The role of heat shock protein 20 and paroxonase 2 in the human placenta: influence of obesity and labour

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Abstract

The mechanisms that control uterine contractions during parturition are better understood, however significant gaps in our knowledge still remain. Until it is fully understood, it will be difficult to understand the causes of idiopathic pre-term labour. The placenta plays an essential role during labour, which involves inflammatory pathways, mechanical stretching, hormonal, and the neurological process. Placental oxidative stress is a feature of human labour and maternal obesity. Heat-shock proteins (HSPs) can protect cells during stress. The small heat-shock protein 20 (HSP20), which belongs to the sHSP family, acts as a chaperone as well as regulating acting cytoskeleton dynamics, and may also regulate contractile protein activation. HSP20 is also known as HSPB6, and has been shown to protect against a number of pathophysiological processes, including apoptosis and oxidative stress. The hypothesis of this work is that placental expression of HSP20 would be changed during labour and obesity. In addition, expression may alter in different zones of the placenta. We recruited 6 women in labour who delivered vaginally (uncomplicated labour), and 6 women who were delivered by Caesarean section (not in labour). Furthermore, women who had normal pregnancies with different body mass indices (BMI)s were also recruited. Four BMI groups were studied: 1) BMI <30 (n=6), 2) BMI 30-35 (n=6), 3) BMI 35-40 (n=6) and 4) BMI>40 (n=6). These women delivered by Caesarean section and were not in labour. Sampling of 4 equally spaced pieces performed for 3 placental zones (inner, middle and outer) of each placenta (total 12 samples per placenta). Western blot analysis and RT-PCR were used to investigate expression of HSP20. The results demonstrated that there was a significant decrease in HSPB6mRNA expression at the middle area in the labour group when compared to the non-labour group (p=0.01). No significant difference was found at the inner and outer areas (p=0.3) for both. For BMI groups no differences were found in HSP20 expression at protein and molecular levels.

Oxidative stress and inflammation are significant features of maternal obesity, and Paroxonase 2 (PON2) has a clear role in oxidative stress and inflammation. It protects cells against oxidative damage and lipid peroxidation, modulation of endoplasmic reticulum stress, and regulation of apoptosis. As a result, the hypothesis was that expression of placental PON2 would change during obesity. PON2 was examined in placentas obtained from women who had normal pregnancies with different BMIs. Four BMI groups were studied as per the HSP20 study. Western blotting and real time PCR were performed to investigate the expression of placental PON2, which has 2 isoforms: one at 62kDa, and another at 43kDa, in all samples. The results showed no spatial differences in PON2 protein or mRNA expression of either isoform at the three sites (inner, middle, and outer) between all BMI groups.

Conclusion: This is the first study to investigate the expression of HSP20 in labour and during maternal obesity. Whether differences in phosphorylation of the protein occur requires future investigation. Similarly, this work is the first to investigate the expression of PON2 in different BMI groups in the human placenta at the three sites (inner, middle, and outer). However, knowledge concerning its role in the reproductive system is still under investigation, and more studies need to be conducted to explain its precise roles. It would be of interest in future work to determine how other PONs 1 and 2 are altered.

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Author’s Declaration

I declare that, except where explicit reference is made to the contribution of others that this thesis and the work described within it was carried out entirely by myself and has not submitted for any other degree at the University of Glasgow or any other institution.

Signature----------

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Dedication

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List of Abbreviations

ACD	Alpha crystalline domin
ALL	Acute lymphoblastic leukemia
Amp	Ampere
ATP	Adenosine triphosphate
BSA	Bovine Serum Albumin
BMI	Body mass index
JNK	jun amino terminal kinase
cDNA	Complementart Deoxy-Ribonucleic Acid
cGMP	Condescend gercyclicguanosine monophosphate
CHOP	C/Enhancer-Binding Protein Homologous Protein
CM	Centimetre
CO2	Carbon dioxide
CRH	Corticotropin-releasing hormone
CRP	C-reactive protein
CS	Caesarean section
CXCR1-3	Chemokine receptors 1-3
CXCL8	Chemokine (C-X-C motif) ligand 8
CXCL10	Chemokine (C-X-C motif) ligand 10
CTE	C- terminal extension
DAMPs	Damage-associated molecular patterns
DHEA-S	Dehydroepiandrosterone-sulfate

DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELC/S	Elective caesarean section
ER	Endoplasmic Reticulum
EMC/C	Emergency caesarean section
GDM	Gestional diabetes mellitus
GLUT	Glucose transporter
Grp78	78 kDa glucose-regulated protein
Grp94	94 kDa glucose-regulated protein
HCG	Human chorionic gonadotrophin
HCL	Hydrochloric acid
HDL	High Density Lipoprotein
HIF1-α	Hypoxia-Induceble Factor 1Alph
HSPB6	Heat shock proteins alpha-crystallin-related B6
HSP20 mRNA	Heat shock protein 20 messenger ribonucleic acid
HSP20	Heat shock proteins 20
HSP27	Heat shock protein 27
HSP40	Heat shock protein 40
HSP60	Heat shock protein 60
HSP70	Heat shock proteins 70
HSP71	Heat shock protein 71
HSP72	Heat shock protein 72

HSP90	Heat shock protein 90
HSP104	Heat shock protein 104
HSP110	Heat shock protein 110
HSPA	Heat shock protein group A
HSPB	Heat shock protein group B
sHSP	Small heat shock proteins
HSF-1	Heat shock factor 1
HPL	Human placental lactogen
HRP	Horseradish peroxidase
IFN-γ	Interferon-gamma
IGF1	Insulin-like growth factor-1
IgG	Immunoglobulin
IKK	Inhibitor of NF-k beta kinase
IRAK	Interleukin 1 receptor associated kinase
IL-1β	Interleukin 1-β
IL-6	Interleukin-6
JNK	c -jun amino terminal kinase
kDa	kilo Dalton
Kg/m^2	kilogram per square metre
Kg/h^2	The weight divided by the square of the height
LDH	Lactate dehydrogenase
L	Litre
LDL	Low Density Lipoprotein

LG	Labour group
mg	Miligram
mg/L	Milligrams per litre
mL	Millilitre
mM	Millimolar
mmHg	Millimeter of mercury
mRNA	Messenger ribonucleic acid
MLCP	Myosin phosphatase
MMP-9	Matrix metallopeptidase 9
MYPT1	Myosin phosphatase target subunit 1
Na+/H+	Sodium–hydrogen
Na^+/K^+	Sodium-potassium
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLG	Non-labour group
NHS	Normal human serum
NO	Nitric oxide
NTD	N-terminal domain
ng	Nanogram
NLG	Non-labour group
O_2	Oxygen
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PGH	Placental growth hormone

PLGF	Placental growth factor.
PKA	Protein Kinase A
PKC	Protein kinase C
PKGI*a*	Proteinkinase G
PON	Paroxonase
PON1	Paroxonase 1
PON2	Paroxonase 2
PON3	Paraoxonases 3
QRT-PCR	Quantitative real time polymerase chain reaction
rpm	Round per minute
RNA	Ribonucleic acid
RQ	Relative quantification of gene expression.
ROS	Reactive oxygen species
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain
SDS-PAGE	Sodium deodecyl sulfate polyacrylamide gel electrophoresis
SE	Standard error
Ser16	Serine 16
Ser59	Serine 59
Ser157	Serine 157
STBMs	Synctiotrophoblast micro particles
STD	Stock standard solution
SVD	Spontaneous vaginal delivery

sHSPs	Small heat shock protein
TEMED	N, N tetramethylethylenediamine
TGF	Transforming growth factor
TNF-α	Tumor necrosis factor alpha
UPR	Unfolded protein response
V	Voltage
VASP	Vasodilator-stimulated phosphoprotein
VEGF	Vascular endothelial growth factor
VLDL	Very-low-density lipoprotein
β-actin	Beta-actin
β-ME	beta-Mercaptoethanol
µg	Microgram
µL	Microlitre

Chapter 1 – Introduction

1. The placenta

1.1 Anatomy of the normal term placenta

In humans the placenta is the main organ which provides the physical attachment of the embryo to the uterus, and interchange between the growing fetus and the mother. During pregnancy it plays an essential role transporting waste and nutrients and in secreting hormones. Figure 1 shows the basic anatomy of the placenta.

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Figure 1-1 Placenta anatomy: 1.Umbilical cord, 2.Amnion, 3.Chorionic plate, 4.Villus 5.Basal plate, 6.Cotyledon, 7.Intervillousspace.Taken from http://www.embryology.ch/anglais/fplacenta/villosite01.html.

1.1.1 Overview of the placenta

The placenta has a disc shape and is a dark reddish-blue in colour. It averages 22 cm in length and its thickness is 2–2.5 cm. It weighs approximately 470 gm (Benirschke et al., 2006). It connects to the fetus by an umbilical cord, which is approximately 55–60 cm in length, has two umbilical arteries and one umbilical vein. The umbilical cord has an eccentric attachment, which inserts into the chorionic plate. The amniotic side is formed of a single layer of epithelium and basement membrane with a base of avascular connective tissues (Rampersad et al., 2011).

The humane placenta is formed of cotyledons which are irregular well-defined lobules (10-14). There are septae which separate cotyledons. Cotyledons contain villous tree

structures embedded in the maternal blood circulation. Figure 1-2 shows the cord insertion side of the placenta and Figure 1-3 shows the maternal side.

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Figure 1-2: The umbilical cord inserts into the fetal surface. Note the vessels radiating out from the cord over the fetal surface in this normal term placenta.

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Figure 1-3: The maternal surface of a normal term placenta is seen here. Note that the cotyledons that form the placenta are reddish brown and indistinct.

1.1.2 Early development of the placenta.

- **Pre-implantation stage**

Fertilization is the process of fertilized egg formation (zygote), which forms a ball of cellsknown as a morula, after mitotic division in the fallopian tubes. It is then transported into the uterine cavity over a period of 3-4 days. The morula develops into a blastocyst, and thereafter differentiation of the trophoblast occurs. The blastocyst is surrounded by a single layer of mononucleated trophoblasts, known as the trophectoderm, which develops to the extraembryonic membrane and placenta. In later pregnancy, the fetal membranes and placenta are formed from the trophoblast, whereas the umbilical cord, the embryo and the placental mesenchyme result from the inner layer. Placenta formation occurs when the blastocyst connects to the uterine epithelium after hatching from the zona pellucid this happens at about 6-7 days post-conception (Aplin, 2000).

- **Pre-lacunar stage**

This stage starts on day 7 post-conception, when the blastocyst is implanted into the uterine wall. If this does not occur, pregnancy failure is the result. Abnormal insertion of the umbilical cord may happen due to blastocyst orientation, which varies during the implantation time (McLennan, 1968). The microvilli arising from trophectoderm create the first communication with the uterus epithelium. An early attachment of the blastocyst results through complex interaction between trophoblast cells and the decidua. Studies from the 1970s (Schlafke & Enders, 1975) revealed that, during implantation, blastocyst apposition occurs first, following that is the attachment of the blastocyst to decidua through the inner cell mass. Consequently, interstitial implantation of the blastocyst deeply in the connective tissue occurs, and near to maternal blood vessels which supply the placenta. The differentiated multinucleated syncytiotrophoblast which is adjacent to

the maternal tissue developed from proliferation and fusion of the cytotrophoblast with the outer layer during invasion stage of the blastocyst. The embryo has inserted into the uterus and a continuous expansion of the epithelial cells over the implantation area has taken place by 6-7 days post-conception (Hertiget al., 1956). Studies have shown (Rampersadetal, 2011) that bilaminar disk of embryoblast and amnioblast are formed due to differentiation of the inner cell mass.

- **Lacunar stage**

A large lacuna is formed by the fusion of multiple fluid-filled spaces inside the syncytiotrophoblast approximately eight days after conception. These spaces will eventually form the intervillous space, which is filled with maternal blood due to erosion of blood capillaries. The placental spiral artery undergoes physiological change, due to wall destruction and endothelium replacement by trophoblast (Kaufmann et al., 2003, Lyall, 2007). Maternal blood is prevented from entering the intervillous space or lacunae for the first 10 weeks of pregnancy (Hustin et al. 1988). The glandular secretions and plasma in the intervillous space are the main source of nutrition for the fetus during the oxygen crisis period, i.e. prior to when blood flow starts. The fetus is protected by a low oxygen environment during the organogenesis time; the low-oxygen environment means that reactive oxygen species are not produced (Lyall, 2007).

- **Villous stage**

The trabeculae branches rapidly as the trophoblasts proliferate. The precursors of the intervillous space are the lacunae, while the trabeculae are the precursors of the chorionic villi. At that time they are called the primary villi, which transform into secondary villi when extraembryonic mesoderm from the primary chorionic plate invades the trabeculae.

The core of villous, called the stroma, is made up of a network of fibroblasts with Hofbauer cells (fetal macrophage) dispersed throughout (Kaufmann, 1977). At day 19 of development, fetal capillaries begin to develop within the villi due to the differentiation of hemangioblastic progenitor cells (Demir et al. 1989). This development changes secondary villi to tertiary villi. The villous cytotrophoblasts form a continual layer below the syncytiotrophoblast surrounding the villi in early pregnancy; however, very few villous cytotrophoblasts can be seen at term; therefore, the maternal circulation is separated from the endothelium of the villous capillaries by the syncytial layer at term. The villous tree is established by the growth of villi throughout the placenta. There are 60-70 main stem villi in the placenta, each of which is at the centre of a villous tree. These are subdivided into 2-5 branches which, in turn, are subdivided into more branches. The diameter of the final stem villi is 80-100 micrometers. They contain a single arteriole, venule and ten capillaries. At term, the area of exchange of the villi is approximately $14m^2$ and the length of the capillaries in the villi is 50km.

- **Trophoblast invasion**

There are two types of villi in the placenta: the first, called floating villi, do not attach to the maternal uterus but instead move freely in the intervillous space allowing exchange between the maternal and fetal circulation. The second are called anchoring villi, which connect the placenta to the uterus. At the tips of the second type of villi, the cytotrophoblast cells proliferate and break through the syncytium, before invading the uterus and its spiral arteries. At this stage, these cells are called extra-villous cytotrophoblast; their invasion is one of the most important processes in placentation. There are many physiological changes which occur as a result of extra-villous cytotrophoblast cell migration into spiral arteries: loss of the endothelial and muscular layer is one (Brosens et al. 1967; DeWolf et al.1973), although in later pregnancy the

endothelial layer is restored. Consequently, changingthe blood flow through the spiral arteries from a low flow-high resistance to a high flow-low resistance circuit is designed to meet the requirements of the growing fetus and placenta. Moreover, maternal vasomotor control over the intervillous circulation is lost through the loss of vessels contraction (Moll et al. 1988). There are two pathways of invasion, the interstitial and the endovascular (Pijnenborg et al. 1980; Pijnenborg et al. 1981a; Pijnenborg et al. 1981b; Pijnenborg et al. 1983; Pijnenborg, 1990, Pijnenborg et al. 1996; Benirschke and Kaufmann, 2000). Invasion of the placental bed to the endometrium, and the first third of the myometrium by these cells, occurs through the interstitial pathway, while the lumen of arteries and the distal parts of some veins are invaded by extra-villous cytotrophoblast in the endovascular pathway.

1.1.3 Functions of placenta

1.1.3.1 Nutrition

The placenta plays an essential function in oxygen and nutrient transfer from maternal blood to the fetus, and the removal of waste products from the fetus to the mother. There are two types of transport. Different plasma concentrations of large molecules are maintained on both sides of the placental barrier, and this is because of an active transport system (Wright et al, 2011).

Obesity and diabetes during pregnancy may affect the nutrient transport level - either increasing or decreasing levels consequently, the overgrowth of the fetus or its restriction occurs (Kappen et al, 2012).

The placenta acts as a very important transport system, where exchange between the fetus and the mother occurs (Sibley & Boyd, 1988; Sibley et al., 1998; Illsely, 2011). The main

fetal needs, for example, oxygen (O_2 concentration 90 mmHg), gases, free fatty acids and urea are transported by passive diffusion between the spiral arteries and intervillous space.

Waste products, such as CO_2, are eliminated to the maternal blood (where O_2 concentration is 30 mmHg) via the uterine vein (O_2 concentration 30-45 mmHg). Moreover, by passive diffusion, the de-oxygenated blood is brought back through the umbilical artery into the intervillous space.

On the other hand, there is an ATP independent way, through which glucose transport is facilitated and diffused by the GLUT group of transporter (mainly the GLUT-1 isoform) that occurs in the fetal membranes of syncytiotrophoblast and maternal microvilli (Illsley 2000). However, there are other isoforms formed during gestation (Donnelly&Campling. 2014).

There are important ions to be transported: for instance, sodium ion transport occurs by other transporters (Na^+/K^+ exchanger) and (Na^+/H^+ exchanger). Channels such as the calcium and potassium channels are also important (Bernucci et al., 2006).

Proteins such as IgG and also lipid transport occurs via endocytosis, which is receptor mediated. IgG bind to the receptors present on microvilli and then is passed to the cytoplasm. Similarly, maternal plasma lipoproteins bind to receptors in this way (Donnelly & Campling. 2014).

1.1.3.2 Excretion

By diffusion, waste products, such as uric acid, creatinine and urea are excreted into maternal blood (Donnelly & Campling. 2011).

1.1.3.3 Immunity

At the 24th week of gestational age, the transfer of antibodies begins (Pillitteri. 2009). Protection is provided to the fetus by IgG passing across the placenta barrier; this immunity lasts for many months after birth. It also prevents microbal transmission, and therefore acts as a selective barrier.

1.1.3.4 Endocrine function

The human placenta is a vital structure in regards to secretion of important hormones during pregnancy. These hormones are secreted from the syncytial layer of the chorionic villi. The placenta produces various hormones, proteins and peptide hormones, which are necessary for the pregnancy to progress. Human chorionic gonadotrophin (hCG), human placental lactogen (hPL), oestrogen, progesterone, and human placental growth hormones are all examples of hormones and proteins secreted by the placenta during pregnancy (Lacroixet al., 2002). However, prolactin, oxytocin, corticotropin-releasing hormone (CRH), and inhibins are peptide hormones. In addition, the placenta produces placental growth factor (PLGF), vascular endothelial growth factor (VEGF), transforming growth factor (TGF), and epidermal growth factor (EGF).

- **Human chorionic gonadotrophin (hCG):**

HCG is secreted immediately after implantation and can be detected early in pregnancy in both blood and urine. It stimulates the corpus luteum to secrete progesterone and oestrogen, as progesterone reduction during pregnancy leads to the endometrium being sloughed off. Furthermore, prevention of fetal rejection is due to its suppressive effect on the maternal immune response (Asher et al., 1973; Cole 2009). This hormone is a glycoprotein hormone and formed of alpha and beta subunits. Luteinizing hormone is secreted by pituitary glands of all ages (males and females) and its analog (Cole 2009, Hoermannet al., 1990).

- **Humane placental lactogen (hPL/ chorionic somatomammotropin):**

Containing lactogenic and growth-promoting properties, this is a polypeptide placental hormone produced by the syncytium. HPL prepares the mother for lactation by stimulation of the mammary glands. This hormone is responsible for protein, glucose and fat regulation in maternal blood that meets the fetal needs during early pregnancy as it impacts on the metabolic rate of the mother, in order to increase energy supply for the embryo. Additionally, this hormone has an insulin-resistance activity, as well as a similar structure and growth hormone action.

- **Oestrogen:**

Oestrogen stimulates the growth of the uterus in order to accommodate the fetus. It also stimulates development of the mammary glands and prepares them for lactation. Additionally, it is essential for the development of secondary female sex characteristics.

- **Progesterone**:

Progesterone levels are high during pregnancy, as the placenta takes over production from the ovary. This hormone is important for decreasing uterine contraction and endometrium maintenance during pregnancy, thereby preventing premature labour.

- **Human placental growth hormone (hPGH):**

PGH is a trophoblast hormone, which is secreted by placenta throughout pregnancy in a continuous manner; its secretion is an essential for physiological adjustment to pregnancy, particularly in controlling maternal IGF1 levels. It also maintains maternal metabolic adaptation to pregnancy. This hormone possesses one glycosylation site and has 13 amino acids; therefore this feature differentiates it from pituitary growth hormone. Moreover, it is characterized by high somatogenic but low lactogenic activities. PGH slightly replaces

pituitary growth hormone, in the maternal circulation from 12-20 weeks up to term, which then disappears completely. Lower maternal levels of PGH can be seen in pregnancies with fetal growth retardation (McIntyre et al., 2000). A study undertaken in 2002 showed that PGH may impact on placental development through an autocrine or paracrine mechanism (Lacroix et al., 2002).

1.2 Normal term parturition

1.2.1 Definition

Expulsion of one baby or more from the uterus is known as delivery, childbirth, labour or parturition. It is a physiological process, and includes an integrated, sequential set of changes in the myometrium, decidua, and uterine cervix which can occur over a period of days or weeks. Before rupture of the fetal membranes, a group of events occur which involve biochemical connective tissue changes in the cervix. These precede uterine contraction and cervical dilatation.

Labour can be divided into three stages:

1. Cervical dilatation and shortening (ripening).

2. Infant descent and delivery.

3. Placenta delivery.

The process of labour starts with strong uterine contractions after cervical remodeling and membrane rupture.

