>VEGF-A₁₄₈</i>	255	98%	4.0E-66	100%	1	NM_001171627.1
<i>VEGF-A₁₆₅</i>	255	98%	4.0E-66	100%	1	NM_001171626.1
<i>VEGF-A₁₈₃</i>	255	98%	4.0E-66	100%	1	NM_001171625.1
<i>VEGF-A₁₈₉</i>	255	98%	4.0E-66	100%	1	NM_001171624.1
<i>VEGF-A₂₀₆</i>	255	98%	4.0E-66	100%	1	NM_001171623.1
<i>VEGF-A_{165b}</i>	255	98%	4.0E-66	100%	1	NM_001171629.1

Download GenBank Graphics		Homo sapiens vascular endothelial growth factor A (VEGFA), transcript variant 6, mRNA A		
Sequence ID: NM_001171628.1		Length: 3422		Number of Matches: 1
Range 1: 1252 to 1389 GenBank Graphics		Next Match Previous Match		
Score	Expect	Identities	Gaps	Strand
250 bits(276)	2e-64	138/138(100%)	0/138(0%)	Plus/Plus
Query 2	ATCTTCAAGCCATCCTGTGTGCCCCTGATGCGATGCGGGGGCTGCTGCAATGACGAGGGC			61
Sbjct 1252	ATCTTCAAGCCATCCTGTGTGCCCCTGATGCGATGCGGGGGCTGCTGCAATGACGAGGGC			1311
Query 62	CTGGAGTGTGTGCCCACTGAGGAGTCCAACATCACCATGCAGATTATGCGGATCAAACCT			121
Sbjct 1312	CTGGAGTGTGTGCCCACTGAGGAGTCCAACATCACCATGCAGATTATGCGGATCAAACCT			1371
Query 122	CACCAAGGCCAGCACATA			139
Sbjct 1372	CACCAAGGCCAGCACATA			1389

Download GenBank Graphics		Homo sapiens vascular endothelial growth factor A (VEGFA), transcript variant 9, mRNA B		
Sequence ID: NM_001204384.1		Length: 3494		Number of Matches: 1
Range 1: 1252 to 1389 GenBank Graphics		Next Match Previous Match		
Score	Expect	Identities	Gaps	Strand
250 bits(276)	2e-64	138/138(100%)	0/138(0%)	Plus/Plus
Query 2	ATCTTCAAGCCATCCTGTGTGCCCCTGATGCGATGCGGGGGCTGCTGCAATGACGAGGGC			61
Sbjct 1252	ATCTTCAAGCCATCCTGTGTGCCCCTGATGCGATGCGGGGGCTGCTGCAATGACGAGGGC			1311
Query 62	CTGGAGTGTGTGCCCACTGAGGAGTCCAACATCACCATGCAGATTATGCGGATCAAACCT			121
Sbjct 1312	CTGGAGTGTGTGCCCACTGAGGAGTCCAACATCACCATGCAGATTATGCGGATCAAACCT			1371
Query 122	CACCAAGGCCAGCACATA			139
Sbjct 1372	CACCAAGGCCAGCACATA			1389

Download GenBank Graphics		Homo sapiens vascular endothelial growth factor A (VEGFA), transcript variant 5, mRNA C		
Sequence ID: NM_001171627.1		Length: 3519		Number of Matches: 1
Range 1: 1252 to 1389 GenBank Graphics		Next Match Previous Match		
Score	Expect	Identities	Gaps	Strand
250 bits(276)	2e-64	138/138(100%)	0/138(0%)	Plus/Plus
Query 2	ATCTTCAAGCCATCCTGTGTGCCCCTGATGCGATGCGGGGGCTGCTGCAATGACGAGGGC			61
Sbjct 1252	ATCTTCAAGCCATCCTGTGTGCCCCTGATGCGATGCGGGGGCTGCTGCAATGACGAGGGC			1311
Query 62	CTGGAGTGTGTGCCCACTGAGGAGTCCAACATCACCATGCAGATTATGCGGATCAAACCT			121
Sbjct 1312	CTGGAGTGTGTGCCCACTGAGGAGTCCAACATCACCATGCAGATTATGCGGATCAAACCT			1371
Query 122	CACCAAGGCCAGCACATA			139
Sbjct 1372	CACCAAGGCCAGCACATA			1389

Figure A.7. Example of BLAST alignment for VEGF-A variants 6, 9 and 5 qPCR primers. Alignment of the sequencing consensus results (Query) extends from 2 to 139 bp (98% cover) and matches VEGF-A variant 6 (VEGF-A₁₂₁) **(A)**, VEGF-A variant 9 (VEGF-A₁₄₅) **(B)**, and VEGF-A variant 5 (VEGF-A₁₄₈) **(C)**, with an 100% identity and 0% gaps between sequences.

