



Middlesex University

The Role of Heparin and Heparin-Binding Growth
Factors in Pre-eclampsia

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Abstract

The aims of this study tested the hypothesis that expression of heparin-binding growth factors (HBGFs) in normal placental development was altered in a specific pregnancy disorder pre-eclampsia. HBGFs bind to heparin, a glycosaminoglycan (GAG) affecting activity. I investigated the role of heparin and HBGFs in pathophysiology of pre-eclampsia. Placental tissue from a cohort study of 87 women was performed following uncomplicated pregnancy at term, but not in labour (TNL, n=26), preterm labour (PTL, n=17), following labour onset (TL, n=21), first trimester (FNL, n=4) and pre-eclampsia (PE, n=19). The HBGFs studied were vascular endothelial growth factor (VEGF), placental growth factor (PLGF), fibroblast growth factor 2 (FGF2), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), heparin-binding epidermal growth factor (HB-EGF), midkine (MK), pleiotrophin (PTN), and cluster differentiation (CD105). The localisation of HBGFs and receptors VEGFR-1, VEGFR-2, PLGFR-1, VEGFR-2 and FGF2R-1 in placenta were detected. The expression of VEGF, PLGF, FGF2, HGF, PDGF, CD105 was confined to villous trophoblast, endothelial cells except for MK, HB-EGF and PTN was specifically to villous trophoblast. The total RNA production in human placentae samples (n=7) from PE and controls were analysed using qRT-PCR. Placental expression of mRNA was extracted for primer assays of PLGF, FGF2, MK, PTN, and endogenous housekeeping gene as Succinate dehydrogenase complex subunit A (SDHA). FGF2 and SDHA mRNA expression was significantly different using Mann-Whitney *U* test. An *in vitro* villous trophoblast invasion model was performed with human fibrosarcoma HT1080 invasive cells (positive control), mouse embryonic fibroblast NIH/3T3 non-invasive cells (negative control) and immortalised human primary villous trophoblastic cell lines TCL-1. The greatest stimulation was by FGF2, PDGF-BB, HGF, MK and co-incubation with heparin enhanced these responses, except for PTN using the Mann-Whitney *U* test. Heparin's role is indicated in mediating the effects of HBGFs. It suggests heparin therapeutic use in the treatment of pre-eclampsia.

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53. Figure 37. Represents the percentage of invasion presented as mean \pm SEM for the following cell lines HT 1080 (+ve control) of (n=3, 57.6% \pm 10.8%, p<0.05) 1000 gain, (n=3, 59.7% \pm 11.1%, p<0.05) 1500 gain, (n=3, 65.5% \pm 12.3%, p<0.05) 2000 gain 3T3/NIH (-ve control) (n=3, 21.9% \pm 5.1%, p<0.05) 1000 gain, (n=3, 22.5% \pm 5.2%, p<0.05) 1500 gain, (n=3, 25.2% \pm 5.8%, p<0.05) 2000 gain and human trophoblast TCL-1 cell line in DMEM at (n=3, 21.5% \pm 5.0%, p<0.05) 1000 gain, (n=3, 22.0% \pm 5.1% , p<0.05) 1500 gain, (n=3, 24.6% \pm 5.7% , p<0.05) 2000 gain. The TCL-1 cell line in RPMI at (n=3, 22.9% \pm 5.2%, p<0.05) 1000 gain, (n=3, 23.2% \pm 5.3%, p<0.05) 1500 gain, (n=3, 25.9% \pm 5.9%, p<0.05) 2000 gain. (*p <0.05 vs. HT1080 and 3T3/NIH, Mann-Whitney *U* test) at 1000,1500 and 2000 gains.

54. Figure 38. Represents the percentage of invasion presented as mean \pm SEM for the following cell lines HT 1080 (+ve control) of (n=3, 100% \pm 20.1%, p<0.05), 3T3/NIH (-ve control) (n=3, 36.5% \pm 7.5%, p<0.05), and human trophoblast TCL-1 cell line with the presence of heparin binding-epidermal growth factor (HB-EGF) expression at (n=3, 28.5% \pm 2.3%, p<0.05) 0nmol/l, (n=3, 28.7% \pm 1.5%, p<0.05) 1nmol/l, (n=3, 30.2% \pm 3.0%, p<0.05) 10nmol/l and (n=3, 29.9% \pm 2.4%, p<0.05) 100nmol/l. (*p <0.05 vs. HT1080 and 3T3, Mann-Whitney *U* test).