1.2.2 Parturition as an inflammatory response:

The human placenta plays an essential role in inflammatory responses during labour, which is a mix of hormonal, mechanical stretch, neurological, and immunity processes (Keelan et al., 2003; Petraglia et al., 2010; MacIntyre et al., 2012). Both immune and endocrine pathways control the start of labour. Further, there are important hormones which have a crucial impact on parturition, such as oxytocin and corticotrophin releasing hormones which act as neuroendocrine mediators during labour (MacIntyre et al., 2012). A number of previous studies illustrated that inflammatory mediators, such as cytokines, result from infiltration of activated leucocytes into the cervix, placenta and myometrium during labour: these mediators participate in initiating the labour process.

Recently, there has been considerable evidence to support the view that, during physiological parturition, two T-cell subsets of memory-like T cells ($CD3^{+}CD4^{+}CD45RO^{+}$ and $CD3^{+}CD4^{-}CD8^{-}CD45RO^{+}$ cells) can be detected in the choriodecidua of placenta delivered by normal labour (Gomez-Lopezet al., 2013). During normal-term parturition, potent mediators groups, which consisted of cytokines (IL-1β and TNF-α), MMP-9 chemokines, cell adhesion molecules, (CXCL8 and CXCL10), and chemokine receptors (CXCR1-3), were discovered in these cell types (Gomez-Lopez et al., 2013). Various pro- inflammatory mediators, including prostaglandins as well as cytokines, have an obvious role during labour (Challis et al., 2000; Keelan et al 2003; MacIntyre et al., 2012; Fernandez & Díaz- Castro et al., 2015).

In a different study, placentas obtained from pregnancies with long (>15 hours) and short periods (< 5 hours) of labour, were compared with non-labour placentas delivered by Caesarean section for expression of inflammatory factors which are released into the maternal circulation, such as pro-inflammatory cytokines and anti-angiogenic factors. This study showed that there was a marked rise in all of these inflammatory (TNF- α and

IL-1β) as well as angiogenic factors in placentas delivered with normal delivery, compared to those delivered by Caesarean section, and this an increase was linked to the length of labour time. For instance, TNF-α, IL-1 β, are increased in placentas with short labour (Cindrova-Davies et al., 2007), however, placental vascular endothelial growth factor (VEGF-A) and HIF-1α increased in placentas with long labour (Ramsey et al., 1980). After labour there is also an increase in Interleukin 1 β, tumor necrosis factor- α (TNF- α), oxidative stress, and activation of the nuclear factor kβ -pathway in placenta; however, there was a decrease in tissue levels of placenta growth factor (Cindrova-Davies et al., 2007). Moreover, apoptosis was also activated in labored placentas and the magnitude of these changes was significantly associated with the duration of labour (Cindrova-Davies et al., 2007).

1.2.3 Parturition and oxidative stress:

Labour is a powerful inducer of oxidative stress, which occurs when an over production of reactive oxygen species (ROS) overwhelms the intrinsic anti-oxidant defences. During oxidative stress there is an increased production of free radicals, which may cause many cellular responses that depend on the insult severity and the compartment where oxidative stress species are produced (Roberts & Hubel, 2009; Burton & Janiaux, 2011). Anti-oxidants have been reported as a control system for the free radicals (Díaz-Castro et al., 2015). A number of studies have shown that there is a relationship between placental changes during labour and oxidative stress (Cindrova-Davies et al., 2007). During labour, intrauterine pressure increases and with this the progression of uterine contractions occurs. As a negative consequence, an intermittent utero-placental blood flow occurs, which leads to ischemic-reperfusion injury (Li & Jackson, 2002).

This is supported by research using Doppler ultrasound, which illustrated a linear inverse relationship between intensity in uterine contractions and uterine artery resistance (Brar et

al., 1988). In addition, previous studies demonstrated that during labour, there is an increase in oxygen consumption. As a result, mitochondrial respiration is raised; electrons formed with the electron transport chain are lost, leading to production of ROS (Stipek et al., 1995; Fernandez-Sanchez et al., 2011; Díaz-Castro et al., 2015). The inflammatory response at parturition time has been reported as a powerful cause of the formation of ROS (Díaz-Castro et al., 2015). Many potent pro-inflammatory mediators including cytokines, prostaglandin, interleukin-6 (IL-6), and TNF-α are produced during labour, which cause formation of ROS; free radicals re-induce the inflammatory process in a closed circle (Fernandez-Sannchez et al., 2011; Díaz-Castro et al., 2015). Our understanding of oxidative during the labour still has many gaps.

1.2.4 Abnormal placenta and apoptosis:

Apoptosis is defined as programmed cell death during in which cell self destruction and DNA fragmentation occurs. Studies have revealed that in the presence of overwhelming endoplasmic reticulum (ER) stress, the unfolded protein response UPR and pro-apoptotic CHOP protein have been activated by ER stress (Oyadomari & Mori, 2004; Zhang et al., 2006; Song et al., 2008). Further, the over production of ROS by mitochondria leads to cell death (Ott et al., 2007). Studies have shown that the apoptotic pathway occurs in placental tissues, facilitating the continuous renewal of villous trophoblast through gestation (Taglaueret al., 2013). Abnormal placentas from patients with GDM, which is linked to maternal obesity, have a significantly raised apoptotic index compared to normal ones (Thomas et al., 2014). Importantly, 13 genes (BCL2, BCL2L1, BCL2L11, CASP4, DAPK1, IκBκE, MCL1, NFκBIZ, NOD1, PEA15, TNF, TNFRSF25, and XIAP) were upregulated, while seven genes (BCL10, BIRC6, BIRC7, CASP5, CASP8P2, CFLAR, and FAS) were down regulated in GDM placentas (Thomas et al., 2014).

1.3 Maternal obesity

1.3.1 Definition:

Body mass index (BMI) is calculated as kg/h^2 (the weight of subjects divided by the square of the height); where k is the subject's weight in kilograms and their height in metres.

Maternal obesity is defined as BMI which exceeds >30kg/m^2. The prevalence of maternal obesity during pregnancy is high and has a relation to the clear incidence of adverse outcome for mother and fetus (Heslehurst et al., 2010; Jarvie & Ramsay, 2012).

1.3.2 Classification of obesity

Accumulation of excess body fat in the body results in obesity which has a negative impact on health. Females can be classified into the following BMI groups:

Underweight	BMI <18.5
Normal	BMI 18.5-24.9
Overweight	BMI 25-29.9
Obese	BMI 30-35
Morbidly obese	BMI >35

Recent studies have shown that obesity impacts on pregnancy, this has been studied using the waist-hip ratio; however, the relevant data for these parameters are rarely available (Wood. 2006).

1.3.3 Complications of obesity:

Obesity during pregnancy and the intrapartum time has many adverse outcomes (Yu et al 2006) such as gestational diabetes, hypertension and thromboembolic disorders. Moreover, women are at risk of suffering an abortion, fetal loss, caesarean section (C/S), haemorrhage, infertility, and instrumental delivery (Yu et al 2006, Heslehurst et al., 2008). Obese women have prolonged labour and reduction in uterine contraction (Vahratian et al. 2004, Kominiarek et al. 2011). Multiparous obese women have fewer labour disorders, such as pre-eclampsia than nulliparous females (Kominiarek et al. 2011, Jensen et al.1999).

Obese females have been found to be at high risk of developing macrosomia, which may lead to shoulder dystocia (Robinson et al. 2003; Overland et al., 2012). Postpartum haemorrhage is by far the most common ailment in obese females (Heslehurst et al., 2008), and is most likely associated with uterine atony (Zhang et al. 2007;Bateman et al., 2010; Fyfe et al., 2012,). Induction of labour is more common in obese women; furthermore, obesity also interferes with using certain types of pain medications, such as an epidural block. Long-term weight gain during the postpartum interval may lead to the development of maternal obesity. Increasing weight throughout pregnancy and continuing to gain weight for 1 year after delivery is linked to being overweight 10 years or later (Rooney & Schauberger 2002).

1.3.3.1 Pre-eclampsia and obesity

Pre-eclampsia is a clinical syndrome associated with pregnancy, and is defined as high blood pressure (>140/90 mmHg), on at least 2 occasions, 6 hours apart and associated with proteinuria (>300 mg/L in a 24 hour urine collection) which occurs after 20 weeks of pregnancy (Jeyabalan 2013; Redman et al., 2014). It occurs in 2%- 4% of all

pregnancies in the developed world and is considered one of the leading causes of maternal mortality and morbidity across the world (Shahzya et al., 2014).

Pre-eclampsia is associated with maternal complications, such as premature labour and fetal growth restriction (Redman & Sargent, 2005). Spiral arteries undergo remodeling during normal pregnancy; they are then dilated, and their responsiveness to vasoconstrictive stimuli is lost. Consequently, blood enters the intervillous space in intermittent manner with low pressure (Lyall, 2006). However, there is a partial remodeling of the spiral arteries during pre-eclampsia (Lyall et al., 2013). Therefore, blood flow with high pressure leads to hydrostatic injury of placental villi. Additionally, oxidative stress occurs due to perfusion of fully oxygenated arterial blood in a non-pulsatile manner and oxygen delivery fluctuations (Roberts & Hubel, 2009; Burton & Janiaux, 2011). The placenta has a critical role in the pathophysiology of pre-eclampsia (Paleiet al., 2013). The risk for pre- eclampsia increases during obesity by about 2-3 fold (Bodnar et al., 2005); this can be reduced by weight loss (Magdaleno et al., 2012). Furthermore, an increase in BMI increases the risk for pre-eclampisa. The relationship between obesity and pre-eclampsia has been demonstrated in different people around the worldwide *(*Mahomed et al. 1998, Hauger et al., 2008). There are suggestions that fluid retention during pre-eclampsia leads to weight gain (Fortner et al., 2009).

Obesity as a feature of metabolic syndrome (obesity, hypertension, impaired glucose tolerance, insulin resistance, and dyslipidemia) can lead to pre-eclampsia by various mechanisms such as releasing angiotensinogen from adipose tissue, reduction in available nitric oxide because of oxidative stress, and increasing sympathetic tone (Dandona et al., 2005). Moreover, leptin secreted by placentas contributes to circulation concentrations, and is associated with an increase in maternal BMI in pre-eclampsia (Laivuori et al., 2000; Teppa et al., 2000; Chappell et al., 2002).

1.3.3.2 Inflammation and obesity

Inflammation is a key feature of obesity, and is a feature of pre-eclampsia and cardiovascular disease. Altered endothelial function occurs due to the release of various inflammatory mediators by adipose tissues, which become more active during obesity. In obese women there is higher production of inflammatory mediators, C-reactive protein (CRP) and also adipocytes (Wolf et al., 2001, Bodnar et al., 2005). Vascular tissue damage occurs due to production of interleukin-6 which increases during obesity, cardiovascular disease as well as insulin resistance (Grimble, 2002). A strong relationship between obesity and pre-eclampsia has been demonstrated: dyslipidemia causes vascular endothelial damage in obesity and pre-eclampsia. There is a strong correlation between lipid concentrations in patients with pre-eclampsia. This suggests that obese women may have a higher risk of pre-eclampsia due to abnormal lipid deposition (Roberts et al., 2011). One study measured serum CRP levels as a marker of inflammation and trigyceride levels; they extended previous findings that pre-pregnancy BMI is a risk factor for pre-eclampsia inflammation and triglyceride levels at <20 weeks may be important mediators of the BMI-preeclampsia association. Inflammation was the most important mediator in their population (Bodnar et al., 2005).

It has also been shown that there is an association between increases in circulating leptin in pre-eclampsia and maternal BMI (Teppa & Ness 2000; Chappell et al., 2002). Adipose tissues produce TNF-α which leads to endothelial damage, insulin resistance, and oxidative stress. It has been shown that there is an association between increasing in circulating leptin in pre- eclampsia and maternal BMI (Teppa & Ness 2000; Chappell et al., 2002). In addition, it has been shown that TNF-α levels (secreted by adipose tissue) relate to the degree of adiposity as well as insulin resistance (Tzanavari et al., 2010).

1.3.3.3 Insulin resistance and obesity

Two thirds of individuals with excess weight have insulin resistance, which strongly predisposes to type 2 diabetes and cardiovascular disease (Jeyabalan 2013). Additionally, it has been detected that development of type 2 diabetes is more rapid in obese woman, suggesting that insulin resistance during obesity leads to an increase in adverse outcomes (Roberts et al., 2011). Insulin resistance is common during pre-eclampsia, and therefore it increases the risk of cardiovascular disease (Kaaja 1998; Laivuori et al., 2000). During dyslipidemia there is an overproduction of free fatty acids by adipocytes, and this has been proposed to contribute to insulin resistance and oxidative stress which are clear signs of obesity (Jeyabalan 2013).

1.3.3.4 Oxidative stress and obesity

The relation between oxidative stress and obesity is partly due to free fatty acids, the decrease of circulating anti-oxidants, and greater inflammation (Wallstrom et al., 2001; Dandona et al., 2005). Therefore, it is clear that the development of pre-eclampsia in obese pregnant women is due to oxidative stress which leads to endothelial function change, followed by vascular injury (Kaaja 1998).

1.4 Heat shock proteins (HSPs)

1.4.1 Background

The placenta has an essential role in several complex mechanisms, such as maintaining pregnancy to term. Placental malfunction is linked to complications involving preterm labour, pre-eclampsia, fetal growth restriction, and miscarriage (Petraglia 2010, Burton & Janiaux 2011).

Heat shock proteins (HSPs) are a group of proteins which are normally expressed by all prokaryotic and eukaryotic (Taylor & Bwnjamin 2005). HSP expression increases

following exposure cells to stress; for example, tissue hypoxia, ischemic-reperfusion injury and endotoxins. Moreover, they respond to multiple stimuli, and so their role in cell protection are significant; one of these is prevention of cell denaturation and degradation under high temperature conditions (Inaguma et al., 1996; Huey 2004) as well as chaperone proteins that have ability to protect the cells from various types of damage and help refold proteins (Borges et al., 2012; Abdulsid 2013; Bakthisaran et al., 2015).

HSPs are involved in the regulation of many complex processes; for example, they protect the cells from oxidative stress by regulating the activity of specific enzymes, which is valuable in neurodegenerative disease development. In apoptosis and proliferation, small HSPs (sHSPs) react with certain proteins and regulate the cell redox state, although the regulation of many cellular processes by sHSP is questionable, while the mechanisms of their action remain unclear. Perspective research for studying sHSP structure, properties and mechanism of action, will explain the necessary information relevant to understanding multiple physiological processes (Bakthisaran et al., 2015).

The level of HSPs in cells is affected by many patho-physiological factors: for example, their levels rise dramatically in congestive heart failure (Dohke et al., 2006) and exercise, while spinal cord isolation and denervation causes HSPs level to drop (Huot et al., 1997; Huey et al., 2004).

1.4.2 Families of HSPs:

HSPs have several important functions, which include helping cells to cope with stress. Some of them are induced by sustained damage; for example, heat shock, oxidative stress, ischemia and hypoxia; however, others are expressed constitutively (Lanneau et al., 2010). According to their molecular weight, size, function, and structure, heat shock proteins have been classified into groups. HSPs are responsible for proteins folding properly in the cells

(chaperone activity); they are classified into sHSPs HSP20, HSP27, HSP40, HSP60, HSP70, HSP90 and HSP110 (Schlesinger 1990) Table 1-1.

A recent study conducted in our laboratory showed that HSP70 protein expression was increased in the middle area compared to the outer area ($p = 0.03$) in non-labour and in both the inner and middle areas compared to the outer area ($p= 0.01$ and $p = 0.02$ respectively) in labour. HSP70 was increased in the preeclampsia non-labour group compared to the control non-labour group in the inner area ($p = 0.003$) and in the control labour group compared to the preeclampsia labour group at the middle area ($p = 0.001$). It was concluded that HSP70 is expressed in a spatial manner in the placenta, and that the physiological and pathological significance of these remain to be elucidated (Abdulsid et al., 2013). In addition, it has been reported that HSP27 protein expression is significantly increased in the preeclampsia labour group compared to the control labour group at the 2- to 4-cm site ($p=0.02$) (Abdulsid et al., 2013).

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Table 1-1: HSP families

Heat shock proteins	**Molecular weight (kDa)**	**Action**
The HSPB group of HSPs. Eleven members in mammals including HSP27, HSPB6 or HSPB1 (Kampinga et al., 2009).	20-30 Da	1. Chaperone activity, stabilization of cytoskeleton, anti-apoptotic and anti-oxidant function. 2. Smooth muscle relaxation, cardioprotection, chaperone activity, anti-apoptotic.(Bakthisaran et al., 2015).
HSP40	40 kDa	Co-factor of Hsp70 (Fan, C.Y et al., 2003).
HSP60	60 kDa	Involved in protein folding after its post-translational import to the mitochondrion/chloroplast (Kleinridders et al., 2012).
The HSPA group of HSPs including HSP71, HSP70, HSPP72, Grp78 (BiP), HSP70 found only in primates	70 kDa	Protein folding and unfolding, provides thermo-tolerance to cells on exposure to heat stress. Also prevents protein folding during post-translational import into the mitochondria/chloroplast. (Henstridge et al., 2014)
The HSPC group of HSP including HSP90, Grp94	90 kDa	Maintenance of steroid receptors and transcription factors (Murphy, et al., 2011).
HSP104, HSP110	100 kDa	Tolerance of extreme temperature (Trivedi et al., 2010).

1.4.2.1 Small heat shock protein (sHSP):

This group has chaperone function and the ability to correct unfolded proteins, and changes to proteasomes. These sHSPs exhibit thermoprotection *in vivo* as well as chaperone activity *in vitro*. The sHSPs exist as oligomers formed of 9-32 subunits in their native state with a monomeric molecular mass of 12-43 kDa (Kaape et al., 2003). The sHSPs contain a highly conserved stretch of 80-100 amino acids including C-terminal domains called the "α-crystallin domain" (ACD), N-terminal domain (NTD) and C-terminal extension (CTE) with molecular masses of 12-43 kDa. These extensions facilitate chaperone function of sHSPs (Franck et al., 2003). Some members of the sHSP family form large oligomeric species such as HSP27, αA- and αB-crystallin (Kappe et al., 2003; Kriehuber et al., 2010). The majority of sHSPs are inducible by heat; some have a crucial role during stressful conditions like cell development (Chowdary et al., 2004).

1.4.2.2 HSP20:

During labour uterine contractions are reported to be associated with intermittent perfusion of the utero-placental blood flow. This may provide a basis to our understanding of ischemia reperfusion type of placental injury. During labour imaging Doppler ultrasound studies have shown a negative association between the intensity of the uterine contractions and uterine artery resistance (Brar et al., 1988). Furthermore, it has been reported that during labour the placenta is affected by many pathways linked to oxidative stress (Cindorva-Davies et al., 2007).

HSP20 is a member of sHSP and called HSPB6. It is a17kDa protein. Human HSP20 contains a α-crystallin domain (Taylor & Benjamin, 2005; Kampinga et al. 2009). Its structure includes a homology sequence of troponin and a domain inhibiting platelet aggregation (Fan et al., 2005; Salinthone et al., 2008). There are two kinds of oligomers (Kato et al., 1994, Van de Klundert, 1998) and HSP20 can become a high molecular

weight molecule after interacting with other proteins to form a complex (Chernik et al., 2007). It has been demonstrated that HSP20 found in both skeletal and smooth muscle form a complex with HSP27, another sHSP that have been identified in the myometrium as a contraction-associated protein (White et al. 2005). Importantly, interaction is a functional one, with HSP20 regulating the function of HSP27 (Brophy et al. 1999). HSP20 can be phosphorylated by both cAMP- and cGMP- dependent protein kinases (PKA/PKG) and increases in phosphorylation are correlated with smooth muscle relaxation (Beall et al. 1999). The addition of phosphopeptide analogues of HSP20 into bovine carotid artery smooth muscle leads to inhibition of agonist-induced muscle contractions (Beall et al. 1999). Experiments on a rat heart revealed that HSP20 is present in a fraction form in the cytoplasm and part of HSP20 was associated with actin (Pipkin et al., 2003.). These authors suggested that phosphorylation of HSP20 can be achieved by treatment with the nitric oxide donor sodium nitroprusside; therefore, phosphorylated HSP20 leads to increasing in the rates of shortening and re-lengthing in rat cardiomyocytes.

1.4.3 Expression and regulation of HSP20:

The expression of HSP20 during labour has been shown to be decreased in rat and human myometrial smooth muscle (Cross et al. 2007, Tyson et al. 2008) and red and white rat skeletal muscle (Huey et al., 2004) and rat heart (Pipkin et al., 2003). A study performed on rat myometrium revealed that, during late pregnancy and labour, HSP20 expression is down-regulated and this may be significant for the onset of labour (Cross et al. 2007).

Heat shock factor-1 (HSF-1) has no an impact on HSP20, (Sugiyama et al. 2000, Taylor & Benjamin 2005; Kirbach et al. 2011). There is a correlation between HSP20 expression and effective cyclic nucleotide-induced relaxation, which suggests that HSP20

Phosphorylation could be a dominant mechanism of smooth muscle relaxation (Beall et al. 1999; Pipkin et al., 2003).