A.8. *VEGFR-2*

Primers:

F: 5'- GGCCCCTGATTATACTACACCA -3'

R: 5'- AATGTAGTCTTTGCCATCCTGC -3'

Consensus sequence (159 bp):

5'- ATGAGGGCCCCTGATTATACTACACCAGAAATGTACCAGACSATGC
TGGACTGCTGGCACGGGGAGTCCAGTCAGAGACCCACGTTTTTCAGAST
TGGTGGAACATTTGGKAAATCTCTTGCAAGCTAATGCTCAGCAGGATG
GCAAAGACTACATTGTT -3'

BLAST results:

VEGFR-2 was the only transcript aligned to the consensus sequence, which confirms the specificity of the primers (Table A.8; Figure A.8).

Table A.8. BLAST results for *VEGFR-2* qPCR primers. Detailed information of Transcript, Total score, Percentage of query cover, Expect value, Percentage of identity, Number of matches, and Accession number.

Transcript	Total Score	Query Cover	Expect Value	Identity	Mat.	Accession
<i>VEGFR-2</i>	278	100%	9.0E-73	97.48%	1	NM_002253.3

Download GenBank Graphics		Homo sapiens kinase insert domain receptor (KDR), mRNA				
Sequence ID: NM_002253.3		Length: 5849		Number of Matches: 1		
Range 1: 3675 to 3833		GenBank Graphics		Next Match Previous Match		
Score	Expect	Identities	Gaps	Strand		
275 bits(304)	6e-72	155/159(97%)	0/159(0%)	Plus/Plus		
Query 1	ATGAGGGCCCCTGATTATACTACACCAGAAATGTACCAGACSATGCTGGACTGCTGGCAC					60
Sbjct 3675	ATGAGGGCCCCTGATTATACTACACCAGAAATGTACCAGACCATGCTGGACTGCTGGCAC					3734
Query 61	GGGGAGTCCAGTCAGAGACCCACGTTTTTCAGASTTGGTGGAACATTTGGKAAATCTCTTG					120
Sbjct 3735	GGGGAGCCCAGTCAGAGACCCACGTTTTTCAGAGTTGGTGGAACATTTGGGAAATCTCTTG					3794
Query 121	CAAGCTAATGCTCAGCAGGATGGCAAAGACTACATTGTT					159
Sbjct 3795	CAAGCTAATGCTCAGCAGGATGGCAAAGACTACATTGTT					3833

Figure A.8. BLAST alignment for *VEGFR-2* qPCR primers. Alignment of the sequencing consensus results (Query) extends from 1 to 159 bp (100% cover) and matches *VEGFR-2* with an 97% identity and 0% gaps between sequences.

A.9. *TIE-2*

Primers:

F: 5'- ATCTCACTGCTTGGACCCTTAG -3'

R: 5'- GCTGAGGAGTGTGTAATGTTGG -3'

Consensus sequence (84 bp):

5'- ATCTCACTGCTTGGACCCTTAGTGACATTCTTCCTCCTCAACCAGA
AAACATCAAGATTTCCAACATTACACACTCCTCAGCTG -3'

BLAST results:

Three confirmed *Tie-2* splice variants presented 100% query cover and identity confirming the specificity of the primers (Table A.9; Figure A.9).

Table A.9. BLAST results for *Tie-2* splice variants qPCR primers. Detailed information of Transcript, Total score, Percentage of query cover, Expect value, Percentage of identity, Number of matches, and Accession number. *Tie-2* X splice variants are predicted using gene prediction method: Gnomon.

Transcript	Total Score	Query Cover	Expect Value	Identity	Mat.	Accession
<i>Tie-2 var 3</i>	156	100%	2.0E-36	100%	1	NM_001290078.1
<i>Tie-2 var 2</i>	156	100%	2.0E-36	100%	1	NM_001290077.1
<i>Tie-2 var 1</i>	156	100%	2.0E-36	100%	1	NM_000459.4
<i>Tie-2 var X2</i>	156	100%	2.0E-36	100%	1	XM_005251563.2
<i>Tie-2 var X1</i>	156	100%	2.0E-36	100%	1	XM_005251561.2