55. Figure 39A. Represents the percentage of invasion presented as mean \pm SEM for the following cell lines HT 1080 of (n=3, 60.95% \pm 15.70%; and 76.9% \pm 6.26%, p<0.05), and TCL-1 with the presence of heparin-binding-epidermal growth factor (HB-EGF) expression at (n=3, 46.43% \pm 4.40%, p<0.05) 0nmol/l, (n=3, 51.794.39%, p<0.05) 1nmol/l, (n=3, 53.06% \pm 2.58% p<0.05) 10nmol/l and (n=3, 54.28% \pm 2.26%, p<0.05) 100nmol/l. In the presence of heparin at (n=3, 55.72% \pm 6.38%, p<0.05) 0ng/ml, (n=3, 68.06% \pm 4.45%, p<0.05) 1ng/ml, (n=3, 76.51% \pm 6.80%, p<0.05) 10ng/ml, and (n=3, 79.30% \pm 6.84%, p<0.05) 100ng/ml. (* p <0.05 vs. 0 growth factor, Mann-Whitney *U* test; † p <0.05 vs. 0 heparin).

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Acronym and abbreviations

ACTH	Adrenocorticotrophic hormone
ABC	Avidin biotin complex
ADAM12	Disintegrin & Metalloproteinase domain-containing protein 12
AFP	Alpha-Fetoprotein
A1M	Alpha-1-Microglobulin
APS	Antiphospholipid syndrome
CC	Cytotrophoblast column
CD105	Cluster differentiation 105 (endoglin)
CRH	Corticotrophin releasing hormone
CT	Cytotrophoblasts
DAB	3,3'-Diaminobenzidene
ECM	Extracellular matrix
EGF	Epidermal growth factor
EVT	Extravillous trophoblast
EnEVT	Endovascular Extravillous trophoblast
InEVT	Interstitial Extravillous trophoblast
FTN	First trimester non-Labour
GnRH	Gonadotropin releasing hormone
GHRH	Somatostatin, and growth hormone-releasing hormone
hACTH	Human chorionic adrenocorticotropin
HbF	Free foetal haemoglobin

HBGF	Heparin-binding growth factor
HB-EGF	Heparin-binding epidermal growth factor
hCT	Human chorionic thyrotropin.
hCG	Human chorionic gonadotropin
hCS	Human chorionic somatomammotropin
HGF	Hepatocyte growth factor
HELLP	Hemolysis, elevated liver enzymes, low platelet count
HIER	Heat induced epitope retrieval
hpl	Human placental lactogen
Hrp	Horseradish peroxidase
HGH-V	Growth hormone variant
Inhibin A	Hormone Inhibin A
IL-11	Interleukin II
kDa	Kilodalton
LIF	Leukaemia inhibitory factor
FGF2	Fibroblast growth factor 2
FGF2R-1	Fibroblast growth factor 2 receptor-2
FGR	Foetal growth restriction
FSH	Follicle stimulating hormone
IgG	Immunoglobulin
IGFs	Insulin-like growth factors
IGF-1	Insulin-like Growth factors I
IGF-11	Insulin-like Growth factors II

IUGR	Intrauterine growth retardation
LH	Luteinizing hormone
MK	Midkine
MMPs	Matrix metalloproteinases
MW	Molecular weight
mRNA	messenger Ribonucleic Acid
PBS	Phosphate buffered saline
PE	Pre-eclampsia
PIH	Pregnancy-induced hypertension
PP13	Placental Protein13
PLGF	Placenta growth factor
PLGFR-1	Placenta growth factor receptor-1
PDGF	Platelet-derived growth factor
PDGF α	Platelet-derived growth factor-alpha
PDGF β	Platelet-derived growth factor-beta
PAPP-A	Pregnancy- associated Protein A
PTL	Preterm labour
PTN	Pleiotrophin
SA	Spiral artery
SGA	Small for gestational age
ST	Syncytiotrophoblast
STBM	Syncytiotrophoblast membrane fragments
TGF	Transforming growth factor

TGF β	Transforming growth factor β
TL	Term Labour
TNF α	Tumor necrosis factor
TNL	Term non-Labour
TRH	Thyrotropin releasing hormone
VEGF	Vascular Endothelial Growth Factor
sVEGFR-1/ sflt-1	soluble vascular endothelial growth factor receptor-1/flt-1
VEGFR-2	Vascular endothelial growth factor receptor-2
uE3	Unconjugated Oestriol
uNK cells	Uterine Natural Killer cells