1.4.4 HSP20 and maternal obesity

During heat stress HSPs have significant roles in cell protection. Obesity is associated with mechanisms such as low-grade inflammation and mitochondrial dysfunction that may have a significant influence on the pathology of type 2 diabetes mellitus, insulin resistance and the expression of sHSPs which increases during these conditions. Calcitonin, epinephrine and gene-related peptide are insulin antagonists and increase phosphorylation of HSP20 on ser16, while decreasing phosphorylation on ser157. therefore, HSP20 can be a prominent mediator in insulin action (Wang et al. 1999a, Wang et al. 1999b). Importantly, obesity may enhance oxidative stress which in turn may lead to a release of inflammatory kinase such as protein kinase C (PKC), inhibitor of NF-k beta kinase (IKK), and c jun amino terminal kinase (JNK), this may inhibit insulin signaling. In addition, mitochondrial dysfunction is involved in the pathogenesis of type 2 diabetes mellitus (Khan et al., 2014) by two mechanisms; the first is the accumulation of abnormal lipid species in the liver and skeletal muscle due to poor fatty acid oxidation. The second is by changing the redox state (Darren et al., 2014 and Khan et al., 2014).

1.4.5 Oxidative stress and HSP20:

It has been reported that HSP20 is detectable in many tissues such as skeletal muscles, cardiac and smooth muscles (Fan et al., 2005; Salinthone et al., 2008). HSP20 is like other HSPs in that it can be expressed by a range of stimuli, including oxidative stress (Zhao et al., 2009). Due to the important roles of HSP20, it might be involved in the placenta in labour; however this has not been investigated. Gestational diabetes mellitus (GDM) is a significant complication related to obesity during pregnancy causing metabolic disturbance, and, consequently, oxidative stress due to ROS (Lappa et al.

2011). Pathological complications of gestational diabetes include abnormal fetal outcome. An imbalance between the systemic manifestations of ROS and the ability to readily detoxify the reactive intermediates or to repair the damage, will affect the degree of damage. Peroxidase and the production of free radicals have a toxic impact on the normal cell state and may lead to total cell damage, including damage to proteins, lipids, and DNA. Furthermore, oxidative stress may lead to the disruption of normal mechanisms of cellular signaling as some ROS act as cellular messengers in redox signaling. Antioxidant enzymes can reduce this damage by breaking down ROS and prevent diabetic embryopathy in rat models of diabetes in pregnancy (Higa et al., 2012).

1.4.6 HSP20 phosphorylation

Phosphorylation of HSP20 has been implicated in many physiological and pathophysiological processes such as platelet aggregation, atherosclerosis, smooth muscle relaxation, insulin resistance, myocardial infarction, Alzheimer's disease and exercise training (Fan & Kranias 2011).

During cell stress HSP20 can be activated to its active phosphorylated state by stress kinase in response to elevation of cyclic nucleotide concentrations. Phosphorylation of HSP20 usually occurs and HSP20 undergoes multisite phosphorylation and can be measured with two-dimensional electrophoresis (Beall et al.1997; Wang et al., 2001). CAMP-dependent protein kinase A (PKA) is responsible for ser16 phosphorylation while ser157 phosphorylated is by a PI3-K-dependent pathway: this phosphorylation can occur on insulin stimulation (Wang et al., 1999). Ser59, which is the third phosphorylation site, was discovered *in vitro* by PKA (Beall et al., 1999). Phosphorylation of HSP20 at sites present on the peptide LPPGVDPAAVTSALSPEG containing residues 123-140 occurs with stimulation of bovine carotid arteries by phorbol esters or forskolin and IBMX application (Beall 1999). HSP20 is phosphorylated *in vivo* in a cAMP-dependent manner

and the phosphorylated form promotes muscle relaxation (Rembold et al. 2001; Frobert et al., 2005, Meeks et al., 2005, Rembold 2007). Studies have been shown that there was a significant relationship between HSP20, alpha-smooth muscle actin and HSP27, furthermore, phosphorylation of HSP20 occurs especially during cAMP-mediated relaxation in human myometrium (Tyson et al, 2008).

Importantly, inhibition of agonist-induced muscle contractions occurs with application of physphopeptide analogues of HSP20 into bovine carotid artery smooth muscle (Beall et al., 1999). Following phosphorylation, HSP20 has been shown to be linked with actin (Rembold et al. 2000), and the actin-binding protein a-actinin (Tessier et al. 2003) that is found at focal adhesions in smooth muscle cells. Therefore, phosphorylated HSP20 may cause maintaining uterine quiescence during pregnancy. Indeed it has been shown that expression of HSP20 in rat myometrium is markedly decreased during late pregnancy and labour (Cross et al., 2007).

Thus it is clear that phosphorylation of HSP20 by PKA is important in modulating contractile activity in the human myometrium. Agonists that stimulate cyclic AMP production may cause relaxation and this could have important beneficial effects in altering the outcome of premature contractions prior to term. It has been found that HSP20 acetylation has a significant influence on activity of human myometrium. The non-nuclear lysine deacetylase such as histone deacetylase 8 has been reported to interact with HSP20 by affecting its acetylation. However, by using of selective histone deacetylase 8 inhibitor the HSP20 acetylation was increased without any increasing of nuclear-resident histone acetylation and with no marked changes in gene expression. These effects may have a relationship with inhibition of spontaneous and oxytocin-augmented contractions of ex vivo human myometrial tissue strips (Karolcak-Bayatti et al, 2011).

1.6. Apoptosis

Under pathological and physiological conditions, programmed cell death can occur (apoptosis). It is associated with a cascade of cysteine proteases being activated and as a result, a subsequent release of cytochrome c occurs due to damage of mitochondria, and also fragmentation of double stranded DNA and cell shrinkage (Buja2005). Recent studies have illustrated that HSP20 can react with key pro-apoptotic signaling proteins to reduce apoptosis (Fan et al. 2005). HSP20 produces its anti-apotoptotic influence by binding to Bax, and therefore Bax translocation from the cytosol into the mitochondria will not occur. Cytochrome c release is inhibited and caspase-3 activity is suppressed, which stops damage to mitochondria and maintenance of their shape (Fan et al., 2005).

1.7. Paroxonases (PON2)

Human labour involves neurological, hormonal, mechanical stretch and systemic inflammation and the placenta plays a significant role (Keelan et al., 2003; Petraglia et al., 2010). The paroxonases (PONs) are multifaceted pleiotropic enzymes, there are 3 genes for the 3 forms PON1, PON2 and PON3 and they are located on chromosome 7q21.3–22.1 (Primo-Parma et al., 1996). Research in the PON family has increased greatly in the last few years, particularly in the cardiovascular disease field (Martinelli et al., 2012). PONs have several functions that involved in different biochemical pathways including protection against lipid peroxidation and oxidative damage, modulation of ER stress and regulation of proliferation and apoptosis as well as contributing to innate immunity and detoxification of reactive molecules of drugs (Primo-Parma et al., 1996, Draganov & La Du 2004). Phylogenetic studies have reported that PON1 and PON3 were produced from PON2 gene duplication (Primo-Parma et al., 1996; Draganov & La Du 2004). PON1 and PON3 are circulating proteins that are bound to high-density lipoproteins; in contrast, PON2 is expressed in many tissues (Ng et al., 2001). Paraoxonases (PONs) are ubiquitous proteins which have a role in cell protection against oxidative stress (Ng et al., 2001), and

against LDL oxidation (Ng et al., 2001, Rosenblat et al., 2003). PON2 has a molecular weight of 44kDa and is expressed in different tissues, such as placenta, heart, lung, liver, stomach as well as intestine (Primo- Parma et al., 1996; Reddy et al., 2001; Ng et al., 2001; Draganov & La Du, 2004). PON2 is also expressed in macrophages, arterial walls and smooth muscles (Ng et al., 2001). PON2 is found attached to the plasma membrane inside the cell. It also has an N-terminal, and so it is similar to both PON1 and PON3, however, PON2 is different from PON1 and PON3, as the later two can be detected in the circulation bound to LDL or HDL (Ng et al., 2001).

A recent study has shown the role of PON2 in placenta. It was demonstrated that PON2 has been found in all placenta samples that recruited from labour and non labour cohorts, one at 43 kDa and one at 62 kDa. Further, there was a significant reduction in PON2 protein expression at the middle zone of placentas in the labour group compared to non-labour group. However, there was a significant increase in mRNA within the middle zone of placentas of labour group only, which may suggest a different response to oxidative stress during uterine contractions (Awarfaly et al, 2014).

There are several diseases linked to PON2 polymorphisms. Two polymorphisms were have been described are a pair of amino acid substitution with alanine or glycine at position 148 (A148 G) and have been linked to variations of birth weight, fasting blood sugar levels, as well as changes in total and LDL cholesterol (Hegele et al., 1997; Hegele et al., 1998; Hegele et al., 1999). Cysteine or serine, which are also a pair of amino acid substitution at position 311 (S311C), is associated with Alzheimer's disease, coronary heart disease, and ischemic stroke in diabetes type 2 patients (Sanghera et al., 1998; Kao et al., 2002; Janka et al., 2002; Chen et al., 2003). Inflammation as well as oxidative stress is clear signs during maternal obesity; PON2 has anti-oxidant properties and protect the cell against oxidative stress. Lactones have been suggested to be the natural substrates of PON2 and PON2

lactonase activity has been shown to be associated with this enzyme's biological antioxidant properties.

1.8. Aims

The mechanisms that regulate the uterine contractions during labour are much better understood however, there are many gaps that need to be clarified. Nothing is known about the role of HSP20 in the placenta or whether it plays a role in labour and obesity. Placental oxidative stress is a feature of human labour and maternal obesity. HSP20 and PON2 have been previously linked to oxidative stress and inflammation. However, there is a lack of data regarding the placental expression of HSP20 and PON2 in labour and during maternal obesity. Also nothing known about the placental expression of HSP20 at different placental zones during labour and obesity. Whether there is any change in expression of placental PON2 during maternal obesity. Therefore, the aims of the present study were to:

(1) examine the spatial expression of HSP20 in placental tissue collected from women who delivered vaginally following normal term labour and to compare the results with the equivalent area of placental tissue collected from women who delivered by cesarean section (not in labour).

(2) examine the spatial expression of HSP20 and PON2 in placentas obtained from women who delivered by caesarean section (not in labour) and who had different BMIs.

(3) examine HSP20 and PON2 protein and mRNA expression in defined placenta zones of obese pregnant women and again to compare the results with normal pregnant women.

Chapter 2 – Material and Methods

2.1 Ethical approval

This project was approved by the West of Scotland research ethics service-REC 4 (REC reference number 13/WS/0149, IRAS ID 130896) appendix 1. Ethics procedures followed the guidelines of the Declaration of Helsinki. Dr Ekram Abdulsid has explained the study to the women and obtained signed consent from the patients before delivery. Placental samples were ultimately stored in the Medical Genetics Institute at Yorkhill Children's Hospital.

2.2 Subjects and placental collection

At the Southern General Hospital (Glasgow) human normal term placentas were collected from (Group 1) healthy pregnant women who had no adverse conditions during pregnancy such as hypertension, pre-eclampsia, or gestational diabetes or any other pathological condition after taking their signed consents prior to delivery. They delivered either by spontaneous vaginal delivery (a tight group with labour ranging from 3-8 hours (labour group, n=6) or by elective C/S which was performed for obstetric reasons such as previous C/S or breach presentation (non-labour group, n=6). The placentas were free of infection and this was confirmed by the pathology report. (Group 2) include women who had normal pregnancies with different BMIs. Four BMI groups were studied: group 1: BMI <30 (n=6), group 2: BMI 30-35 (n=6), group 3: BMI 35-40 (n=6) and group 4: BMI>40 (n=6).

Table 2-1: Patient demographics of clinical data.

Sample	Age (years)	Parity	Smoker	Gestation (weeks and days)	Birth weight (grams)	Placenta weight (grams)	Mode of delivery	BMI
NLG 1	22	G3P2	NO	38 WKS+4	3840	680	ELC/S	37
NLG2	34	G2P1	NO	41WKS+3	3100	450	ELC/S	30.6
NLG3	26	G2P1	YES	39 WKS	2860	683	ELC/S	23
NLG4	22	PG	YES	39WKS+2	3050	465	ELC/S	21
NLG5	34	G3P2	NO	39WKS+1	3555	685	ELC/S	32.4
NLG6	32	G2P1	NO	39 WKS	4255	605	ELC/S	27
LG1	27	PG	NO	40 WKS+2	4192	585	SVD	24
LG2	28	PG	NO	40WKS+4	3646	595	SVD	28
LG3	22	PG	NO	41WKS+3	3940	700	EMC/S	34
LG4	25	PG	NO	41WKS+3	3310	486	SVD	24
LG5	28	G3P2	NO	37WKS+6	3354	636	SVD	26
LG6	26	G4P3	NO	41WKS+1	3870	535	SVD	33.9
BMI 1-1	26	G2P1	YES	39	2860	683	ELC/S	23
BMI 1-2	22	PG	YES	39+2	3050	465	ELC/S	21
BMI 1-3	32	G2P1	NO	39	4255	605	ELC/S	27

BMI 1-4	25	G2P1	NO	40	5250	980	ELC/S	29.1
BMI 1-5	32	G2P1	NO	39	3510	716	ELC/S	24.4
BMI-1-6	36	G2P1	NO	39+1	4220	630	ELC/S	23
BMI 2-1	34	G2P1	NO	41+3	3100	450	ELC/S	30.6
BMI 2-2	34	G3P2	NO	39+1	3555	685	ELC/S	32.4
BMI 2-3	33	G2P1	YES	38+6	3450	625	ELC/S	33.2
BMI 2-4	29	G2P1	NO	37+3	3210	675	ELC/S	32.2
BMI 2-5	43	G4P3	NO	37	3515	750	ELC/S	32.8
BMI 2-6	37	PG	NO	38+3	3460	595	ELC/S	33.1
BMI 3-1	25	G2P1	NO	39+5	3770	690	ELC/S	36.7
BMI 3-2	40	G3P2	NA	38+4	4850	940	ELC/S	36.6
BMI 3-3	32	PG	NA	38+5	2740	430	ELC/S	37.9
BMI 3-4	36	G2P1	NA	39+3	4035	790	ELC/S	35.9
BMI 3-5	33	G3P2	YES	40+1	3725	722	ELC/S	37.4
BMI 3-6	22	G3P2	NO	38+4	3840	680	ELC/S	37
BMI 4-1	29	G3P2	NO	39+3	4015	995	ELC/S	40.3
BMI 4-2	46	G3P1+1	NO	38	2845	590	ELC/S	42.3
BMI 4-3	42	G3P2	NO	39+3	3550	685	ELC/S	44.7

BMI 4-4	25	G3P1+1	NO	38+4	3575	645	ELC/S	49
BMI 4-5	38	G2P1	NO	39	3736	695	ELC/S	47.2
BMI 4-6	33	G2P1	NO	39+2	2940	494	ELC/S	52

NLG; Non-labour group, LG; Lbour group, EMC/C; Emergency caesarean section, ELC/S; Elective caesarean section, SVD; Spontaneous vaginal delivery, WKS; Weeks, BMI; Body mass index

2.3 Sampling methods

All placentas were collected immediately after delivery. They were then washed under continues tap water and then the amnion was cut off. Following that each placenta was divided into three zones by an imaginary line. From the cord insertion point measurements for the placental samples (~1cm^3) were taken for every control patient. These measurements were as following; 0-2 cm (inner zone), 2-4 cm (middle zone) and 4-6 cm (outer zone) as shown on Figure 2.1. Four samples were obtained from each zone i.e. four quadrants per zone (Abdulsid & Lyall, 2013; Abdulsid et al., 2013, Abdulsid et al., 2014). Placentas had a central cord insertion. All samples were rinsed and immediately flash frozen in liquid nitrogen.

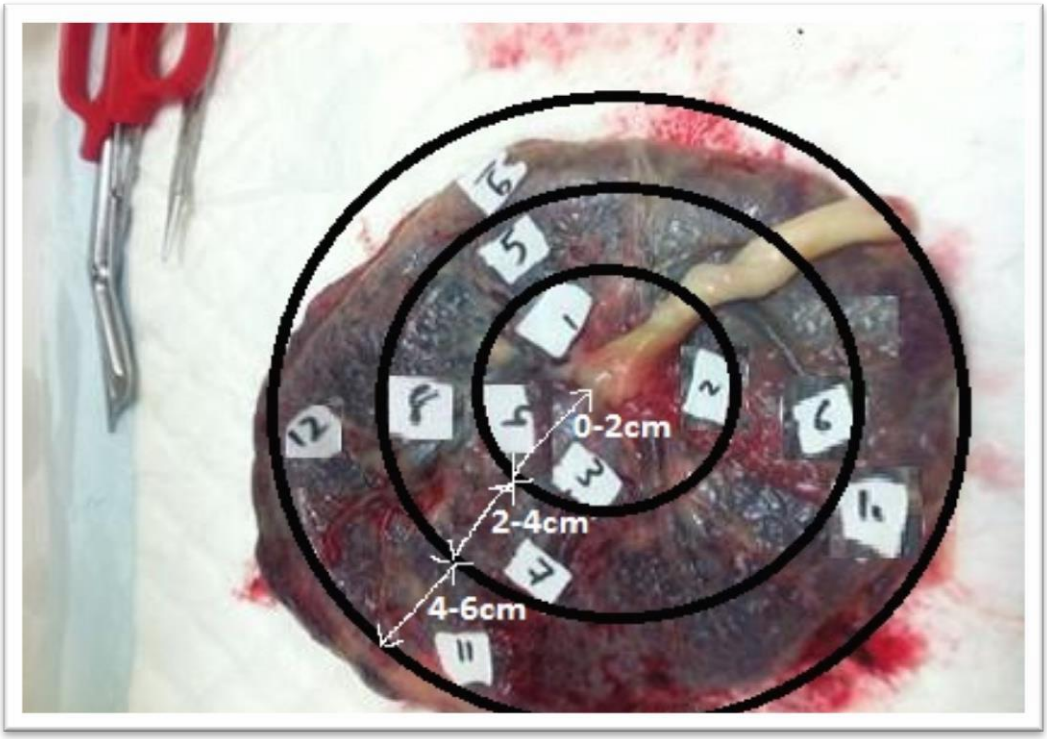

Figure 2. 1: Placental samples sites of collection

2.4 Placental tissue collection and processing

2.4.1 Protein and molecular analysis of collected tissues

The equipment used for tissue collection were sterile. An appropriate number of cryostat containers were placed inside dry ice for staying cold. The tissue samples were washed in phosphate buffered saline (PBS) three times until they were free from blood. Following that samples were immediately flash frozen in liquid nitrogen. After freezing the samples were transported to the laboratory on dry ice and stored in storage -80 C.

2.5 Preparation of placental tissue for protein expression analysis

2.5.1 Placental tissue homogenisation

Homogenising buffer consisted of: (25 Mm Tris + 1 mM EDTA + 25 mMsucrose) and was kept at 4°C. Before the experiment mammalian cell protease inhibitor cocktail 12.5 ul/ml was added to homogenizing buffer. Next, placental tissue samples were removed from -80 ºC. The samples were ground down using a pestle and mortar in the presence of liquid nitrogen until they became a powder (Alwarfaly et al., 2014). After that homogenizing buffer mixed with protease inhibitor cocktail was added to the sample powder. A rotor-stator homogeniser (polytron® PT 1600E, Lucerne, Kinematica) was used for homogenisation of all tissue samples which was set up at speed 20, with 5x10s intervals and 1 minute cool down on wet ice in between for every sample. Following centrifugation of the tissue homogenate at 4860g for 10 minutes at 4°C the supernatant containing the protein (particular and cytosolic fraction) was removed, allocated and stored -80°C. The pellet debris was discarded.

2.5.2 Summary of homogenisation protocol

- Homogenising buffer was prepared (25 Mm Tris, 1mM EDTA, 25mM sucrose

(stored at 4 °C).

- Prior to the experiment protease inhibitor cocktail 12.5 ul/ml was added and kept on wet ice.
- Sterile bijous for the number of the samples and two sterile spatulas were placed on dry ice.
- A sterile pestle and mortar was prepared then the liquid nitrogen was poured into it.
- A number of bijous were labelled each one was equivalent to the specific sample name. Their weights were recorded after removal of the lids.
- The samples were retrieved from -80°C storage then ground down rapidly in the mortar and pestle.
- The tissue powder was then placed in the cooled relevant container and reweighted again in order to determine the tissue weight (gram).
- To the weight of the tissue samples the 3x the volume of buffer (ml) was added.
- Homogenisation was performed using the tissue homogeniser, which was set up at 20 speeds for every sample and 5x 10 seconds burst were used with 1 minute cooling down between each. After that the sample was kept on wet ice.
- For separation of the supernatant protein, the samples were put in labelled 15 ml conical tubes and centrifugation was performed (4860g for 10 min at 4 °C). The supernatant protein was pipetted into 1.5 sterile eppendorfs tubes after ensured it was labelled and the junk protein was disposed in the bin.

2.5.3 Bradford assay for protein estimation

This assay is used for quantify protein concentration, the method described by (Bradford, 1967). In this method Bradford reagent which is acidic brown comassie-dye reagent was added to protein solutions in order to measure the sample protein concentration. Alteration of the colour changes

the absorbance maximum of the protein from 465 nm to 595 nm. The colour response of the sample protein is compared to that of standard samples that is usually bovine serum albumin (BSA). The stock standard solution (STD) BSA (1mg/ml) was used to create the standard curve Table 2-2.

Table 2-2: Dilutions of the BSA which were then used for the protein Standard curve

STD concentration (µg/ml)	Vol. stock STD (µl)	Vol. of d H2O (µl)
0	**N/A**	**50**
200	**10**	**40**
400	**20**	**30**
600	**30**	**20**
800	**40**	**10**
1000	**50**	**N/A**

2.6 Steps and protocol

Initially, dilution of placental samples was performed at 1:40 (1 µl of homogenate and 39µl of dH_2O).A BioPhotometer® was used for measurement of the samples. A standard curve was formed from the measurements obtained from the standards and the samples concentrations were automatically measured against this curve. The sample loading was 50 µg total proteins per lane in Western blots. The final protein calculation was worked out from (the reading value /1000x40). Dividing by 1000 converts µg to mg (mg/ml) then dividing by the dilution factor of 40 gives the final value.