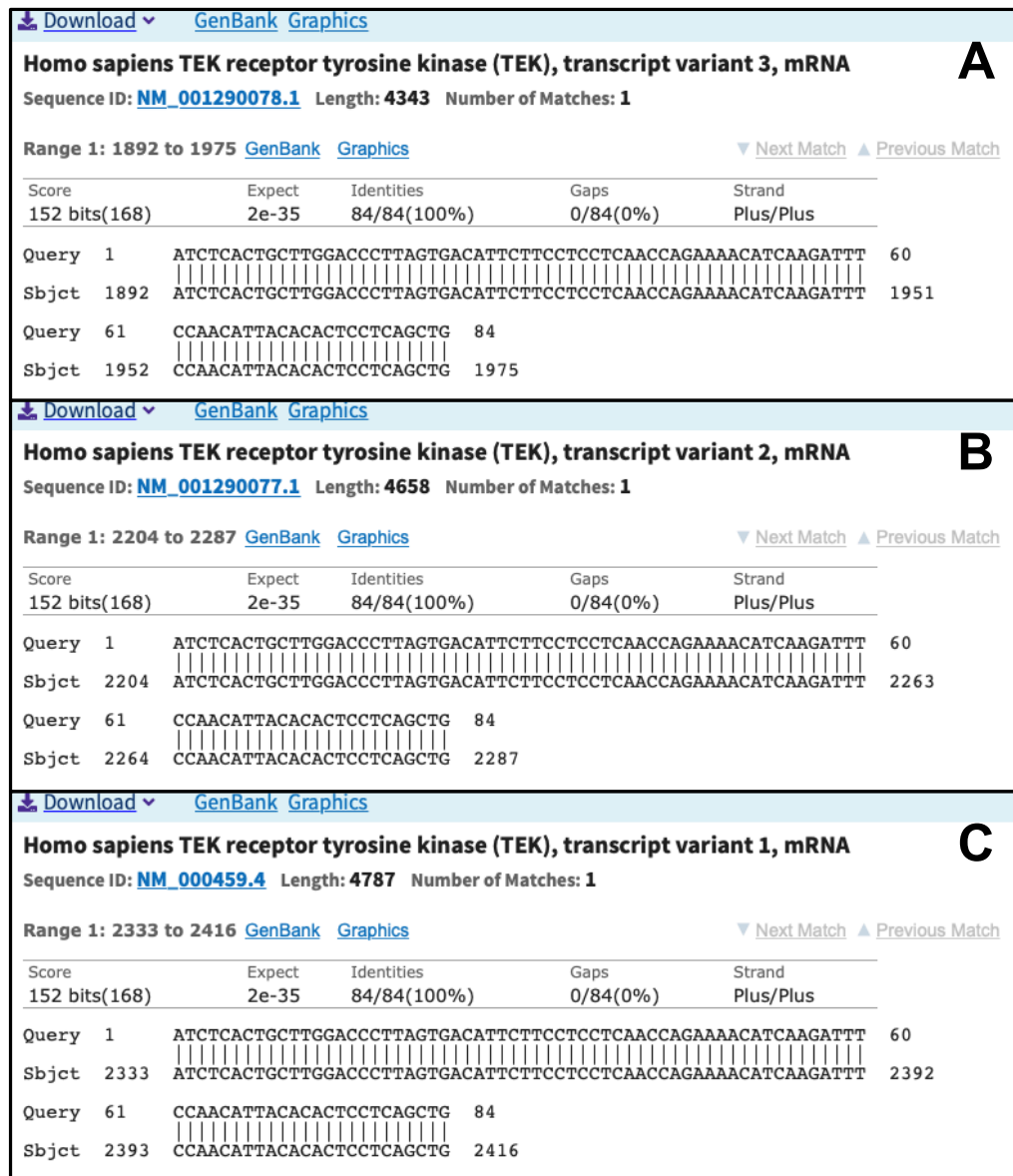


Figure A.9. BLAST alignment for *Tie-2* variants 1, 2 and 3 qPCR primers. Alignment of the sequencing consensus results (Query) extends from 1 to 84 bp (100% cover) and matches *Tie-2* variant 3 (A), *Tie-2* variant 2 (B), and *Tie-2* variant 1 (C), with an 100% identity and 0% gaps between sequences.

A.10. ANGIOPOIETIN-1

Primers:

F: 5'- GGAACCGAGCCTATTCACAGTA -3'

R: 5'- CTGCTCTGTTTTCTGCTGTC -3'

Consensus sequence (116 bp):

5'- GGAAGGGAACCGAGCCTATTCACAGTATGACAGATTCCACATAGG
AAATGAAAAGCAAACTATAGGTTGTATTTAAMAGGTCACACTGGGA
CAGCAGGAAAACAGAGCAGCCTGA -3'

BLAST results:

Four confirmed *Angiopoietin-1* splice variants presented 100% query cover and 99% identity (Table A.10; Figure A.10). The remaining two *Ang-1* splice variants present lower than 45% query cover and 98% identity. The lower percentage of cover could be due to splicing events. Nonetheless, the specificity of the primers was confirmed.