Chapter1

Introduction

1.1. *Pre-eclampsia.*

Pre-eclampsia (PE) is a complex multifactorial pregnancy-specific disorder originating in the placenta. Pre-eclampsia is the leading cause of maternal and foetal morbidity, and mortality (Lyall and Belfort, 2007; Meis *et al*, 1998; Duley *et al*, 2006; Steegers, *et al*, 2010; Tuffnell *et al*, 2010; Uzan *et al*, 2011; and Munir *et al*, 2013). Even without progression to eclampsia, the syndrome presents substantial risk to mother and baby. Each year, worldwide ≥ 4 million women will develop pre-eclampsia and approximately 100 000 women will have eclamptic convulsions, with over 90% occurring in developing countries (RCOG, 2010). Estimates of incidence in developed countries vary. Pre-eclampsia complicates 2–3% of all pregnancies and 2% of women with pre-eclampsia will develop eclampsia, a potentially fatal condition (RCOG, 2010). Eclampsia affects 700,000 pregnancies each year, leading to 43,000 maternal deaths worldwide. Many factors, including genetic predisposition, immunological interactions, maternal endothelial function and environmental factors interact and culminate in the disease manifestation (RCOG, 2010).

The main clinical characterization is recognised by onset of hypertension (blood pressure $\geq 140/90$ mm Hg) developing after the 20th week of pregnancy and proteinuria (urine protein level ≥ 300 mg/L) (Lyall and Belfort, 2007; and Williams, 2010). Symptoms include high blood pressure, headaches, blurred vision, abdominal pain, nausea, vomiting, confusion, shortness of breath and excessive swelling (oedema) of the hands and feet (Lyall and Belfort, 2007). Pre-eclampsia can progress to seizures such as eclampsia, renal impairment, thrombocytopenia, liver dysfunction or foetal compromise and coagulopathy (Akercan *et al*, 2008; and Lyall and Belfort, 2007). Occasionally, PE may progress to another illness; such as the HELLP syndromes were haemolysis, elevated liver function tests and platelets. This condition is characterised by nausea, malaise, abdominal pain, affecting women of severe PE and those who have progressed to eclampsia (Lyall and Belfort, 2007; and Williams, 2010). Pre-eclampsia can quickly develop into a life-threatening condition for both mother and foetus (Kingdom *et al*, 2000; and Lyall and Belfort, 2007).

1.1.1. Normal development and the Human placenta.

The placenta is a vital specialized organ connecting the mother and the foetus and has an essential role for the maintenance of a healthy normal pregnancy (Johnson, & Everitt, 2007; Wang & Zhao, 2010; Williams, 2010; and Kay *et al*, 2011). The placenta forms an interface between the maternal and foetal circulations (Johnson, & Everitt, 2007; Wang & Zhao, 2010; Williams, 2010; and Kay *et al*, 2011). The mother is the supplier of oxygen and essential nutrients to the foetus via the placenta (Johnson, & Everitt, 2007; Wang & Zhao, 2010; Williams, 2010; and Kay *et al*, 2011). This is the site for physiological exchange of, metabolites, carbon dioxide and waste products (Johnson, & Everitt, 2007; Wang & Zhao, 2010; Williams, 2010; and Kay *et al*, 2011). The placenta performs a remarkable range of functions at various stages of foetal development until the foetal organs become functional (Johnson, & Everitt, 2007; Wang & Zhao, 2010; Williams, 2010; and Kay *et al*, 2011). During early pregnancy, the placenta synthesizes glycogen, cholesterol and fatty acids, which serve as sources of nutrients and energy for the embryo and foetus (Johnson, & Everitt, 2007; Wang & Zhao, 2010; Williams, 2010; and Kay *et al*, 2011). In addition to its crucial transporting role, the placenta is also an important endocrine organ that secretes a wide variety of hormones (Johnson, & Everitt, 2007; Wang & Zhao, 2010; Williams, 2010; and Kay *et al*, 2011). It produces numerous growth factors, secretes peptides, synthesizes steroids, protein hormones, transfers nutrients and prevents immune rejection of the foetal allograft (Johnson, & Everitt, 2007; Wang & Zhao, 2010; Williams, 2010; and Kay *et al*, 2011). Foetal survival, foetal growth and successful development are dependent on a normal functional placenta (Johnson, & Everitt, 2007; Wang & Zhao, 2010; Williams, 2010; and Kay *et al*, 2011). There are many growth factors that are required in foetal growth and development of the normal functional placenta that act via different mechanism of action (Ruoslahti and Yamaguchi, 1991; Wang & Zhao, 2010 Williams, 2010; and Kay *et al*, 2011). Adequate trophoblast invasion, increased uteroplacental blood flow and proper physiological remodelling of uterine spiral arteries ensure growth of the uterus, placenta, and foetus (Johnson, & Everitt, 2007; Wang & Zhao, 2010; Williams, 2010; and Kay *et al*, 2011).