Figure 2-2 shows a typical standard curve. The Y axis shows the reading and the x axis shows the protein concentration.

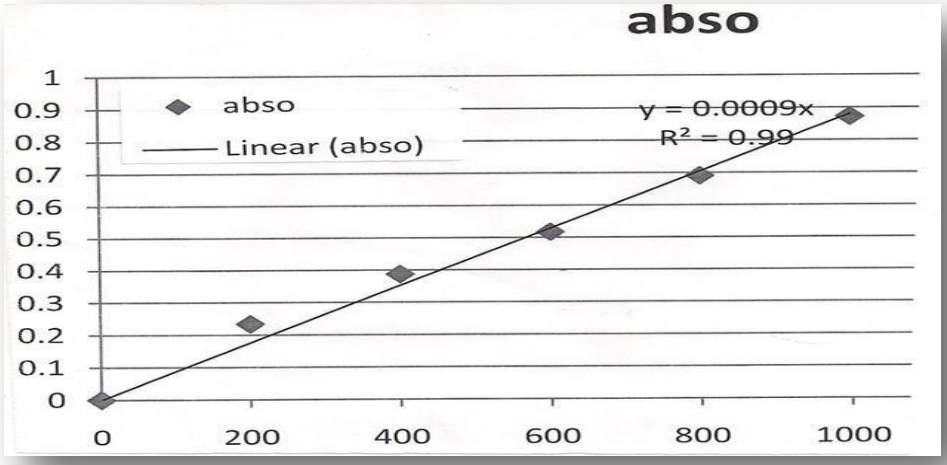

Figure 2-2: The standard curve reading

2.6.1 Western blot

In order to reduce and denature the protein, sodium deodecyl sulphate (SDS) and mercaptoethanol were used. SDS gives the protein a net negative charge for the sodiumdeodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

2.6.2 Gel electrophoresis

First of all, preparation of the samples and including_control were carried out by mixing the samples with loading buffer 1:1 (Abdulsid *et al*., 2013). They were then boiled for 5 minutes at 95°C on heat block. 50µl of protein sample was loaded in each well. To demonstrate the size of proteins 20µl pre-stained SDS-PAGE Standards (Low range, BIO-RAD, cat.no. 161-0305) were also run on a separate well. Moreover, 50 µl of the same positive control (placenta homogenised) known to express HSP20 and PON2 were loaded into the gel and used as a reference sample for every gel. The Protean II® xi Cell System (Bio-Rad Laboratories, Hemelhempstead, UK) was

used and gels were run at 300 V, 27 Amp for the first hour then up to 30 Amp for a total time period of about 5 hours Figure 2-3.

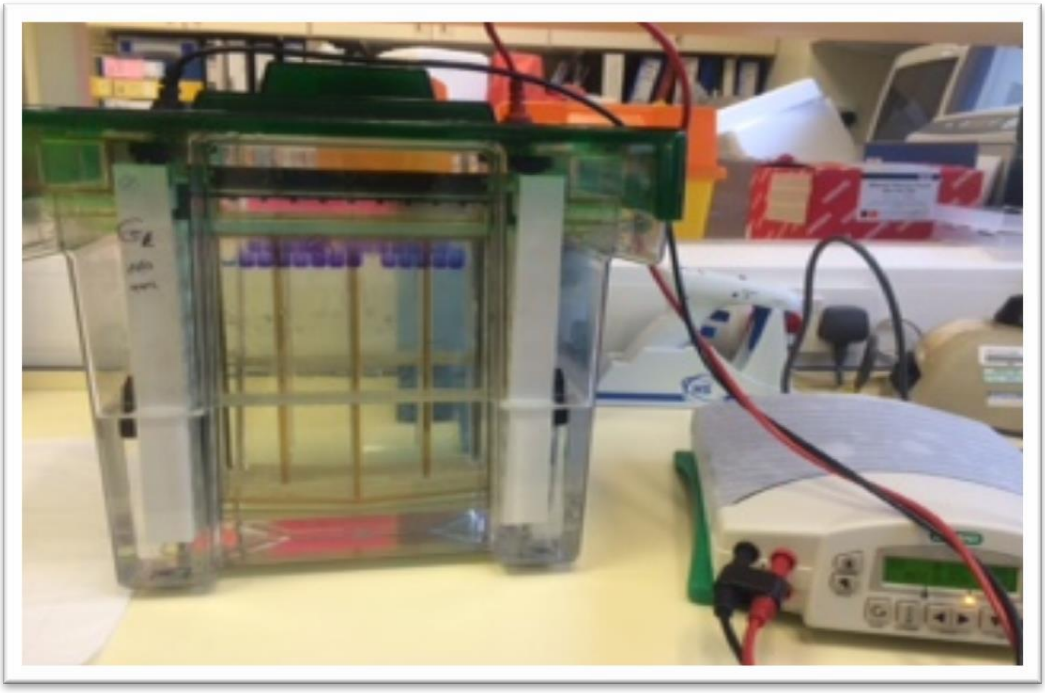

Figure 2-3: SDS-PAGE set up

2.6.3 Western blot protocol summary

Reagents for gel electrophoresis

Buffer A for resolving gel:

- 181.65 g of Tris was added into 1 liter (L) of dH_2O; shaken until dissolved, then 40 ml of SDS (final concentration of 0.4% in 1L was 10%) was added slowly. It was allowed to dissolve. The final pH was adjusted to 8.8 with 5mmol of HCL.

Buffer B for stacking gel:

- 60.55g of Tris was added into 1L of dH_2O, shaken until dissolved, and then 40 ml of 10% SDS solution (final concentration was 10%) was added slowly. It was allowed to dissolve then the pH was adjusted to 6.8 with 5mmol of HCL.

Running buffer (5x):

- To 3.8L of dH_2O 60.4 g of Tris, 288 glycine and 200 ml of 10% SDS solution was added. The pH was adjusted to 8.3 with 5mmol HCL.

Running buffer was diluted as follows:

- 0.5L running buffer (5x), add 2L of dH2O.

Table 2-3: The composition of the resolving and stacking gels.

Reagent	Resolving gel (2 gels) (10%)	Stacking gel (2 gels)
	Volume	Volume
30% acrylamide	24 ml	2.7 ml
50% glycerol	4.8 ml	N/A
Buffer A	18 ml	N/A
Buffer B	N/A	5 ml
Distilled water	24.8 ml	12.2 ml
10% AMPS	360 µl	100 µl
TEMED	36 µl	20 µl
Total	36x2 ml	20 ml

10% AMPS solution (AMPS-ammonium per sulphate)

- 0.1 g of ammonium persulfate and 1 ml of dH_2O (Sigma- Aldrich, cat. No. A3678-100G).
- TEMED: N, N, N, N tetramethylethylenediamine. (Sigma-Aldrich, cat.no.T9281-100ML).

2.6.4 Preparation of samples

- Samples and control were put on heat blocker 95°C for 5 minutes.
- Next the running buffer was poured in the central reservoir of the cooling tank (PROTEAN II xi system by Bio-Rad Laboratories, UK). The apparatus was checked for leaks.
- Next 20 µl of molecular weight ladder (BIO-RAD, 161-0305) was added, and then 50 µl of each sample was loaded into the appropriate gel well.
- Once the sample loading was completed, the gel run was started at 300 V, 27Amp for the first hour then up to 30 Amp for a total time period of about 5 hours.

2.6.4.1 Protein transfer to Nitrocellulose paper (semi-dry transfer)

The Trans-Blot SD Semi-Dry® Electrophoretic Transfer Cell (Bio-Rad, cat.no. 170-3940) was used for the transfer process. WhatmanProtran® nitrocellulose transfer membranes (Sigma-Aldrich, cat.no. Z61360) were used for transfer of the proteins. Nitrocellulose paper and two blotting papers were put in transfer buffer for 20 minutes. The process of transfer was done as follows:

The blotting papers were set up as in step 4 below the gel dimensions (current= gel area x2.5) were taken for calculation of the current (mAmp) and the Volts was set up at 22. The transfer was performed for 30 minutes. After the transfer was completed Ponceau S solution was used to ensure transfer of proteins to the nitrocellulose paper was completed. Then nitrocellulose was destained in 0.1% NaOH solution.

2.6.5 Transfer of protein protocol summary

Reagents

1. Transfer buffer: 800 ml H_2O, 3 g Tris, 14.4 g glycine and 200 ml methanol were mixed.

2. After the run was completed, the stacker gel and the bottom of the resolving gel under the ladder were cut off. The gel dimensions were taken (cm) then it was placed in the transfer buffer box, which contained the two blotting papers.

3. A piece of nitrocellulose paper (20 cm x 15 cm) was placed in the transfer buffer in a box for 20 minutes.

4. After 20 minutes the first piece of blotting paper was placed on the semi dry transfer cell (BIO 0RED, 170-3940) then it was rolled out. Nitrocellulose paper was put on top of it, then the gel on the top and lastly, the second blotting paper was placed on the top and rolled out.

5. Transfer was performed at 20 V for 30 minutes and the current (mAmp) was set according to the gel dimensions.

The last step was detection of the presence of protein by nitrocellulose illumination using Ponceau S solution as in Figure 2-4, then 0.1% NaOH solution was used for nitrocellulose de-staining.

Figure 2-4: A nitrocellulose membrane stained with Ponceau S dye for protein detection.

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2.6.6 Immune-blotting (Immuno-detection of proteins)

Reagents:

TBSTB (1L): 1L of dH2O, 2.4g Tris, 3ml of concentrated HCL (pH 7.5), 29.2g NaCL, 4ml Tween 20 and 2.5g BSA.

The protocol summary for immune detection of protein is shown in Table 2-4.

Membranes were blocked in 5% normal donkey serum (Serotec, cat.no CO6SBZ) for HSP20 or 5% normal goat serum (Sigma-Aldrech, cat.G9023) For PON2 Table 2-4.

Table 2-4: The protocol summary of immune blotting

HSP20	PON2
Blocker: 5% normal donkey serum in TBSTB for 1 hour at room temperature (RT). Primary antibody: 5% normal human serum (NHS) in TBSTB with monoclonal anti human HSP20 primary antibody (1:200) for 1 hour at RT. Secondary antibodies: horseradish peroxidase conjugated donkey anti- rabbit secondary antibody (1:3000) for 1 hour at RT.	Blocker: 5% normal goat serum in TBSTB for 1 hour at RT. Primary antibody: 5% normal human serum (NHS) in TBSTB with mouse monoclonal anti human PON2 primary antibody (1:200) for 1 hour at RT. Secondary antibodies: Goat anti-mouse secondary antibodies (1:2000) for 1 hour at RT.

During the blocking stage, the primary antibodies were pre-absorbed for 30 minutes in 5% normal human serum (Sera Laboratories International, cat.no. S-123-H) in TBSTB at RT. Membranes were incubated with appropriate primary antibodies solution, for instance: HSP20 (Abcam cat.no. ab13491) and PON2 (Santa Cruz cat.no. sc-373981).

Membranes were rinsed and washed in TBSTB (3X5 minutes), and then incubated with appropriate horseradish peroxidase conjugated secondary antibodies for HSP20 (HRP-donkey anti rabbit ab7083) for 1 hour at RT, and PON2 (Goat anti-mouse IgG-HRP, Santa Cruz, cat.no. sc-2005) for 1 hour at RT.

2.6.6.1 Detection

The Amersham ECL® Western Blot detection system (Amersham Pharmacia Biotech, GE healthcare, cat. No RPN2106) was used for detection of reactive proteins. The membranes were fixed in the cassette and x-ray film (SIGMA cat. no. Z373508-50EA) was placed in the top and the cassette was sealed. This was done in the darkroom. In

order to obtain good.bands (not saturated) which can be scanned by Bio-Rad GS-700® imaging densitometer, the membranes were exposed in the dark room for several intervals of time such as 30 seconds, 20 seconds and 5 seconds until the best exposure was obtained for subsequent scanning. All bands densities were expressed relative to the placental control sample density which was considered as an internal control.

2.6.7 Phosphorylated HSP20

The antibody was purchased from Abcam and was used to detect phosphorylated (Pho) HSP-20. Antibody optimization was performed using several methods and in conjunction with advice from the company. None of the methods revealed a band.

1. Chemicals: All chemicals were purchased from Sigma-Aldrich (U.K.) unless stated otherwise.
2. Tissue Homogenising for Western Blot:

This was performed as shown earlier.

3. Bradford assay:

This was performed as shown earlier.

4. Western Blotting for Pho-HSP20

Western blotting was performed as described previously (Abdulsid et al., 2013, Abdulsid et al., 2014) with some modifications. A volume containing 50 µg of each sample was separated by SDS-PAGE electrophoresis on 10% SDS-polyacrylamide resolving gels. Pre-stained low range molecular weight markers (BioRad, UK) were loaded onto each gel.

Transfer of proteins to Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech, UK) was performed at 22V and 200 mA for 30 min. Membranes were blocked in

5% donkey serum in TBSTB buffer (20 mM Tris, pH 7.5, 0.5 M NaCl, 0.4% Tween and 0.25% BSA) for 1 h at RT. Primary antibodies were pre-absorbed in 5% human serum in TBSTB at RT during the blocking process. Membranes were incubated for 1hr at RT with primary antibody solution. The Pho-HSP20 (Rabbit polyclonal antibody) was obtained from Abcam (cat. no. ab58522, rabbit polyclonal) and used at concentration of 1:2000. Membranes were washed and then incubated for 1h at RT with horseradish peroxidase conjugated donkey anti-rabbit secondary antibody from Abcam (cat. no. ab7083 diluted 1:3000 in TBSTB). Membranes were rinsed with TBSTB (2×5 min) and once with distilled water. For each group of experiments the same loading control placenta sample was added to every gel and the densitometry units were normalized to that. We previously confirmed that this method of analysis gives similar findings to other quantitative methods of densitometry (Abdulsid et al., 2013, Abdulsid et al., 2014).

2.7 RNA extraction from placentas

2.7.1 RNA extraction

The RNeasy Midi Kit (Qiagen, cat. no.75142) Figure 2-5 was used for RNA extraction from placenta samples; this was done in three steps according to the manufacture's instructions:

1. Working solution preparation: This was prepared before use according to the Qiagen kit instructions. β--Mercaptoethanol (β-ME) was added to RLT buffer (10µl β-ME per 1ml of RLT Buffer). Buffer RPE was supplied as a concentrate thus 11ml of this plus 44ml of ethanol (96-100%) were combined to obtain 55ml of Buffer RPE.

2. Tissue homogenisation: After removing the frozen tissue samples from storage at -80 ^{o}C placental samples were each placed on a pre-weighed plate (no more than 250 mg of the tissue was used). After the placenta tissue was ground down to a powder it was placed

in 4 ml of RLT buffer (buffer provided with kit). A Polytron PT 1600E, Lucerne) Rotor-stator homogeniser (Kinematica) with setting speed 3x20 seconds was used for tissue homogenisation. Samples were spun in a centrifuge (4000g, 25°C for 10 minutes). After that, the supernatant was added to new 15ml Falcon tubes (Sigma-Aldrich, cat.no.Z617849). 4.0ml of ethanol (70%) was added to the supernatant and the tubes were shaken.

3. Total RNA extraction : a suitable number of RNasey® Midi kit spin columns already included within the 15 ml collection tubes (provided in kit) were retrieved and then added 4 ml of mixed supernatant (ethanol and supernatant) to the column, which was then spun in centrifuge (4000g, 25°C for 5 minutes). The flow-through was disposed. This was repeated two times. After that the column was cleaned three times as follows:
RW1buffer 4.0ml (provided with kit) was added to the RNeasy column. Spun down in centrifuge (4000g, 25°C, for 5 minutes), and the flow-through was discarded.

RPE buffer 2.5ml (provided with kit) was added to each RNeasy column. Then they were spun down in the centrifuge (4000g, 25°C for 2 minutes). RPE buffer 2.5ml was added to each column again.

Then they were spun down in the centrifuge (4000g, 25°C for 5minutes).

The RNeasy column was transferred to a new 15 ml collection tube (provided with the kit) in preparation for RNA elution.

RNase-free water 250 ul was added into the RNeasy silica-gel membrane and allowed to stand for one minute.

After 1 minute the columns were spun (4000g, 25°C for 3 minutes). This was repeated. This step was repeated with the same volume of RNeasy-free water.

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Figure 2-5: RNeasy Midi Kit (Qiagen .cat.no. 75142).

2.7.2 Quantification of RNA

A spectrophotometer NanoDrop 1000™ (Thermo Scientific, USA) was used for calculating the quantity of total RNA (ng/µl) present in the sample. This was done as follows:

- The Nanodrop was "blanked" with 1.5µl of RNAase-free water all sample readings were read against the blank.
- Measurement of all RNA samples was performed one at a time.
- Estimation of the amount of RNA was obtained from the reading at 260 nm.
- The ratio of absorbance at 260/280 nm indicated the purity of RNA in the sample. Therefore, optimal purity was a ratio close to 2. The final measurements were in ng/µl (Figures 2-6, 2-7).

Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
	Sandy	24/04/2013	19:05	0.48	0.012	0.027	0.45	0.38	40.00	230	0.032	0.027
1BMI1	Sandy	24/04/2013	19:05	223.01	5.575	2.694	2.07	2.14	40.00	230	2.603	0.028
1BMI6	Sandy	24/04/2013	19:06	366.50	9.162	4.481	2.04	2.15	40.00	230	4.266	0.025
1BMI11	Sandy	24/04/2013	19:07	442.61	11.065	5.461	2.03	2.17	40.00	230	5.094	0.028
2BMI1	Sandy	24/04/2013	19:08	329.68	8.242	3.999	2.06	2.21	40.00	230	3.730	0.025
2BMI6	Sandy	24/04/2013	19:09	19.22	0.481	0.260	1.85	1.28	40.00	230	0.375	0.017
2BMI11	Sandy	24/04/2013	19:09	504.87	12.622	6.117	2.06	2.08	40.00	230	6.078	0.176
3BMI1	Sandy	24/04/2013	19:10	311.24	7.781	3.788	2.05	2.21	40.00	230	3.519	0.018
3BMI6	Sandy	24/04/2013	19:11	312.75	7.819	3.808	2.05	2.20	40.00	230	3.546	0.021
3BMI11	Sandy	24/04/2013	19:12	402.33	10.058	4.935	2.04	2.18	40.00	230	4.619	0.029

Figure 2-6: Nanodrop reading in ng/ul, indication of RNA purity was from the ratio of absorbance at 260 nm and 280 nm (260/280).

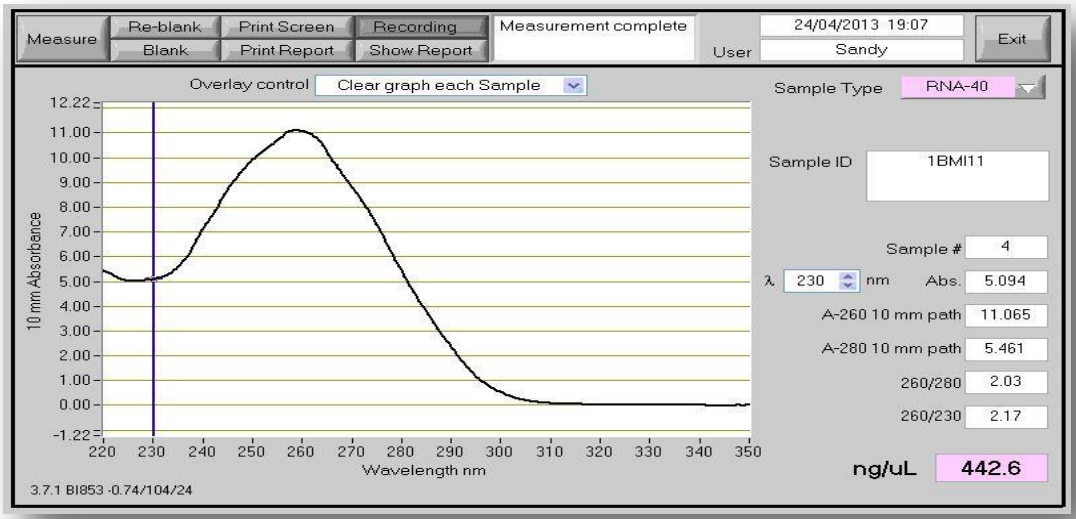


Figure 2-7: Print out showing the RNA concentration of placenta sample number 1BMI 11 in ng/µl (442.6) as a typical result.

2.8 Reverse transcription

This step converts mRNA into cDNA. It is an essential step as RNA cannot serve as a template for PCR. The method used the QuantiTech Kit (Qiagen, cat.no 205310) Figure 2-8 and GoScript™ reverses transcriptase (Promega, cat.no. A5003). Excellent quality of cDNA was obtained using GoScript™ reverse transcriptase.

Image was removed because of copyright restrictions.