Table A.10. BLAST results for *Angiopoietin-1* (*Ang-1*) splice variants qPCR primers. Detailed information of Transcript, Total score, Percentage of query cover, Expect value, Percentage of identity, Number of matches, and Accession number. *Angiopoietin-1* X splice variants are predicted using gene prediction method: Gnomon.

Transcript	Total Score	Query Cover	Expect Value	Identity	Mat.	Accession
<i>Ang-1 var 0.9 kb</i>	211	100%	4.0E-58	99.14%	1	U83508.1
<i>Ang-1 var 1</i>	211	100%	4.0E-58	99.14%	1	NM_001146.4
<i>Ang-1 var 2</i>	211	100%	4.0E-58	99.14%	1	NM_001199859.2
<i>Ang-1 var 3</i>	211	100%	4.0E-58	99.14%	1	NM_001314051.1
<i>Ang-1 var 0.7 kb</i>	91.6	43%	5.0E-22	98.04%	1	U83508.1
<i>Ang-1 var X1</i>	91.6	43%	5.0E-22	98.04%	1	XR_928319.2
<i>Ang-1 var 1.3 kb</i>	86.1	41%	2.0E-20	97.92%	1	U83508.1

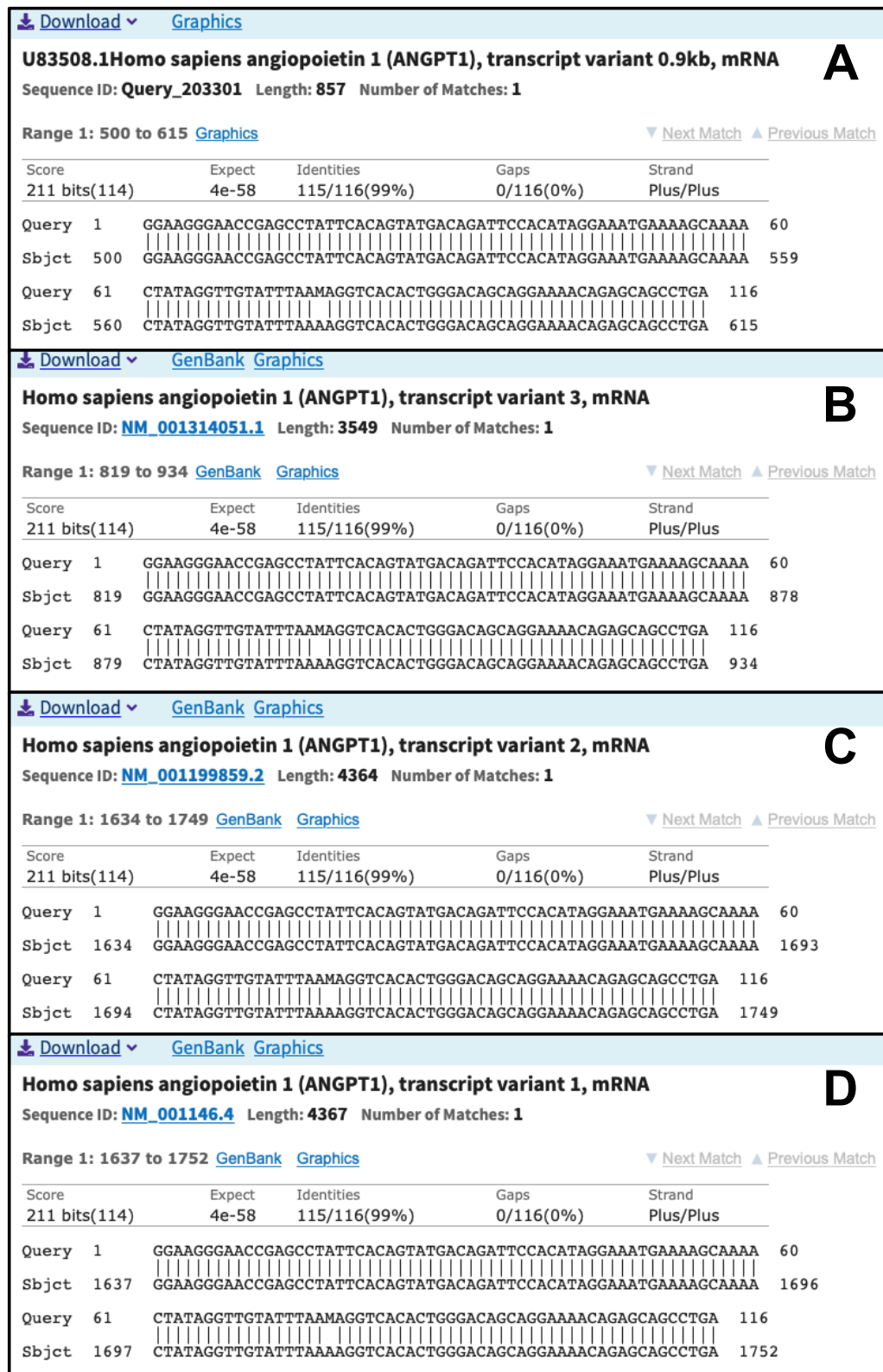


Figure A.10. BLAST alignment for Angiotensinogen-1 variants 1, 2, 3 and 0.9kb qPCR primers. Alignment of the sequencing consensus results (Query) extends from 1 to 116 bp (100% cover) and matches Angiotensinogen-1 variant 0.9 kb (A), Angiotensinogen-1 variant 3 (B), Angiotensinogen-1 variant 2 (C) and Angiotensinogen-1 variant 1 (D), with a 99% identity and 0% gaps between sequences.