1.1.2. The mature placenta.

The human placenta is termed haemochorial villous organ because it provides direct contact between maternal blood and the chorionic (foetal) villi in the intervillous space (Williams, 2010). The structure of the placenta provides a very large surface area by 28 weeks and progressively increases throughout pregnancy (Williams, 2010). At term, it has a diameter of 22 cm, a central thickness of 2.5 cm, and weighs approximately 500g, with a surface area of almost 11 square metres (Williams, 2010). Five to ten percent of this surface area is thin, measuring a few microns (Williams, 2010). Foetal membranes are attached to the uterus in the placenta (Williams, 2010). A discoidal placenta is shown in Figure 1. As can be seen, the placental villi are surrounded by blood from the maternal circulation (Williams, 2010).

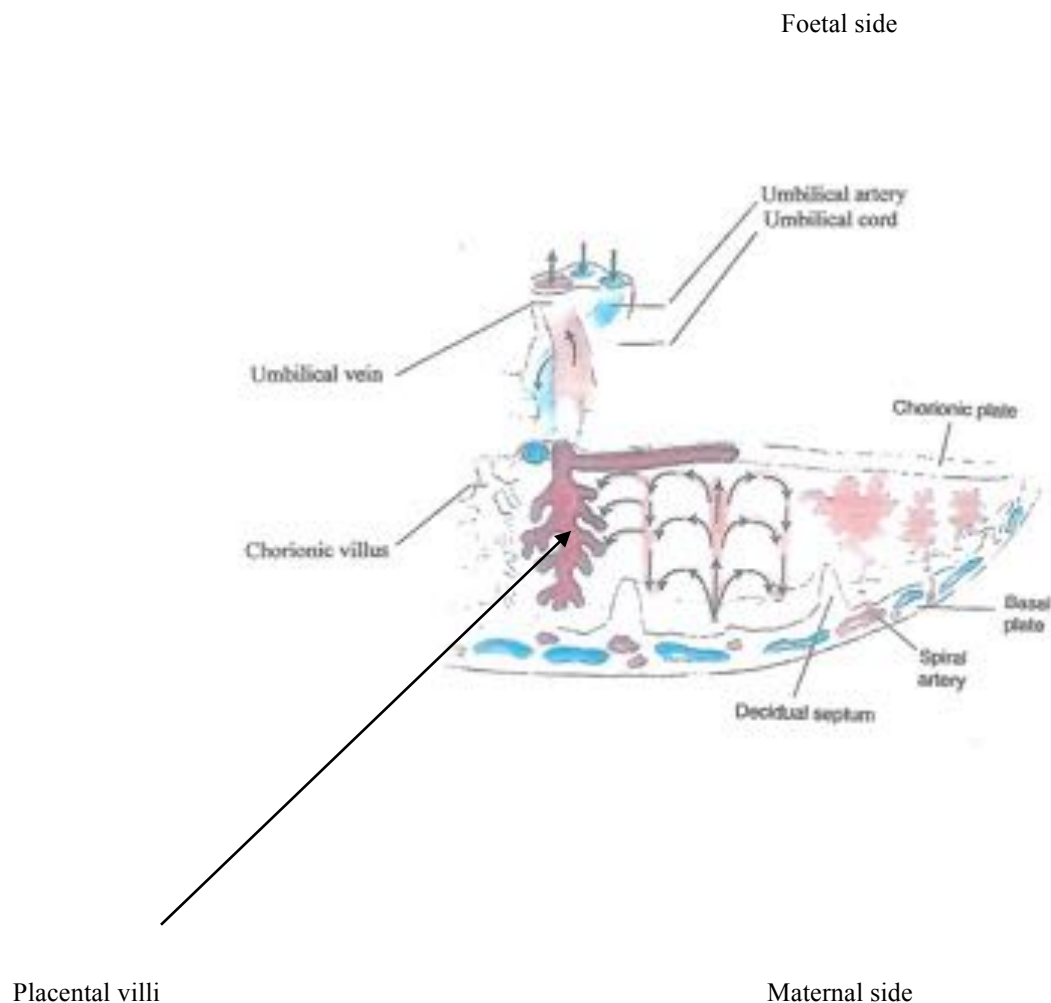


Figure 1. represents a schematic drawing of a normal section through a full-term placenta. The physiology of maternal-placental circulations is shown. The embryonic circulation consists of umbilical arteries in blue and umbilical vein is red. The arrow in black presents maternal blood that circulates in spaces between the villi. Modified from Williams, (2010).