Figure 2-8: The QuantiTech Kit (Qiagen, cat.no 205310)

2.8.1 Genomic DNA elimination reaction:

First of all, mRNA and the QuantiTect® kit were put in wet ice after retrieval from storage at -80°C and -20°C respectively. The calculation for the amount of RNA required for 100ng was done as following:

$$\text{Required RNA (µl)} = \frac{100\text{ng} \times 1\text{µl}}{\text{Original concentration (ng/µl)}}$$

Every PCR tube in every DNA elimination reaction contained:

1. Wipe out buffer 2ul

2. RNA (100ng) X ul and RNase free water X ul (total volume 12 ul)

3. The final total reaction volume was 14ul (2+12)

The tubes were put through the gOUT programme after the reaction was set up. A thermal cycler (DNA Engine®, Bio-Rad) (Applied Biosystems, Singapore) was used for this step.

4. 3 minutes at 42°C.

2.8.2 Reverse transcription reaction (RT)

For this reaction the master mix was prepared as follows: GoScript Reverse transcriptase 1ul (not from QuantiTect®kit).

RT Buffer 4ul

RT primer Mix 1ul

The PCR tubes were removed after the DNA elimination reaction was finished then 6ul of already prepared master mix was added to every tube containing 14ul of RNA The total reaction volume was 20ul. Next, the PCR tubes were placed again in the thermal cycler and the RT4 programme was used:

42°C for 30 minutes

95°C for 5 minutes (deactivation of enzyme). The cDNA was then kept at -20°C storage.

2.9 Quantitative real time polymerase chain reaction (qRT-PCR)

Prior to starting the experiment, sterilization of the bench and all equipment was performed using 70% ethanol. The cDNA was placed on wet ice after removal from storage at -20ºC. For this study the TaqMan Master Mix (Applied Biosystem, cat.no 4369514) and the StepOnePlus® qPCR machine was used (Applied Biosystems). TaqMan gene assays (Applied Biosystems) were used, and endogenous control was β-actin as it has been previously validated in the placenta by our group in many published studies (Abdulsid et al., 2013). The reference sample was placenta cDNA (Figure 2-9) which was diluted the same the samples i.e. 1/5 (1ul cDNA+ 4ul dH_20) and it was diluted 1/5 as previously validated by two previous students (Abdulsid et al., 2013& Alwarfaly et al., 2014). For each primer in a single experiment master mix was prepared (10ul).

Master mix and cDNA (20ul total volume) were prepared as follows:

- 10 ul master mix
- 4ul dH_2O
- 5ul diluted cDNA
- 1ul TaqMan Gene Expression Assay

HSPB6 TaqMan Gene Expression Assay (Applied Biosystems, cat.no: Hs0O328933-m1). PON2 TaqMan Gene Expression Assay (Applied Biosystems, cat. no: Hs0100165563-m1).

Microamp® fast optical 96 well plates (Applied Biosystems, cat.no. 4346906) were used for this experiment. The plate was set up then the cover was placed on it. To remove any bubbles centrifusion of the plate was carried out at 4860g for 90 seconds. Following that the plate was placed in a qPCRStepOnePlus machine and the following qPCR programme was used:

Holding stage:

2 minutes at 50°C

10 minutes at 95°C

Cycling stage X50

30 seconds at 95°C.

1 minute at 60°C

Once the programme was over the plate was kept in the fridge.

The comparative C^t methods of Applied Biosystem software® v2.1 were used to compare several gene expression to attain a relative quantification (RQ) of gene expression. In order to determine the amount of specific transcripts present in cDNA, firstly, CT values for each sample should be normalised to obtain Δ CT. For each group; subtracting the mean CT value of endogenous reference gene β-actin from the corresponding mean CT value of specific gene (SG) (ΔCT = CT SG - CT β-actin). Then the concentration of a specific gene in the samples relative to the positive control was normalised by subtraction to obtain ΔΔCT values (ΔΔCT = ΔCT sample - ΔCT of positive control). Finally, the relative expression, which is called the RQ value was determined by raising 2 to the power of the negative value of ΔΔCT (2-ΔΔCT) for each sample (Amount of target = 2-ΔΔ CT).

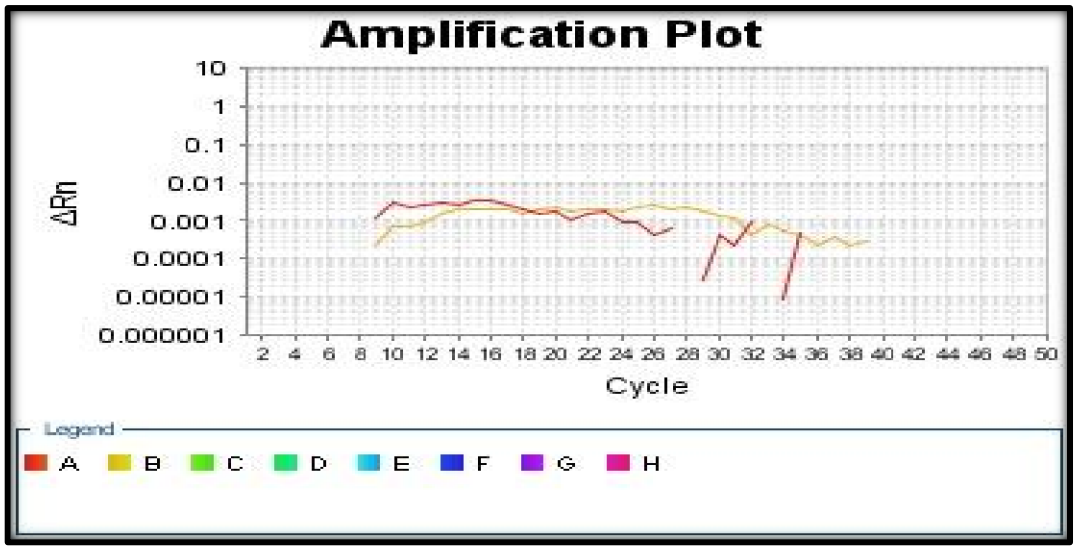


Figure 2-9: The negative control (RNA free water) showed no expression.

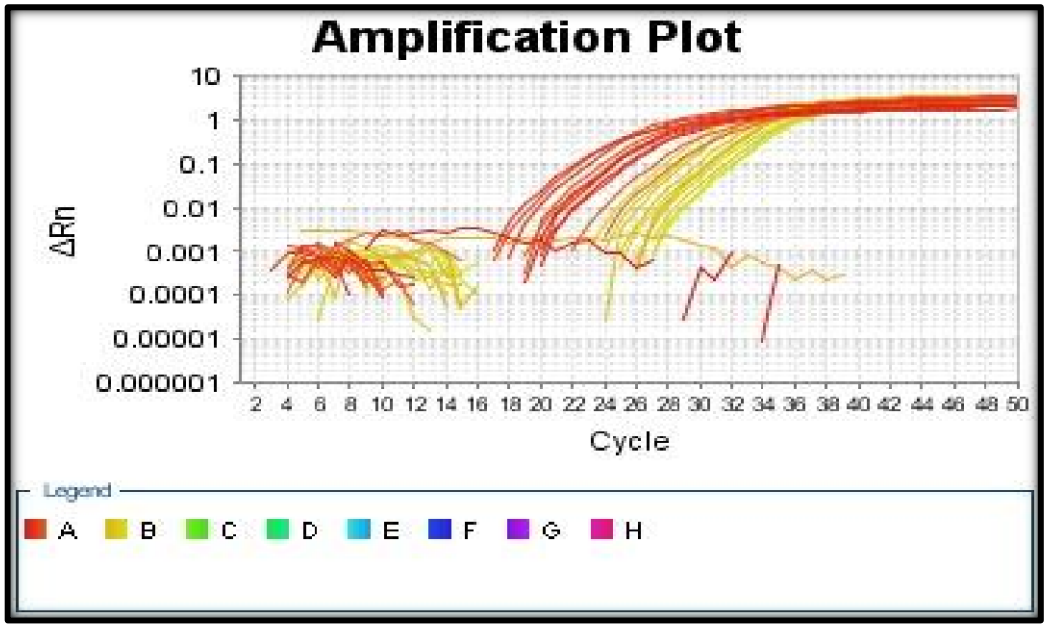


Figure 2-10: Placenta cDNA (positive control) showed clear expression from cycle number 16.

2.10 Statistical analysis

Statistical analysis was performed using Graph Pad prism 5 using analysis of variance for non-parametric. Data was analysed using the Friedman test and Kruskal–Wallis test for 3 groups. Comparison of two groups was performed by the Mann Whitney U test. Graphs show box and whiskers plots.

Comparison of HSP20 mRNA expression between individual placental areas in labour and non-labour group was performed using the Friedman test.

Comparison of HSP20 and HSP20 mRNA expression between labour and non-labour group at the inner, middle and outer areas of the placenta was performed using the Mann-Whitney test.

Comparison of HSP20 and PON2 mRNA expression between different BMI groups at the inner, middle and outer areas of the placenta was performed using the Kruskal-Wallis test. Comparison of HSP20 expression between the BMI <30

group and the BMI >40 group at the inner, middle and outer areas of the placenta was performed using the Mann-Whitney test.

Comparison of HSP20 and PON2 protein expression between different BMI groups at the inner, middle, and outer areas of the placenta was performed using the Friedman test.

Statistical significance was defined as a p-value <0.05. Graphs were drawn on a p c using graph pad prism 5.

Chapter 3 – Results

3.1 Analysis of the patient demographics according to BMI

Comparison between all BMI groups was performed. No significance difference was found between them as in Table 3-1.

Table 3-1: The demographics for patients used for placenta collection

CATEGORY	BMI<30 (n=6)	BMI 30-35 (n=6)	BMI 35-40 (n=6)	BMI>40 (n=7)	p-value
Maternal age	28.83±5.3	35.00±4.69	31.33±6.74	35.50±7.97	p=0.2
Placenta weight	679.8±170.7	630.0±103.1	708.7±166.8	684.0±169.3	P=0.8
Birth weight	3858±894	3381±183	3827±676	3444±459	p=0.4
N0. Primigravida	1	1	1	0	
Gestation age at delivery	39.21±0.39	38.60±1.54	39.03±0.70	38.86±0.54	P=0.6
No. smokers	2	1	1/3 NA	0	
BMI	24.58±2.7	32.38±0.95	36.91±0.69	45.92±4.34	P=0.000

3.2 HSB6 (HSP20) mRNA expression in labour and non-labour groups

Experiment and Figure 3.2.1: This experiment was designed to test if there was a spatial difference in the expression of HSPB6 (HSP20) mRNA within individual placentas obtained from women who were not in labour (non-labour group) and from women who were in labour (labour group). Comparison between areas was performed using the Friedman test analysis. There was no significant difference in mRNA expression between the three areas of the non-labour and labour groups (p=0.74, p=0.95 respectively).

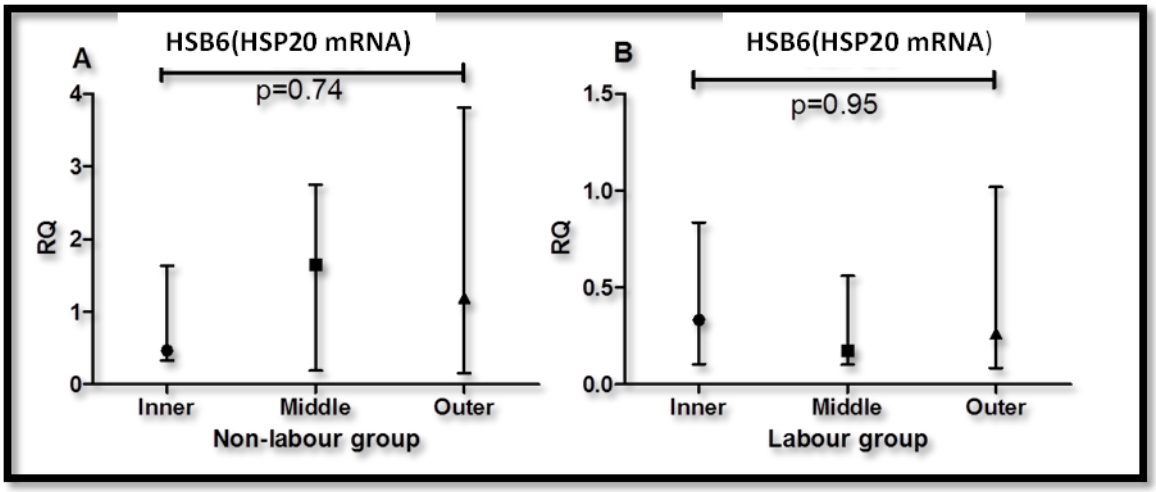


Figure 3.2.1: RQ values expressed relative to β-actin for HSPB6 (HSP20) mRNA measurements in inner, middle and outer areas of individual placentas (non-labour and labour groups). Four quadrants were sampled in each area. Graphs show median and S.E (Standard error) HSPB6 (HSP20) mRNA expressions. Comparison between areas was performed using Friedman analysis

Experiment and Figure 3.2.2: This experiment was designed to test if there was any difference in HSPB6 (HSP20) mRNA expression between labour and non-labour groups at the inner, middle or outer areas of placentas. Comparison between areas was performed using the Mann Whitney test analysis. There was a significant decrease in HSPB6 (HSP20) mRNA expression in the labour group when compared to the non-labour group at the middle area (p=0.01). No significance difference was found at the inner and outer areas (p=0.3 for both).

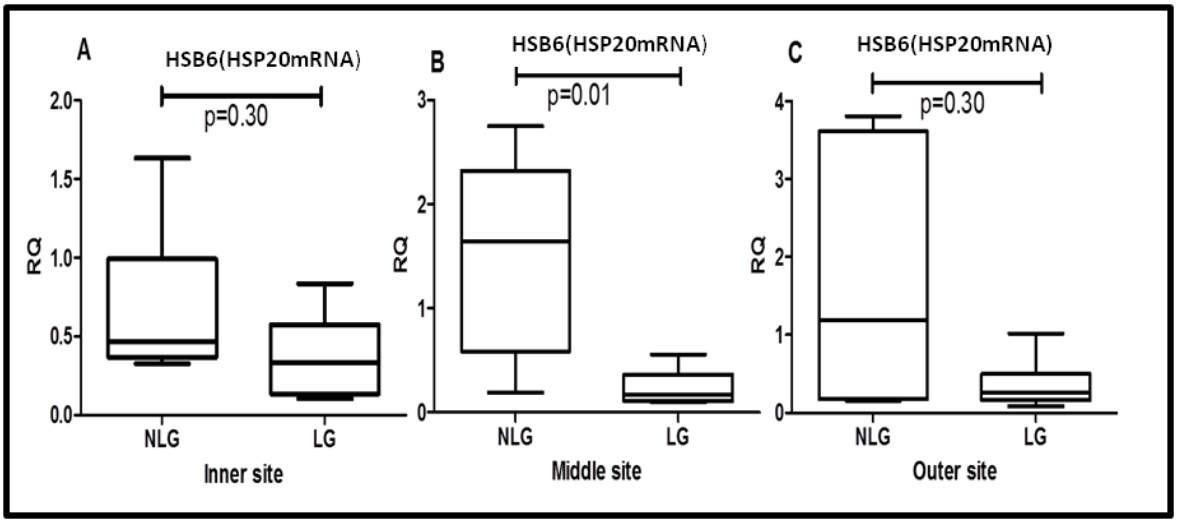


Figure 3.2.2: RQ values expressed relative to β-actin for HSPB6 (HSP20) mRNA measurements in the non-labour compared to the labour group at the inner, middle and outer placental area. Comparison between areas was performed using Mann-Whitney analysis.

3.3 HSB6 (HSP20) mRNA expression in different BMI group

Experiment and Figure 3.3.1: This experiment was designed to test if there was any difference in HSPB6 (HSP20) mRNA expression between BMI <30, BMI 30-35, BMI 35-40 and BMI > 40 groups at the inner area of placentas. Comparison between areas was performed using Kruskal-Wallis test analysis. No significant differences were found in HSPB6 (HSP20) mRNA expression between all BMI groups at the inner site (upper panel of Figure 3.3.1; p=0.13). The lower panel of Figure 3.3.1 shows the sub-analysis and that there was no significant difference when the all BMI <30 were compared to the BMI >40 group (p=0.08).

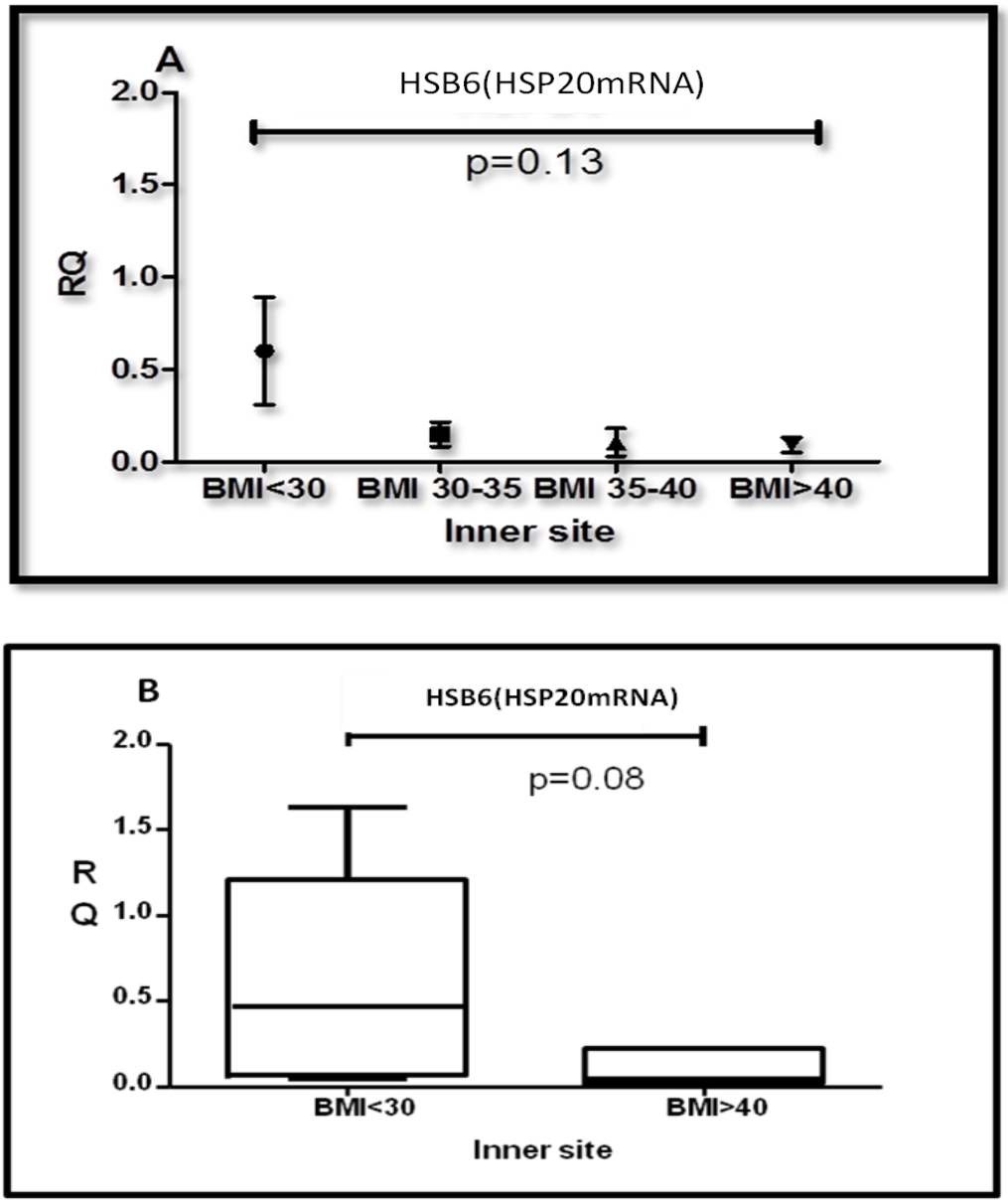
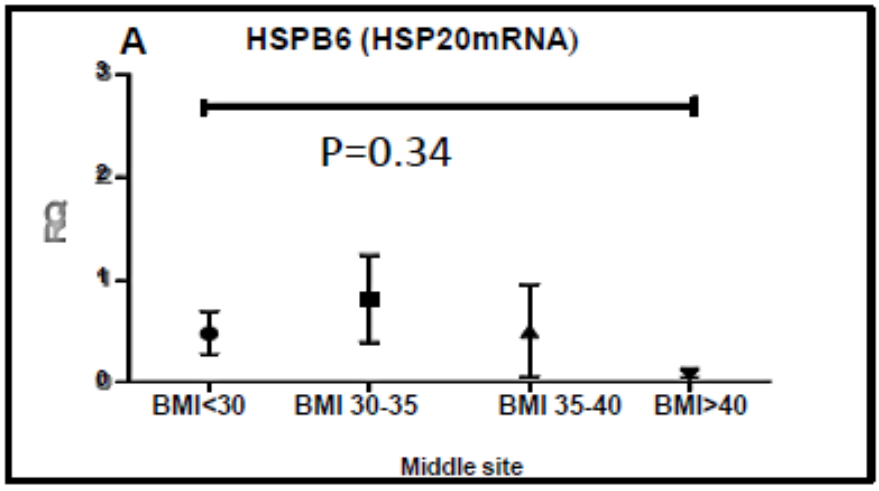


Figure 3.3.1: RQ values expressed relative to β-actin for HSPB6 (HSP20) mRNA measurements in BMI <30, BMI 30-35, BMI 35-40 and BMI >40 at the inner placental area (upper panel). BMI<30 versus BMI >40 comparison between zones was performed using Mann-Whitney test (lower panel).