A.11. ANGIOPOIETIN-2

Primers:

F: 5'- GACCACGAGACTTGAACCTTCAG -3'

R: 5'- TGTCTTCCATAGCTAGCACCTT -3'

Consensus sequence (142 bp):

5'- CCACGAGACTTGAACCTTCAGCTCTTGGAACTCCCTCTCGACAAA
CAAATTGGAAAAACAGATTTTGGACCAGACCAGTGAAATAAACAAAT
TGCAAGATAAGAACAGTTTCCTAGAAAAGAAGGTGCTAGCTATGGAA
GA -3'

BLAST results:

Three confirmed *Angiopoietin-2* splice variants presented 100% query cover and identity, ratifying the specificity of the primers (Table A.11; Figure A.11).

Table A.11. BLAST results for *Angiopoietin-2* (*Ang-2*) splice variants qPCR primers. Detailed information of Transcript, Total score, Percentage of query cover, Expect value, Percentage of identity, Number of matches, and Accession number. *Angiopoietin-2* X splice variant was predicted using gene prediction method: Gnomon.

Transcript	Total Score	Query Cover	Expect Value	Identity	Mat.	Accession
<i>Ang-2 var 3</i>	263	100%	2.0E-68	100%	1	NM_001118888.1
<i>Ang-2 var 2</i>	263	100%	2.0E-68	100%	1	NM_001118887.1
<i>Ang-2 var 1</i>	263	100%	2.0E-68	100%	1	NM_001147.2
<i>Ang-2 var X1</i>	263	100%	2.0E-68	100%	1	XM_017013318.1

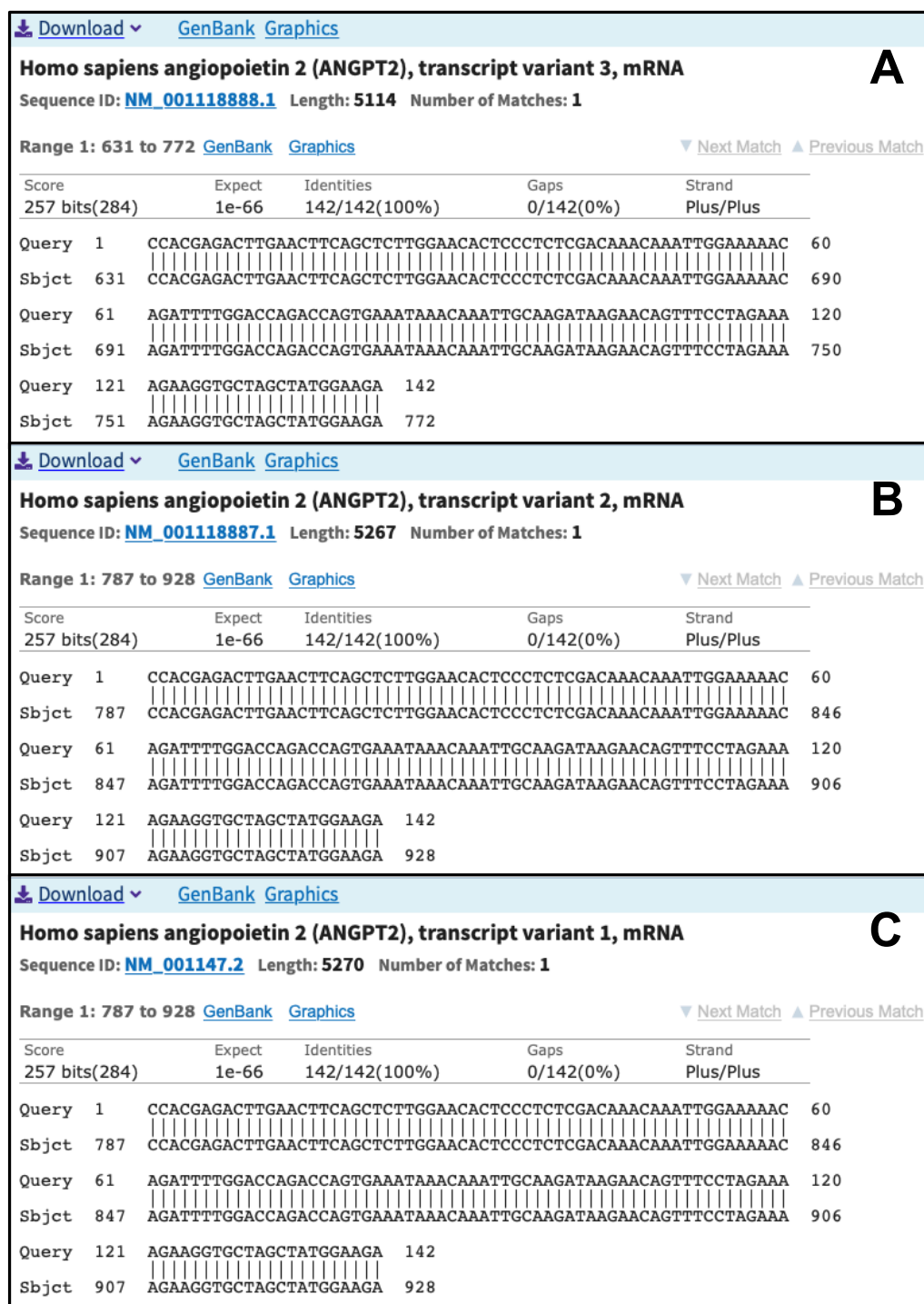


Figure A.11. BLAST alignment for Angiotensin-2 variants 1, 2 and 3 qPCR primers. Alignment of the sequencing consensus results (Query) extends from 1 to 142 bp (100% cover) and matches *Angiotensin-2 variant 3* (A), *Angiotensin-2 variant 2* (B) and *Angiotensin-2 variant 1* (C), with an 100% identity and 0% gaps between sequences.

A.12. *MMP-9*

Primers:

F: 5'- CGCTACCACCTCGAACTTTG -3'

R: 5'- CCGGCACTGAGGAATGATCTAA -3'

Consensus sequence (128 bp):

5'- CGCTACCACCTCGAACTTTGACAGCGACAAGAAGTGGGGCTTCTGC
CYGGACCAAGGATACAGTTTGTTCCTCGTGGCGRCGCATGAGTTCGGC
CACGCGCTGGGCTTAGATCATTCTCAGTGCCGG -3'

BLAST results:

MMP-9 was the only transcript aligned to the consensus sequence, which confirms the specificity of the primers (Table A.12; Figure A.12).

Table A.12. BLAST results for *MMP-9* qPCR primers. Detailed information of Transcript, Total score, Percentage of query cover, Expect value, Percentage of identity, Number of matches, and Accession number.

Transcript	Total Score	Query Cover	Expect Value	Identity	Mat.	Accession
<i>MMP-9</i>	230	100%	2.0E-58	98.44%	1	NM_004994.2

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Homo sapiens matrix metalloproteinase 9 (MMP9), mRNA

Sequence ID: [NM_004994.2](#)
Length: 2387
Number of Matches: 1

Range 1: 1138 to 1265
[GenBank](#)
[Graphics](#)

[Next Match](#)
[Previous Match](#)