Experiment and Figure 3.3.2: This experiment was designed to test if there was any difference in HSPB6 (HSP20) mRNA expression between BMI <30, BMI 30-35, BMI 35-40 and BMI > 40 groups at the middle area of placentas. Comparison between areas was performed using Kruskal-Wallis test analysis. There was no significant differences in HSPB6 (HSP20) mRNA expression between all BMI groups at the middle site (p=0.34); shown in Figure 3.3.2 upper panel. The sub-analysis (Figure 3.3.2 lower panel) showed that there was no significant difference when all BMIs <30 were compared to the BMI >40 group (p=0.08).



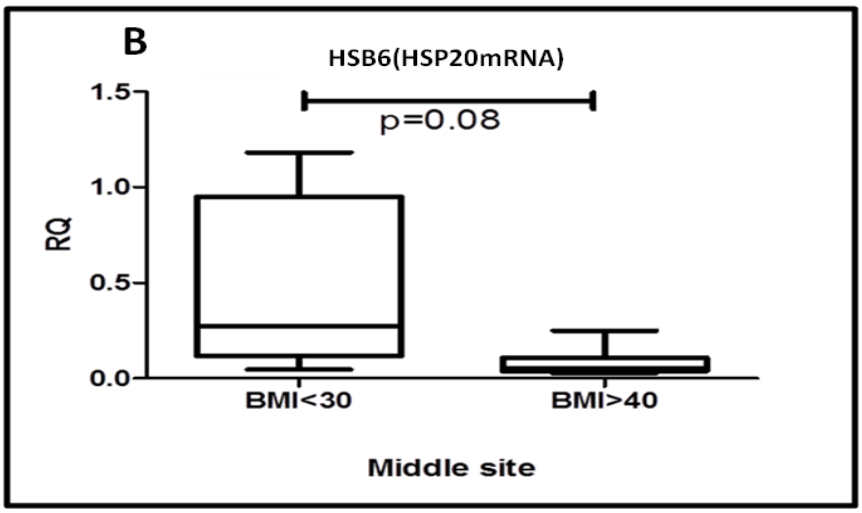


Figure 3.3.2: RQ values expressed relative to β-actin for HSPB6 (HSP20) mRNA measurements in BMI <30, BMI 30-35, BMI 35-40 and BMI >40 at the middle placental area (upper panel) . BMI<30 versus BMI >40 comparison between the zones was performed using Mann-Whitney analysis (lower panel).

Experiment and Figure 3.3.3: This experiment was designed to test if there was any difference in HSPB6 (HSP20) mRNA expression between BMI <30, BMI 30-35, BMI 35-40 and BMI > 40 groups at the outer area of placentas. Comparison between areas was performed using Kruskal-Wallis test analysis. There was no significant differences in HSPB6 (HSP20) mRNA expression between all BMI groups at the outer site (p=0.23, Figure 3.3.3 upper panel). The sub-analysis showed that there was no significant difference when the BMI <30 compared to the BMI >40 group (p=0.08; Figure 3.3.3 lower panel).

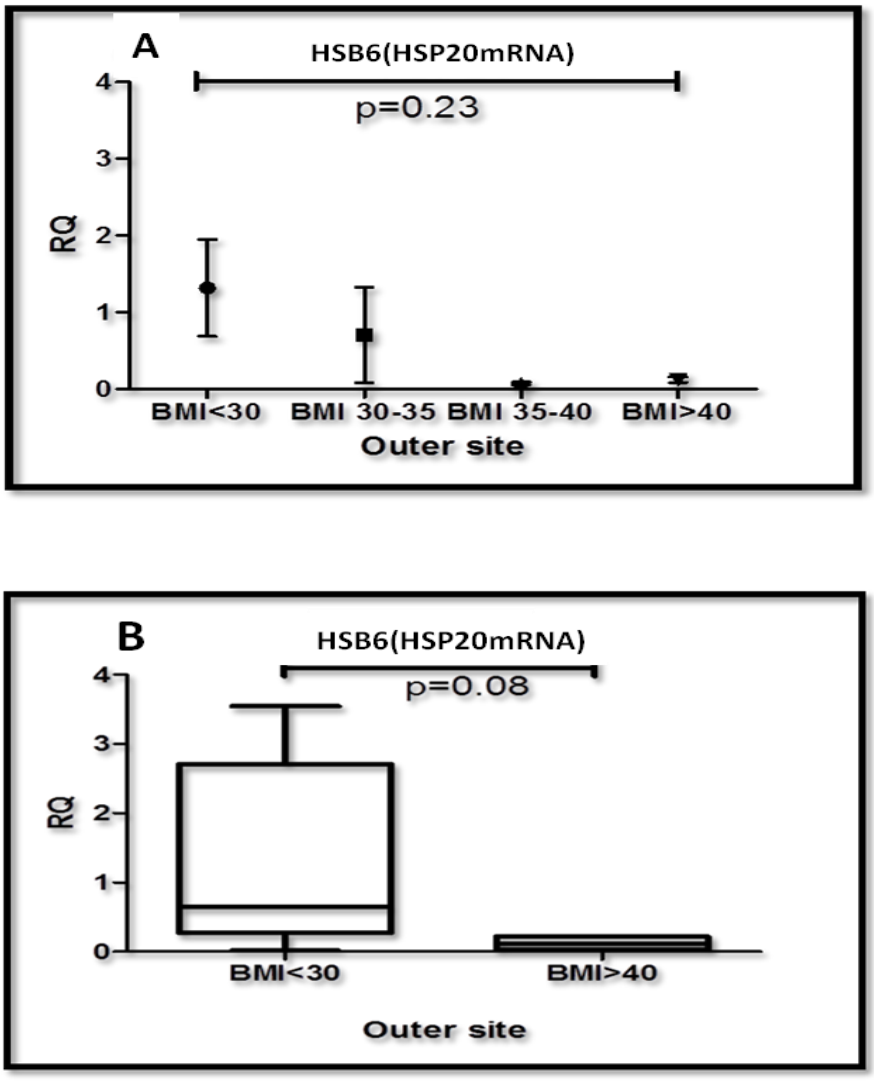


Figure 3.3.3: RQ values expressed relative to β-actin for HSPB6 (HSP20) mRNA measurements in BMI <30, BMI 30-35, BMI 35-40 and BMI >40 at the outer placental area (upper panel). BMI<30 versus BMI>40 comparison between the zones was performed using Mann-Whitney analysis (lower panel).

Summary of section 3.3; Overall statistical analysis showed no difference between groups. It appeared the BMI>40 were different from the non-obese groups. Thus a second analysis was performed where the 2 extremes were compared ie BMI<30 versus BMI>40. There was no significant difference in inner, middle and outer zones (p=0.08 for each).

3.4 HSP20 (HSB6) protein expression in the labour and non-labour groups

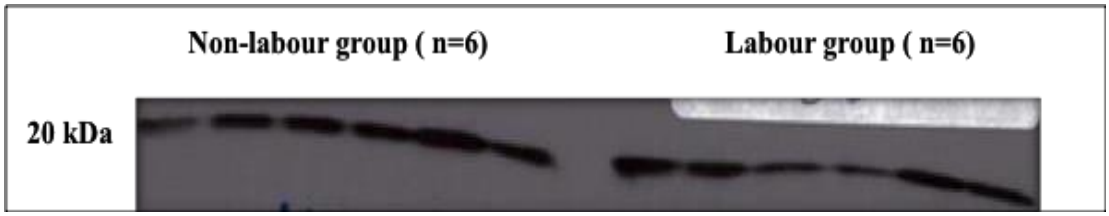


Figure 3.4.1a: Western blot showing the expression of HSP20 in the inner placental area of both labour and non-labour.

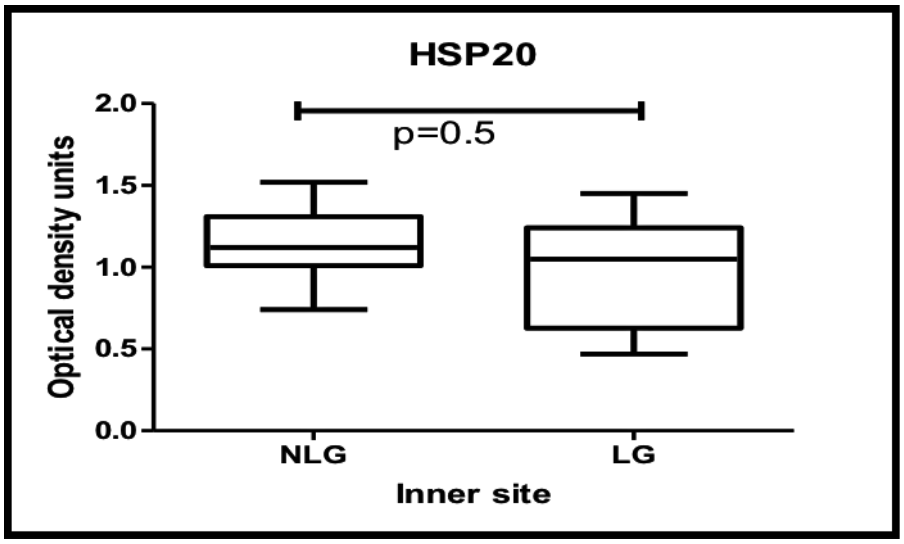


Figure 3.4.1b: Comparison between HSP20 expression at the inner area of labour and non-labour was performed using Mann-Whitney analysis.

Experiment and Figure 3.4.2: This experiment was designed to test if there was a spatial difference in expression of HSP20 at the middle placental area of the labour versus the non-labour group. Western blots showing HSP20 expression in the middle area of placentas of the non-labour and labour groups (n=6 each) are shown in Figure 3.4.2a. No significance differences were found following statistical analysis Figure 3.4.2b.

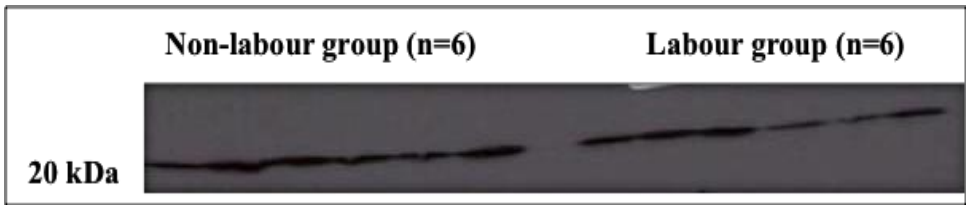


Figure 3.4.2a: Western blot showing the expression of HSP20 in the middle placental area of both labour and non-labour.

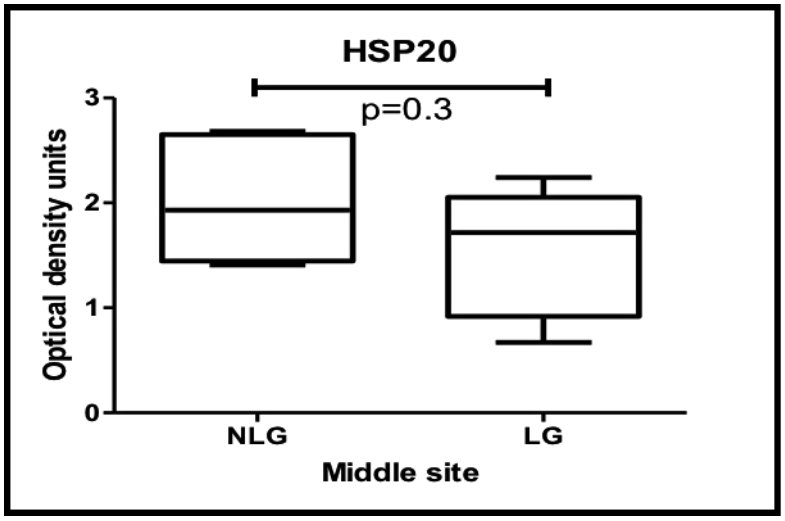


Figure 3.4.2b: Comparison between HSP20 expression at the middle area of labour and non-labour was performed using Mann-Whitney analysis.

Experiment and Figure 3.4.3: This experiment was designed to test if there was a spatial difference in expression of HSP20 at the outer placental area of both labour and non-labour groups. Western blots showing HSP20 expression in the outer area of placentas of all non-labour and labour groups (n=6 each) are shown in Figure 3.4.3a. No significant differences were found following statistical analysis Figure 3.4.3b.

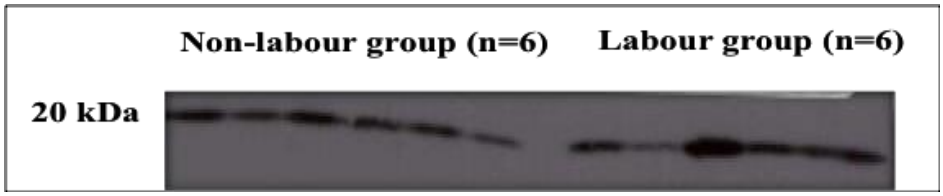


Figure 3.4.3a: Western blot showing the expression of HSP20 in the outer placental area of both labour and non-labour.

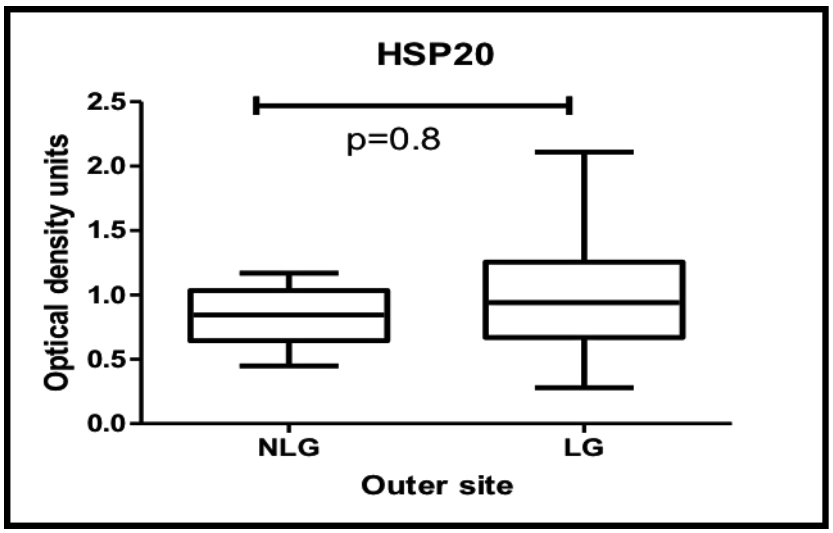


Figure 3.4.3b: Comparison between HSP20 expression at the outer area of labour and non-labour was performed using Mann-Whitney analysis.

In summary there was no spatial difference in expression of HSP20 in the three placental areas when comparing non-labour with labour groups. Due to the fact this work was performed and written within a year there was not enough time to compare zones within individual placentas as was done for HSPB6 (HSP20). This could be a future experiment.

3.5 HSP20 (HSB6) protein expression in BMI groups

Experiment and Figure 3.5.1: This experiment was designed to test if there was a spatial difference in expression of HSP20 (HSPB6) within placentas obtained from women who have BMI <30, BMI 30-35, BMI 35-40, and BMI >40. Western blots showing HSP20 expression in the inner area of placentas groups (n=6 in each group) is shown in Figure 3.5.1 (upper panel). No significant differences were found following statistical analysis Figure 3.5.1 (lower panel)

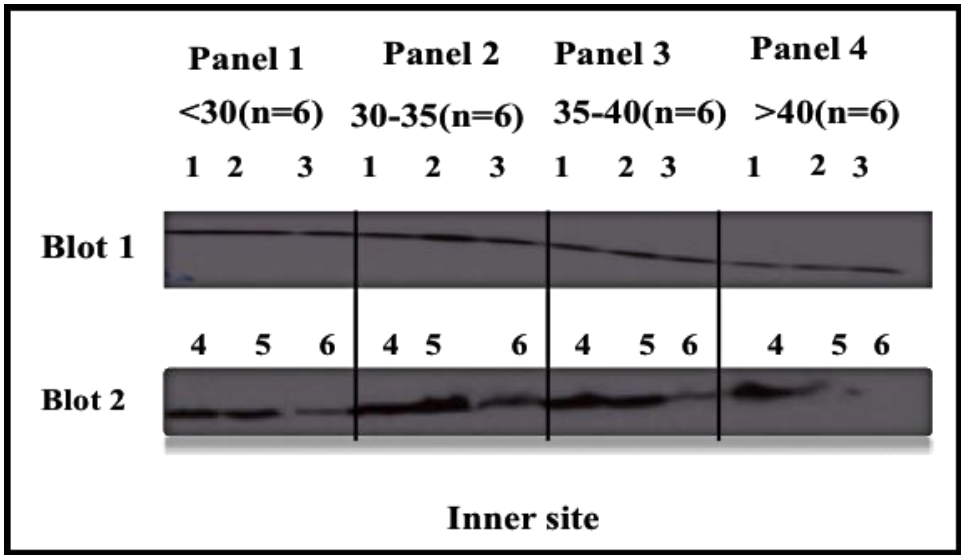


Figure 3.5.1 (upper panel): Western blots show HSP20 (HSPB6) expression at the inner site of the placentas. Blot 1 shows samples (1,2,3) and blot 2 below shows samples (4,5, 6) from a total of 6 patients. Panel 1 displays BMI group <30, panel 2 displays BMI group 30-35, panel 3 shows BMI group 35-40 and panel 4 shows BMI >40.

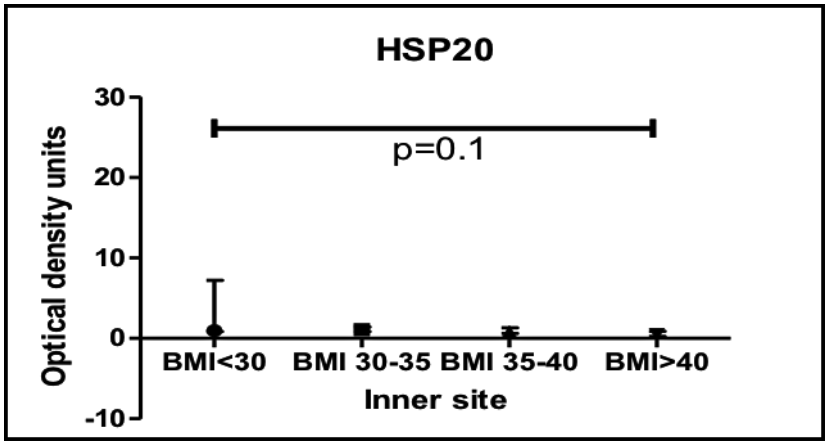


Figure 3.5.1 (lower panel): Comparison between HSP20 expression at the inner area of different BMI groups (<30, 30-35, 35-40, and >40) was performed using the Friedman test.

Experiment and Figure 3.5.2: This experiment was designed to test if there was a spatial difference in expression of HSP20 (HSPB6) within placentas obtained from women who have BMI <30, BMI 30-35, BMI 35-40, and BMI >40. Western blots showing HSP20 (HSPB6) expression in the middle area of placentas of groups (n=6 in each group) are in the Figure 3.5.2 (upper panel). No significance differences were found following statistical analysis Figure 3.5.2 (lower panel).

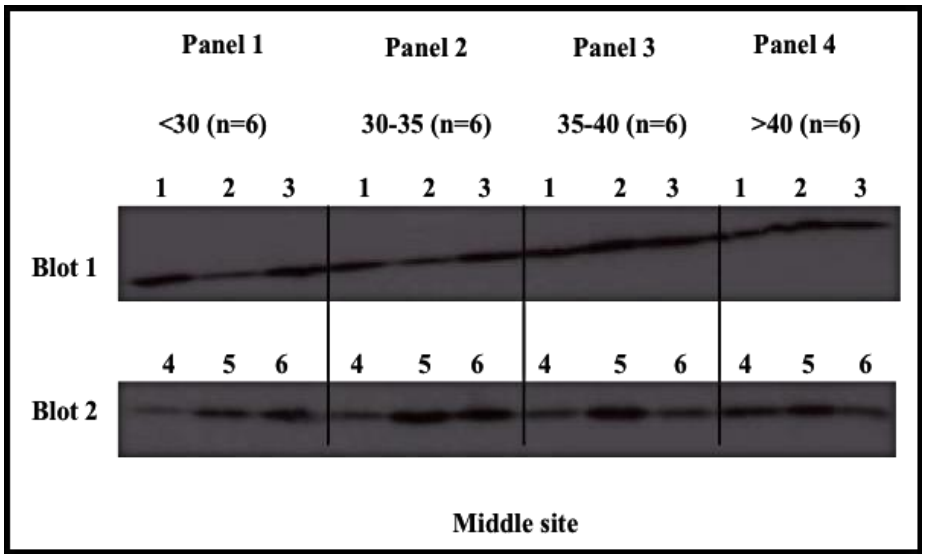


Figure 3.5.2 (upper panel): Western blots show HSP20 (HSPB6) expression at the inner site of the placenta. Blot 1 shows samples (1, 2, 3) and blot 2 below shows samples (4,5, 6) from a total of 6 patients. Panel 1 displays BMI group <30, panel 2 displays BMI group 30-35, panel 3 shows BMI group 35-40 and panel 4 shows BMI >40.