Score	Expect	Identities	Gaps	Strand
226 bits(250)	2e-57	126/128(98%)	0/128(0%)	Plus/Plus
Query 1	CGCTACCACCTCGAACTTTGACAGCGACAAGAAGTGGGGCTTCTGCCYGGACCAAGGATA			60
Sbjct 1138	CGCTACCACCTCGAACTTTGACAGCGACAAGAAGTGGGGCTTCTGCCCGGACCAAGGATA			1197
Query 61	CAGTTTGTTCCTCGTGGCGRCGCATGAGTTCGGCCACGCGCTGGGCTTAGATCATTCTC			120
Sbjct 1198	CAGTTTGTTCCTCGTGGCGGCGCATGAGTTCGGCCACGCGCTGGGCTTAGATCATTCTC			1257
Query 121	AGTGCCGG	128		
Sbjct 1258	AGTGCCGG	1265		

Figure A.12. BLAST alignment for *MMP-9* qPCR primers. Alignment of the sequencing consensus results (Query) extends from 1 to 128 bp (100% cover) and matches *MMP-9* with an 98% identity and 0% gaps between sequences.

APPENDIX B

OCCLUDIN LOCALISATION IN ENDOTHELIAL CELLS ISOLATED FROM PLACENTAE

B.1. SUCCESSFUL ISOLATION OF HUVECS AND HUAECs

Isolation of HUVEC was obtained following a routine protocol from our group (Section 2.4.1). These cells reached confluency after three to four days of seeding. Once confluent, cells presented a cobblestone appearance (Figure B.1A). Furthermore, isolation of HUAEC was obtained after modifications to published protocols (Section 2.4.2). Cannulation of umbilical arteries presented a challenge due to their reduced elasticity. Nonetheless, isolation of HUAEC was possible after various attempts. These cells presented a lower proliferation ratio than HUVEC, as it took them longer to reach confluency (five days) (Figure B.1B).

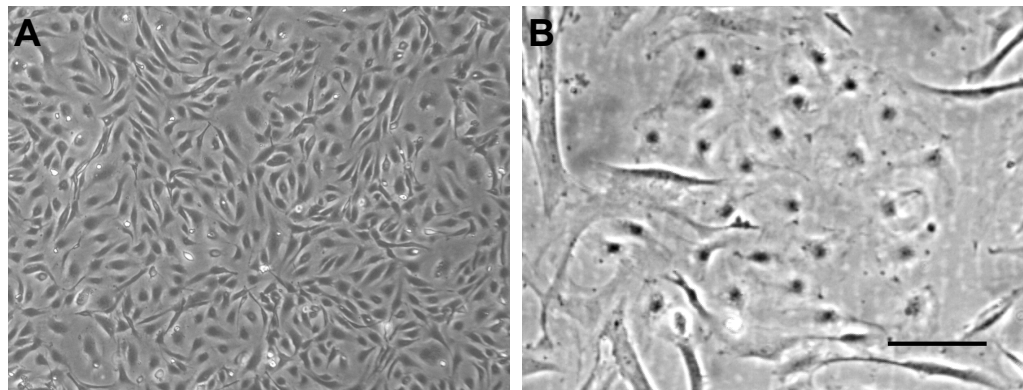


Figure B.1. Micrograph (phase contrast microscopy) of human umbilical endothelial cells (HUVEC). (A) Human umbilical vein endothelial cells (HUVEC) isolated from normal pregnancy, passage 2, after four days of seeding. **(B)** Human umbilical arterial endothelial cells (HUAEC) isolated from normal pregnancy, passage 2, after five days of seeding. Both cell types formed a monolayer on gelatine-coated tissue culture flasks. Cells were cultured at 37°C humidified incubator with 5% CO₂. Scale bar: 100 µm.

B.2. TECHNICAL CHALLENGES IN THE ISOLATION OF HPMECs

Isolation of HPMEC was troublesome due to the low number of cells that could be isolated from placental chorionic villi. Different published protocols were employed (Section 2.4.3); however, due to the low yield, cells died before reaching confluency (Figure B.2).

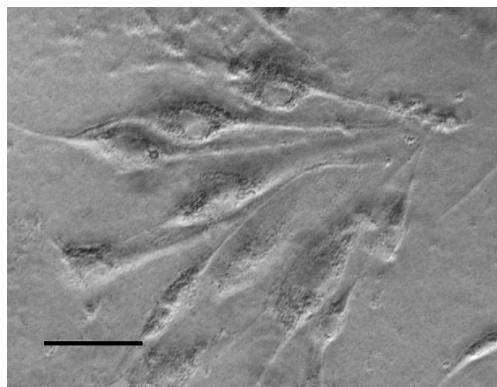


Figure B.2. Micrograph (phase contrast microscopy) of human placental microvascular endothelial cells (HPMEC). Micrograph of HPMEC after 20 days of seeding. Cells were cultures at 37°C humidified incubator with 5% CO₂. Scale bar: 100 µm.

B.3. HUVEC AND HUAEC DID NOT PRESENT JUNCTIONAL OCCLUDIN IN THEIR MEMBRANES

HUVEC and HUAEC were harvested and seeded in coverslips at a 5 x 10⁴ cells/ml concentration in a 24-well plate. Upon confluence, Occludin immunostaining was performed as described by Babawale and collaborators (Babawale *et al.*, 2000) with some modifications. Briefly, cells were washed with pre-warmed 1X PBS, fixed with a volume of acetone: methanol (1:1), and incubated at -20°C for 15 minutes. The following steps continued as described in Section 2.21. PECAM-1 (red) was used as a control for endothelial cell-cell adhesion (Figure A.3A, D). Occludin (green) was found in the cytoplasm (Figure B.3B, E). Therefore, double localisation of PECAM-1 and Occludin (yellow) was not found in HUVEC or HUAEC (Figure B.3C, F).

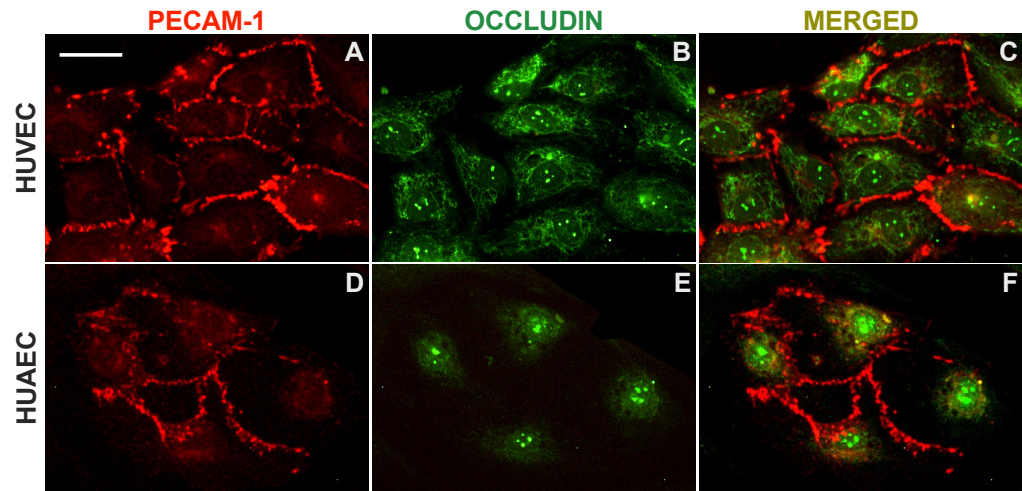


Figure B.3. Representative micrographs of occludin localisation in human umbilical endothelial cells (HUVEC and HUAEC). Cells were isolated from normal pregnancies. Samples were double-stained with PECAM-1 (red; panels A, and D) and Occludin (green; panels B, and E). Panels C, and F show double-staining with PECAM-1 and Occludin. Occludin was found in the nucleus and cytoplasm of HUVEC and HUAEC. Scale bar: 50 μ m.

Our study aimed to use endothelial cells as an *in vivo* model to determine the effect of *miR-181a-5p* on Occludin gene expression and localisation in the TJs. Therefore, we needed to confirm the localisation of Occludin in endothelial monolayers. Our study showed that Occludin was not part of the TJs of HUVEC and HUAEC. Contradictory to our results, a study determined that Occludin localised in the membranes of these cells; displaying a weak, diffuse, less organised staining in HUVEC, while in HUAEC, Occludin displayed a strong, more continuous intercellular staining (Kevil *et al.*, 1998). In this study, Kevil maintained both endothelial cells in EGM media (Clonetics) and fibronectin-coated flasks. Our study differed to theirs by using M199 media (Gibco) supplemented with 20% FCS and gelatine-coated flasks. A study showed there are no differences in HUVEC phenotype or in the expression of adhesion molecules (PECAM-1, CD44, and ICAM-1) between cells cultures on gelatine-coated flasks or fibronectin-coated flasks (Relou *et al.*, 1998). Hence, the differential localisation of Occludin might depend on the growing media. Furthermore, a study showed that treatment of endothelial cells with a cyclic-AMP cocktail made Occludin staining stronger and more continuous at cell-cell borders (Dye *et*

al., 2001). However, the use of c-AMP to culture cells will generate changes in their gene expression profile, which would interfere in our experiments. Future studies that aim to use HUVEC for quantification of junctional Occludin should find adequate culture conditions that will bring the protein to the membranes without repercussions at a gene level.