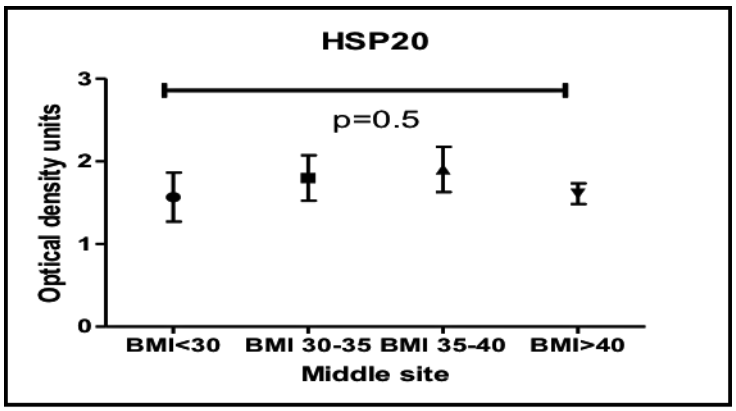


Figure 3.5.2 (lower panel): Comparison between HSP20 (HSPB6) expression at the middle area of different BMI groups (<30, 30-35, 35-40, and >40) was performed using the Friedman test.

Experiment and Figure 3.5.3: This experiment was designed to test if there was a spatial difference in expression of HSP20 (HSPB6) within placentas obtained from women who have BMI <30, BMI 30-35, BMI 35-40, and BMI >40. Western blots showing HSP20 (HSPB6) expression in the outer area of placentas of groups (n=6 in each group) are in Figure 3.5.3 (upper panel). No significance differences were found following statistical analysis.

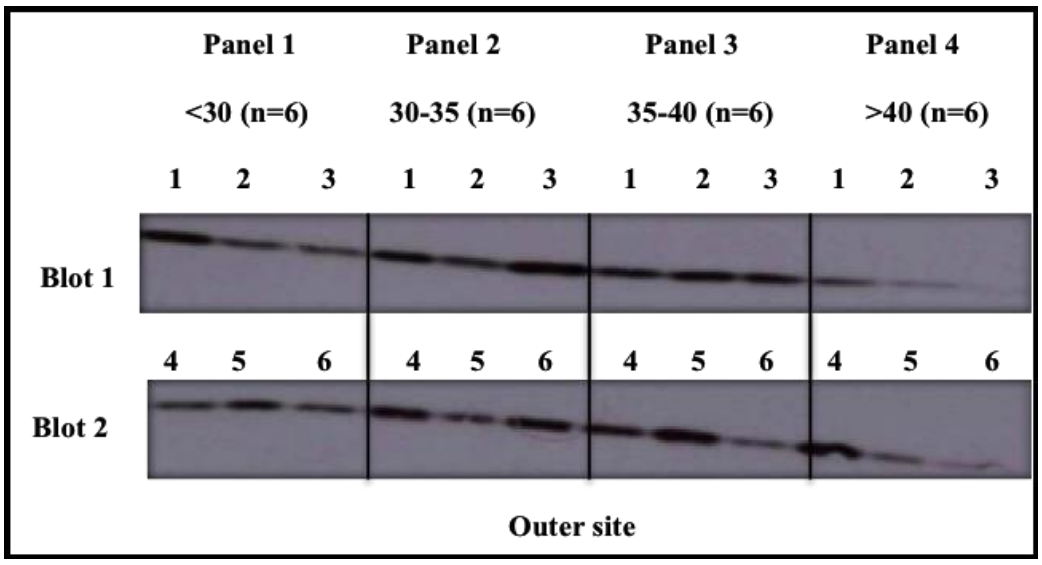


Figure 3.5.3 (upper panel): Western blots show HSP20 (HSPB6) expression at the inner site of the placenta. Blot 1 shows samples (1, 2, 3) and blot 2 below shows samples (4,5, 6) from a total of 6 patients. Panel 1 displays BMI group <30, panel 2 displays BMI group 30-35, panel 3 shows BMI group 35-40 and panel 4 shows BMI >40.

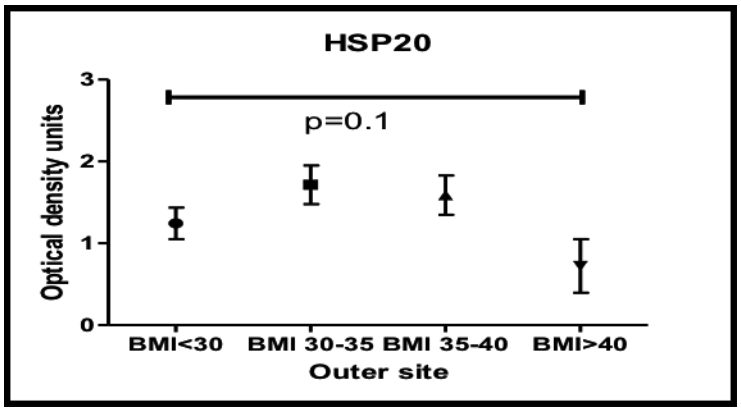


Figure 3.5.3: Comparison between HSP20 (HSPB6) expression at the outer area of different BMI groups (<30, 30-35, 35-40, and >40) was performed using the Friedman test.

In summary, for the above section, there was no spatial difference in expression of HSP20 (HSPB6) within between the three sites of placentas of all BMI groups.

3.6 **PON2 mRNA expression in different BMI groups**

Experiment and Figure 3.6.1: This experiment was designed to test whether there was any difference in PON2 mRNA between the MBI group <30, the BMI group 30-35, the BMI group 35-40, and the BMI group >40 at the inner site of the placenta. The results are shown in Figure 3.6.1. No differences were found.

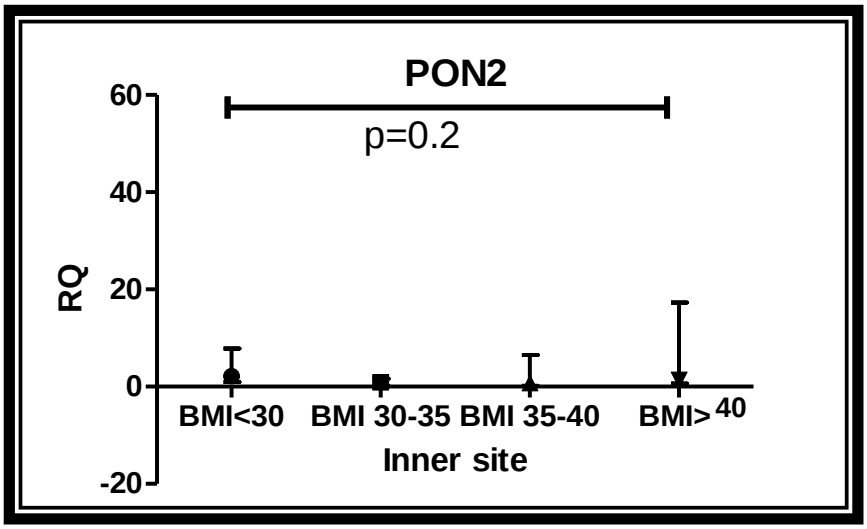


Figure 3.6.1: PON2 mRNA expression at the inner site of placentas for all BMI groups (n=6 per group). Statistical analysis using the Kruskal-Wallis was used for comparison between the groups.

Experiment and Figure 3.6.2: This experiment was designed to test whether there was any difference in PON2 mRNA between the MBI group<30, the BMI group 30-35, the BMI group 35-40, and the BMI group >40 at the middle site of the placenta. The results are shown in Figure 3.6.2 and no differences were found.

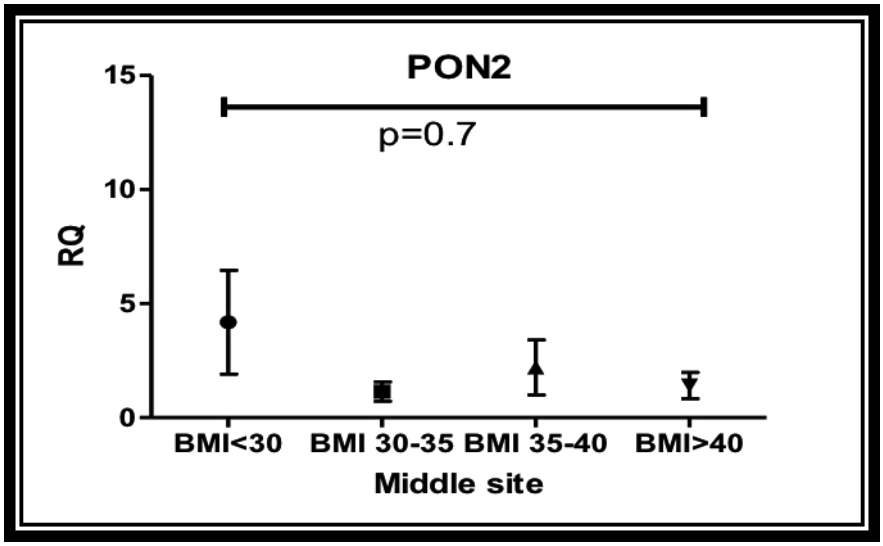


Figure 3.6.2: PON2 mRNA expression at the middle site of placentas for all BMI groups. Statistical analysis was performed using the Kruskal-Wallis test (n=6 per group).

Experiment and Figure 3.6.3: This experiment was designed to test whether there was any difference in PON2 mRNA between the MBI group <30, the BMI group 30-35, the BMI group 35-40, and the BMI group >40 at the outer site of placenta. The results are shown in Figure 3.6.3. No differences were found.

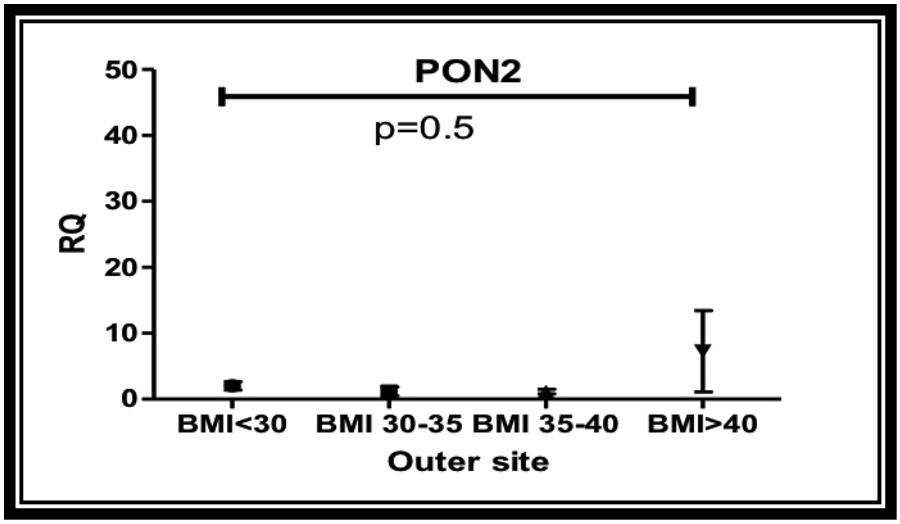


Figure 3.6.2 shows PON2 mRNA expression at the outer site of placentas for all BMI groups. Statistical analysis was performed using the Kruskal-Wallis test for comparison between the groups.

3.7 PON2 protein expression in BMI groups

Experiment and Figure 3.7.1 : This experiment was designed to test if there was a spatial difference in expression of PON2 within placentas obtained from women who had normal pregnancy outcomes but with different BMIs. Group1: BMI <30 (n=6), group 2: BMI 30-35 (n=6), group 3 BMI 35-40 (n=6) and group 4 BMI>40 (n=6). Western blots showing PON2 expression in the inner area of placentas for each BMI group are shown in Figure 3.6.1 (upper panel). PON2 has two isoforms, isoform 1 is at 62 kDa and the isoform 2 is at 43 kDa. Examples of Western blots showing PON2 (isoform 1, 62 kDa) and (isoform 2, 43kDa) shown in Figure 3.7.1 (upper panel). The graphs and statistical analysis are shown below the blots Figure 3.7.1 (lower panel). No significant differences were found.

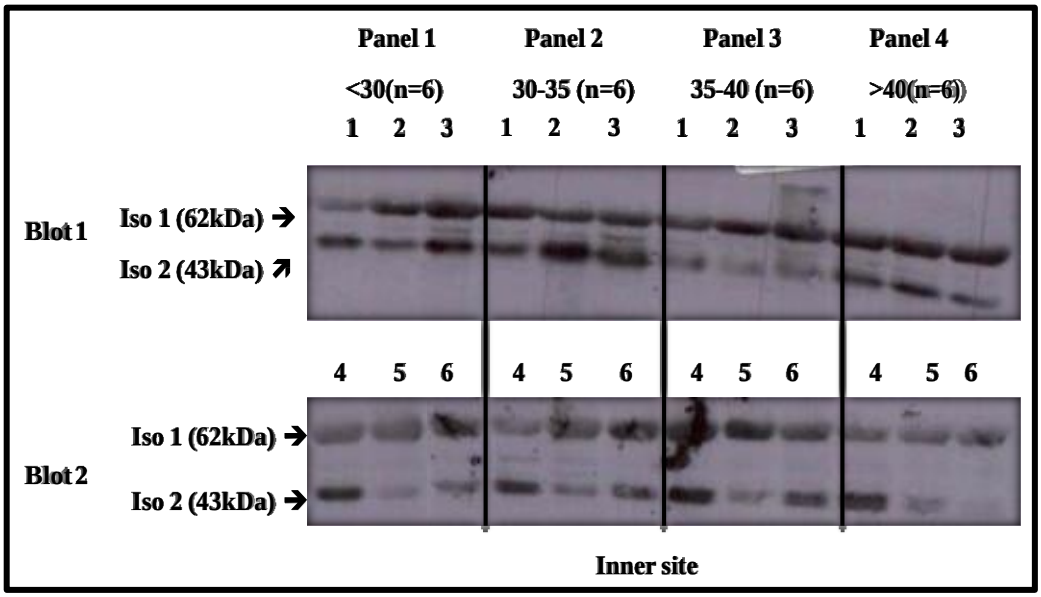
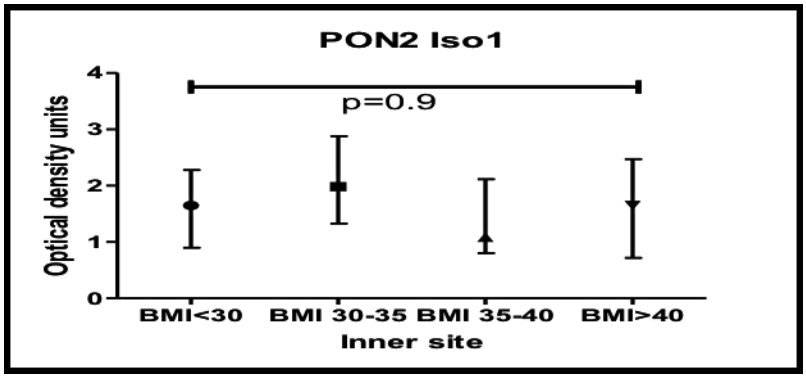


Figure 3.7.1: Western blots showing PON2 expression in inner site of all BMI groups. Panel 1 shows BMI <30 and samples (n=6) in blots 1& 2. Panel 2 shows BMI 30-35 and samples (n=6) in blots 1&2. Panel 3 shows BMI 35-40 and samples (n=6) in blots 1&2. Panel 4 shows BMI >40 and samples (n=6) in blots 1&2. Blots 1&2 show the 62 kDa isoform 1& 43 kDa isoform 2.



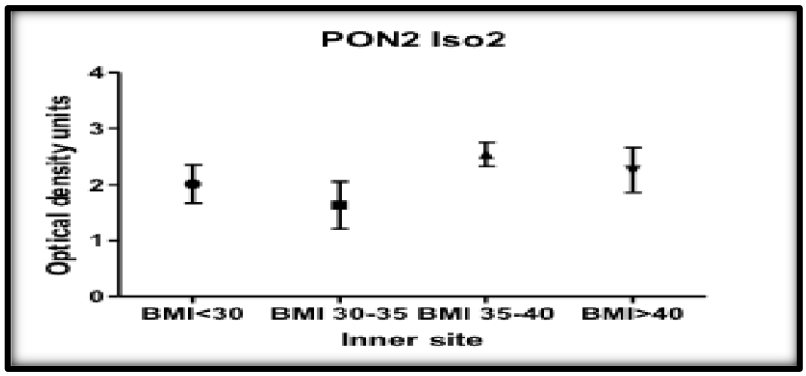


Figure 3.7.1(lower panel): The graphs show the statistical analysis. Comparison between different BMI groups at the inner site was performed using the Friedman test.

Experiment and Figure 3.7.2: This experiment was designed to test if there was a spatial difference in expression of PON2 within placentas obtained from women who had normal pregnancy outcomes with different BMIs. Group1: BMI <30 (n=6), group 2: BMI 30-35 (n=6), group 3 BMI 35-40 (n=6) and group 4 BMI>40 (n=6). Western blots showing PON2 expression in the middle area of placentas for the BMI groups are shown in Figure 3.7.2 (upper panel). The graphs and statistical analysis are shown below the blots Figure 3.7.2 (lower panel). No significance differences were found.

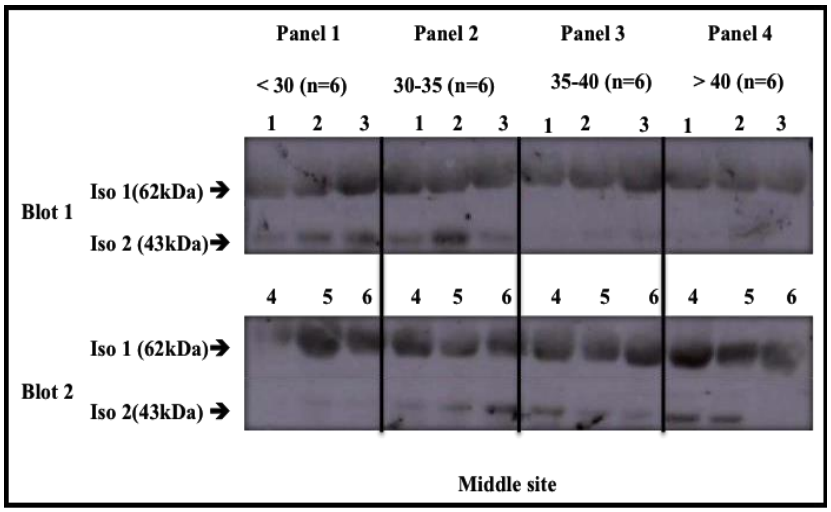


Figure 3.7.2 (upper panel): Western blots showing PON2 expression in middle site of all BMI groups. Panel 1 shows BMI <30 and samples (n=6) in blots 1& 2. Panel 2 shows BMI 30-35 and samples (n=6) in blots 1&2. Panel 3 shows BMI 35-40 and samples (n=6) in blots 1& 2. Panel 4 shows BMI >40 and samples (n=6) in blots 1& 2. Blots 1&2 show the 62 kDa isoform 1& 43 kDa isoform 2.

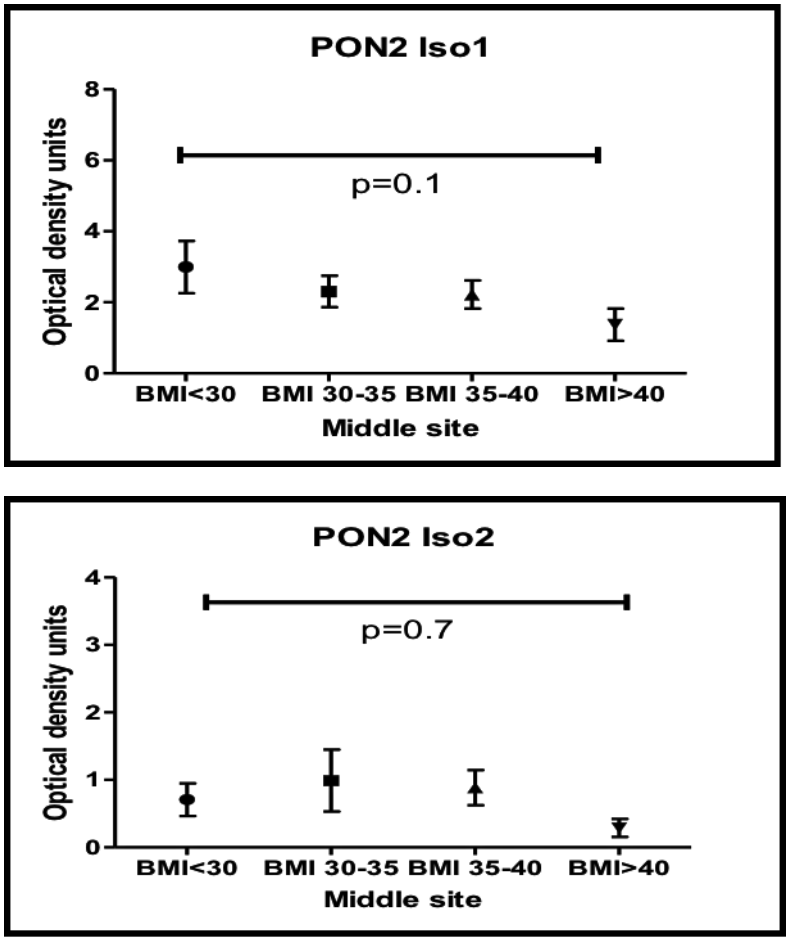


Figure 3.7.2 (lower panel): The graphs show the statistical analysis. Comparison between different BMI groups at the middle site was performed using the Friedman test.

Experiment and Figure 3.7.3: This experiment was designed to test if there was a spatial difference in expression of PON2 within placentas obtained from women who had normal pregnancy outcomes with different BMIs Group1: BMI <30 (n=6), group 2: BMI 30-35 (n=6), group 3 BMI 35-40 (n=6) and group 4 BMI>40 (n=6). Western blots showing PON2 expression in the outer area of placentas for BMI group and PON2 (isoform 1, 62 kDa) & (isoform 2, 43kDa) expression for placentas of all BMI groups are shown in Figure 3.7.3 (upper panel). The graphs and statistical analysis are shown below the blots Figure 3.7.3 (lower panel). No significance differences were found.

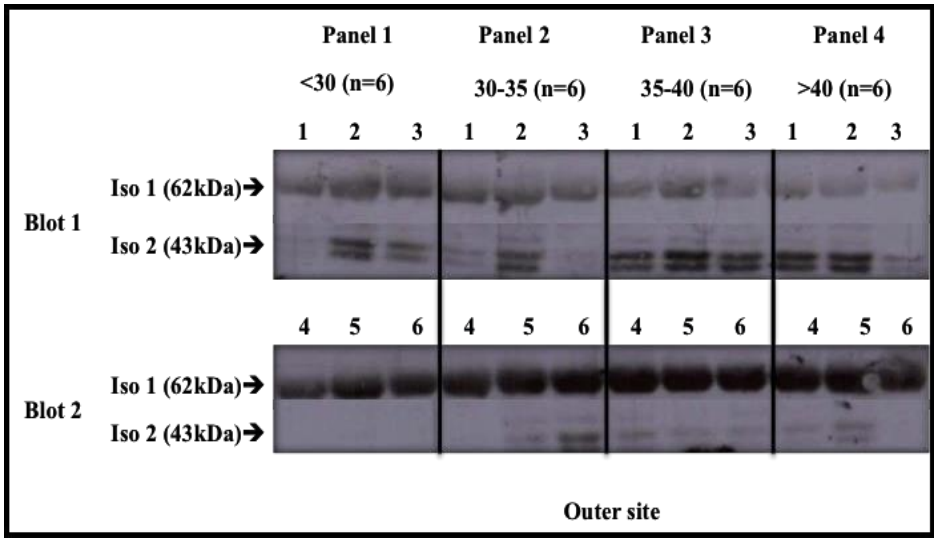


Figure 3.7.3 (upper panel): Western blots showing PON2 expression in outer site of all BMI groups. Panel 1 shows BMI <30 and samples (n=6) in blots 1& 2. Panel 2 shows BMI 30-35 and samples (n=6) in blots 1&2. Panel 3 shows BMI 35-40 and samples (n=6) in blots 1&2. Panel 4 shows BMI >40 and samples (n=6) in blots 1&2. Blots 1&2 show the 62 kDa isoform 1& 43 kDa isoform 2.

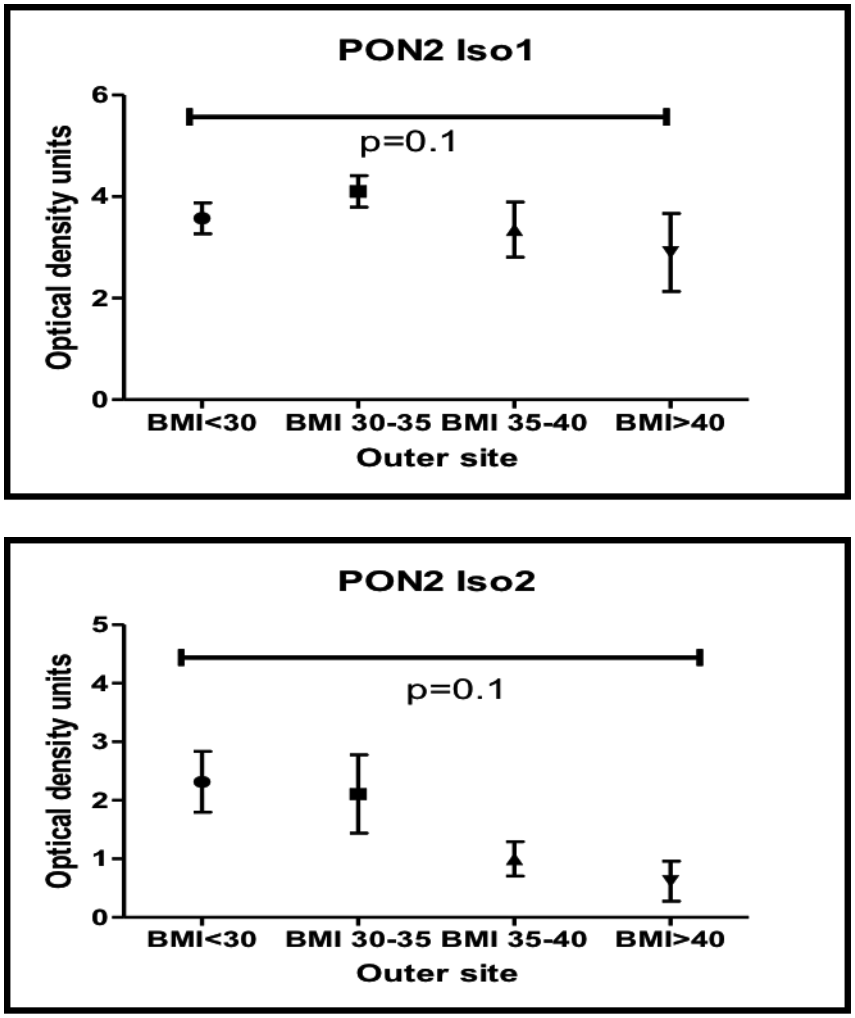


Figure 3.7.3 (lower panel): The graphs show the statistical analysis. Comparison between different BMI groups at the outer site was performed using Freidman test analysis.

In summary for the above section there was no spatial difference in expression of either PON2 isoform or PON2 mRNA between the three placental areas of all BMI groups. A study has been performed in our laboratory revealed that there were spatial differences in PON2 expression between labour and non-labour groups (Alwarfaly et al. 2014).

Chapter 4 – Discussion

4.1 HSB6 (HSP20) findings in labour

Collection of human tissue, particularly from women in labour is always difficult. The collection of material was overseen by consultant Obstetrician and Gynaecologist, Dr Kevin Hanretty. The labour group were all spontaneous labour and were a tight group (labour time minimum 3 hours maximum 8 hours). All placentas were free of infection, confirmed by the pathology report of every placenta. The non-labour group were all definitely without labour, membranes were intact with no cervical dilatation. All were planned Caesarean sections performed for obstetric reasons: breach presentation (2) previous caesarean section (2) or maternal request (2). Since 12 pieces are analysed for every placenta this means the number of tissues to process 12 x 6 for each group made performing Western blots a challenge. The numbers of cases chosen were consistent with previous studies of this type in the field. None of the changes found correlated with gestation.

In the current study there were no significant differences in expression of HSB6 (HSP20) mRNA between the three zones (inner, middle and outer) of individual placentas of the labour group and individual placentas of the non-labour group Table 4-1.

For the first time this work shows that there was a significant decrease in HSPB6 (HSP20) mRNA expression at the middle zone of the labour group when compared to the non-labour group. No difference was found at the inner and outer zones Table 4-2. There was a reduction in HSP27 mRNA and protein at the inner and middle sites in the labour group compared to the non-labour group. HSP70 was increased at the middle zone in the labour group compared to the non-labour group and HSP70 was increased in the inner zone in the non-labour group compared to the labour group.

The present results addressed whether there was a significant variation in expression depending on the placenta zone where the sample piece of tissue was taken from. However, the overwhelming majority of published studies do not control for sampling area. Studies on the placenta often show conflicting results and this may be due to sampling errors and methods; part on this work therefore attempted to try and understand if these conflicting results could be explained by sampling approaches.

Table 4.1 summarises the expression of HSPB6 (HSP20), HSP27 and HSP70 at different zones of individual placenta in labour and non-labour groups. Table 4.2 summarises the findings of comparing comparable zones of labour versus non-labour.

Table 4-1: The expression of HSPB6 (HSP20), HSP27, HSP70 at different zones of individual placentas in labour and non-labour groups. Increased, Decreased, no changes ⇔

Heat shock Protein or mRNA	Labour group			Non-labour group		
	Inner zone	Middle zone	Outer zone	Inner zone	Middle zone	Outer zone
HSPB6 (HSP20) protein	⇔	⇔	⇔	⇔	⇔	⇔
HSPB6 (HSP20) mRNA	⇔	⇔	⇔	⇔	⇔	⇔
HSP27 protein	↓	⇔	⇔	↓	⇔	⇔
HSP27 mRNA	⇔	⇔	⇔	⇔	⇔	⇔
HSP70 protein	↑	↑	⇔	⇔	↑	⇔
HSP70 mRNA	⇔	⇔	⇔	⇔	⇔	⇔

Table 4-2: Comparison between the three HSPs for labour and non-labour groups.

Heat shock protein or mRNA	**Labour group**			**Non-labour group**		
	Inner zone	Middle zone	Outer zone	Inner zone	Middle zone	Outer zone
HSP20 protein	⇔	⇔	⇔	⇔	⇔	⇔
HSB6 (HSP20) mRNA	⇔	↓	⇔	⇔	⇔	⇔
HSP27 protein	↓	↓	⇔	⇔	⇔	⇔
HSP27 mRNA	↓	↓	⇔	⇔	⇔	⇔
HSP70 protein	⇔	↑	⇔	↑	⇔	⇔
HSP70 mRNA	⇔	⇔	⇔	⇔	⇔	⇔

In order to protecting from stress that occurs in many diseases, the body responds by altering the expression of several HSPs (Garrido et al., 2012). The current study showed that the overall trend was for a decreased in the expression of the HSPs investigated during labour. Whether this means that down-regulation is linked to initiation of labour or a result of labour cannot be answered; this is a limit of working with humans. Ischemic-reperfusion injury due to uterine contractions can cause changing in protein expression in placenta (Cindrova-Davies et al., 2007) and so it is possible that labour itself could cause this change.

At the protein level, the immunoblots analysis demonstrated that there were no significant differences in HSP20 expression at the inner, middle and outer zones of placentas of the labour compared to the non-labour group. However, immunobloting for of HSP20 protein in individual placentas of both labour and non-labour groups was not carried out because of lack of time as this piece of work was carried out and completed within the year. Oxidative stress occurs when the production of ROS overwhelms the intrinsic anti-oxidant defences (Abdulsid et al., 2013). It has been reported that there is association between placental changes and oxidative stress during labour (Sitras et al., 2008; Lee et al., 2010; Peng et al., 2011; Lim et al., 2012). Moreover, decreased antioxidant capacity, can lead to production of ROS, and these will have an impact on the function of placenta involving vasoactive effects, as well as proliferation and differentiation of trophoblast (Myatt. .2004). HSP20 protects against apoptosis, decreases oxidative stress, reduces the pro-inflammatory cytokine balance, stabilizes actin, and inhibits NFκB activation (Garrido et al., 2012).

However, the opposite effect of these events needs to occur during labour. As the present study was based on human patients, it would be difficult to separate the endocrine and hemodynamic influence of labour on the placenta. However, there is no doubt that the uterine contractions associated with labour provide the basis for ischemia-reperfusion type injury to the placenta. Doppler ultrasound studies have demonstrated a linear inverse

relationship between uterine artery resistance and the intensity of the uterine contractions during labour (Brar et al., 1988).

One of the key mechanisms involved in regulating vascular tone is via endothelial cell nitric oxide (NO) release. The effects of NO are partly mediated by activation of soluble guanylate cyclase in underlying smooth muscle cells resulting in an increase in cyclic guanosine monophosphate (cGMP), activation of the serine/threonine protein kinase G(PKGI*a*) and subsequent interaction with downstream effect or proteins (Schlossmann et al., 2003; Morgado et al., 2012). The potent vasodilatation of the cGMP/PKG pathway has been shown to be due to a decrease in calcium through the activation of calcium mechanisms and/or to a decrease in the calcium sensitivity of smooth muscle myofilaments (Wu et al, 1996; Lee et al, 1997; Schlossmann et al, 2003; Morgado et al, 2012). Several myofilament-associated proteins have been identified as potential downstream targets for PKG actions and among these is HSP20 as well as VASP (vasodilator-stimulated phosphoprotein), telokin and the targeting subunit of myosin phosphatase (MLCP) termed MYPT1 (Rembold et al., 2000; Choudhury et al., 2004; Batts et al., 2005; Khromov et al., 2006; Lu et al., 2008; Fischer, 2010; Ying et al., 2012). In animal studies the variance in tissue sensitivity of relaxation to PKG stimulants have been attributed to differences in expressions of such targets and/or of the PKGI isoform itself (Rembold et al., 2000; Choudhury et al., 2004; Batts et al 2005; Khromov et al., 2006; Lu et al., 2008; Fischer, 2010; Ying et al 2012).

Of the myofilament-associated proteins tested by Dordea et al (2003), only HSP20 expression correlated with the tissue-specific PKG sensitivity. HSP20 expression was increased in myometrial arteries compared with placental arteries.

One group (Batts et al., 2005) has previously shown a correlation between low HSP20 expression in rabbit bladder smooth muscle and insensitivity to relaxation by PKG stimulation. Nothing was stated about the location of placental expression of HSP20.

4.2 HSP20 findings with BMI group

The present data aimed to examine if there was a spatial difference in HSP20 expression in placentas obtained from placentas of women with 1) BMI <30, 2) BMI 30-35, 3) BMI 35-40 and 4) BMI >40 and who were delivered by caesarean section. There was no significance difference in expression of HSP20 at the inner, middle and outer zones of the placentas between all BMI groups. This was the first study to investigate the expression of HSP20 during maternal obesity. Whether changes in phosphorylation occur requires further investigation and so a definite role in obesity in pregnancy cannot be excluded. Several studies provided evidence linking obesity and inflammation and metabolic disorders such as diabetes mellitus type 2 which has an association with chronic inflammation known as low grade inflammation (Hotamisligil, 2006).

Drugs such as calcitonin, epinephrine and gene related peptides are insulin antagonists and increase phosphorylation on ser16 while decreasing phosphorylation on ser157, therefore, HSP 20 can be a prominent mediator in insulin action (Wang et al. 1999a, Wang et al. 1999b). The precise mechanism by which HSP20 protects against obesity-induced insulin resistance remains to be elucidated.

4.3 PON2 findings

The present study investigated whether there was a spatial difference in PON2 expression in the three zones of placentas (inner, middle, outer) obtained from women who have different BMI groups; no significant differences were found in protein expression of either PON2 isoforms in the inner, middle and outer areas of the placentas of all BMI groups.

Also there were no significance differences in PON2 mRNA in the inner, middle and outer areas of placentas of all BMI groups. PON2 undergoes glycosylation with high-mannose-type sugars, which are important for maintaining protein structure but they are not necessary for their enzymatic activities (Dragomir et al., 2005). PON2 is present intra-cellularly; not in serum. It is expressed at perinuclear sites, mitochondria and ER. It has defense antioxidant abilities and it is able to prevent ER stress-induced apoptosis as well as cell-mediated oxidative modification of low density lipoprotein (LDL) (Ng et al., 2001, Horke et al., 2010). It is expressed in many tissues. PON2 is able to protect the cell, arteries and macrophage from oxidative stress by reducing reactive oxygen species (Ng et al., 2001, Horke et al., 2007, Aviram and Rosenblat 2004).

Obesity is one of the major causes of adverse outcome during pregnancy in western societies. Deregulated cell death is common in excess fat woman (Higgins et al, 2013), with a significant contribution of (1)- oxidative stress during which intrinsic anti-oxidant defences are overwhelmed by ROS production and (2)- inflammatory events. These two processes form a vicious cycle and regulate cell death pathways in either direction. Recently, it has been demonstrated that oxidative stress can be reduced due to PON2 attached to mitochondrial membranes. Consequently, decrease of ROS-triggered mitochondrial apoptosis and cell death occur (Schweikert et al., 2012). Research on PON2 reported that the correlation between metabolic disorders and genes is limited. PONs are essential detoxifying and anti- oxidative enzymes with roles being described in diabetes and obesity (Ng et al., 2006; Shih et al., 2007; Shih et al., 2009).

Studies from our laboratory have shown that PON2 protein is spatially expressed in the human placenta and changed in labour (Alwarfaly et al., 2014). No difference in protein expression of either isoform was found between the three sites in either the labour or non-labour group. At the middle site there was a highly significant decrease in PON2 expression in the labour group when compared to the non-labour group for both the 62 kDa

form (p=0.02) and the 43 kDa form (p=0.006). No spatial differences were found within placentas at the mRNA level in either labour or non-labour. There was, paradoxically, an increase in PON2 mRNA in the labour group at the middle site only. This was the first report to describe changes in PON2 in the placenta in labour. Another previous findings revealed that an important association of PON2 with cancer. For instance, microarray studies showed PON2 overexpression in some tumours such as prostate carcinoma and hepatocellular carcinoma (Li, et al., 2002, Ribarska et al., 2010), and various others including ovarian, cervical and endometrial tumours (Kang et al., 2010). It has been reported that elevation levels of PON2 may reduce redox-triggered induction of pro-apoptotic CHOP through JNK pathway during ER stress this may lead to prevention of mitochondrial cell death signaling (Witte et al., 2011).

Mitochondria have a role during apoptosis by overproduction of ROS (Ott et al., 2007). PON2 in human can decrease ROS as well as ER stress-induced apoptosis in vascular cells (Horke et al., 2007). PON2 was identified as one of a small family of up regulated genes in pediatric acute lymphoblastic leukemia patients who have very poor outcome prognosis (Oyadomari & Mori, 2004; Zhang et al., 2006; Song et al., 2008). Additionally, an over expression of PON2 was identified in lymphocytes infected with T-cell leukemia virus (Pise-Masison, et al., 2002).

Insulin resistance and atherosclerosis have a strong relation with obesity, dyslipidemia, oxidative stress, hypertension, and chronic inflammation. The liver is an important organ for glucose absorption, synthesis, and storage as well as the first site for lipid metabolism. There are many factors, which may have a significant impact on glucose metabolism in the liver, such as inflammatory stimuli and systemic and local oxidative stress (Klover. 2003; Shoelson, 2006). Consequently, whole-body insulin responsiveness is affected (Cai et al., 2005). A number of studies revealed that PON2 plays a profound role in insulin sensitivity

through its ability to modify ROS probably due to linking of PON2 to mitochondrial action (Bourquard et al. 2011).

4.4 Future directions

HSPB6/HSP20 shares structural and functional characteristics with other sHSPS. However, the exact role of HSPB6 /HSP20 remains to be elucidated and many questions remain. It would be of interest in a future study to investigate if there is a relationship between the area distribution of HSP20 and the way the placenta separates although this would be technically difficult to do. Collection of placentas at term by C/S are not exposed to the stress of labour, however, one possibility is that area differences in HSP20 might reflect the fact that labour is not far off and that the molecular steps to allow labour to proceed have started. Thus it would be interest to do comparison between placentas in the second trimester where labour is not soon to determine if such differences still present. Collection of such early material always presents challenges due to ethical considerations but is achievable as shown by many of the group's previous studies on placental bed biopsies from 8-18 weeks of gestation. It will be also of interest to investigate HSP20 expression in particular with regard to the role in pre-term labour and long labour. In addition, studies using placental explants *in vitro* and cell cultures might help to understand the steps involved in changing HSP20 expression. It would also be of interest to examine the expression of HSP20 protein and PON2 in individual placenta in both labour and maternal obesity with labour.

In summary using a systematic approach to sampling the placenta this study has shown that inflammatory markers vary between labour and non-labour and this depends on the site of sampling. No clear differences were found during obesity however future studies looking at post-translation modification may shed further light.

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Appendix

WoSRES West of Scotland Research Ethics Service

Dr Kevin Hanretty Senior University Clinical Teacher/ Honorary Consultant Obstetrician & Gynaecologist GGC NHS Board Reproductive & Maternal Medicine Level 2, McGregor Building Western Infirmary, Glasgow G11 6NT

West of Scotland REC 4

Ground Floor - Tennent Building Western Infirmary 38 Church Street Glasgow

Dear Dr Hanretty

Study title:

REC reference: Protocol number: IRAS project ID:

An investigation of the regulation and functions of the inflammation, stress and apoptotic pathways in pregnant women and in the human placenta. In particular, the impact of pre-eclampsia and fetal growth restriction on these pathways will be investigated. 13/WS/0149

VERSION 2 130896

Thank you for your letter received 17 July 2013 responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the Co-ordinator Ms Evelyn Jackson, evelyn.jackson@ggc.scot.nhs.uk.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

G11 6NT Date

Direct line Fax E-mail

22 July 2013

0141 211 2482 0141 211 1847 rose.gallacher@ggc.scot.nhs.uk

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site

concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Covering Letter		23 May 2013
Investigator CV		22 May 2013
Other: Academic Supervisor CV: Lyall		22 May 2013
Other: Student CV: Abdulsid		22 May

		2013
Participant Consent Form	3	08 July 2013
Participant Information Sheet	3	08 July 2013
Protocol	2	16 May 2013
REC application		23 May 2013
Response to Request for Further Information		

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website. Further information is available at National

Research Ethics Service website > After Review We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/ With the Committee's best wishes for the success of this project. Yours sincerely

13/WS/0149 Please quote this number on all correspondence

R Galbacher

For Dr Brian Neilly Chair

Enclosures: Copy to:

"After ethical review – guidance for researchers"

Ms Joanne McGarry, Greater Glasgow and Clyde NHS