1.1.3. Placental function.

1.1.3.1. Haemochorial placental blood flow.

There is no intermingling of maternal and foetal blood in the placenta. The placenta is a unique vascular organ. It receives blood supplies from both the maternal and the foetal circulatory systems, which are separated by several tissues called the placental membrane or placental barrier. Some villi anchor the villous tree to the basal plate whereas the bulk of the placenta comprises trees of gas-exchanging terminal villi floating in the maternal blood.

The placental circulation consists of two distinctly different systems (Wang & Zhao 2010). The uteroplacental blood circulation is essential for sufficient exchange of gases as oxygen, water, nutrients and waste products. It's the terminal villi that are grape-like structures that are important as these are characterized by capillarization and dilated sinusoids. The terminal villi are linked to stem villi that connect to chorionic plate and are characterized by condensed fibrous stroma containing both large vessels with microvessels. As maternal blood flows around terminal villi and into the intervillous space this process occurs via decidual arteries. The in-flowing maternal arterial blood pushes the deoxygenated blood into the endometrial veins. However, it is the uterine veins that drain the deoxygenated blood. The maternal rate of placental blood flow at term is approximately 600–700 ml/minute (80% of the uterine perfusion). The foetoplacental blood circulation involves two umbilical arteries that carry deoxygenated and nutrient-depleted foetal blood into the villous core foetal vessels to the placenta. It is the umbilical vein, which supplies fresh oxygenated and nutrient-rich blood from the placenta to the foetus. Wang & Zhao, (2010), estimated that the surface area of syncytiotrophoblasts is approximately 12m^2 and the length of foetal capillaries is approximately 320 kilometres. The terminal villi are essential components of the functional unit of maternal-foetal exchange of oxygen and nutrients (Wang & Zhao, 2010).

1.1.3.2. Transfer between mother and foetus.

The mechanisms by which transfer of substances across the placenta between the mother and foetus occurs include passive diffusion, facilitated diffusion, active transport, endocytosis and exocytosis. The placental transfer or transport involves bidirectional movement of gases, nutrients, waste materials, drugs and other substances across the placenta between maternal and foetal circulations. The process of transfer of substances across the placenta requires blood flow from the foetus via the umbilical vein into the foetal capillary epithelium across the placental membrane, entering the intervillous space and then into the endometrial spiral arteries. Blood flows from the mother via the endometrial spiral arteries, and enters the intervillous space across the placental membrane into the foetal capillary via the umbilical vein. A counter current mechanism exists whereby blood from the maternal circulation as shown in red in Figure 1. (modified from Williams, 2010) is separated from foetal blood containing foetal waste products, as shown in blue, returning back to the maternal circulation via the umbilical vein that carries oxygenated blood to the foetus.

Transport across the placenta increases during the course of gestation due to changes in placental structure (decreasing the distance between maternal and foetal blood), increased foetal and maternal blood flow, and greater foetal demands. Placental transfer increases as the foetal growth rate increases (Wang & Zhao 2010; and Kay *et al*, 2011).

Syncytiotrophoblasts are an important site of transport with substances moving from the apical membrane close to the maternal blood in intervillous space, and across the cell wall to the basal membrane of foetal capillary epithelium. Several mechanisms are summarised by which specific substances across are transferred the placenta. Protein carriers and other transporters mediate facilitated diffusion and active transport. For pregnancy the placental syncytiotrophoblast represents the barrier between the maternal and foetal circulations, (Wang & Zhao 2010; Williams, 2010; and Kay *et al*, 2011).

1.1.3.3a. Simple (passive) diffusion. Diffusion is movement of a substance from higher to lower concentration down electrochemical gradients and is a passive process. Diffusion is the major mechanism of placental transfer. For example water, electrolytes, oxygen, carbon dioxide, urea, simple amines, creatinine, fatty acids,

steroids, fat-soluble vitamins, narcotics, antibodies, barbiturates, and anesthetics. Simple diffusion allows most substances with a molecular mass ≤ 5000 Da to pass through the placental tissue (Johnson, & Everitt, 2007; Wang and Zhao, 2010; and Kay *et al*, 2011).

1.1.3.3b. Facilitated diffusion. This involves transport via protein carriers and other transporters to move substances like glucose and oxygen across the placental membrane. Transporters are located on both the maternal-facing border syncytiotrophoblast and the foetal-facing basal membrane (Johnson, & Everitt, 2007; Wang and Zhao, 2010; and Kay *et al*, 2011).

1.1.3.3c. Active Transport. This utilizes energy-dependent carrier systems and other transporters to move substances against concentration or electrochemical gradients. For example: amino acids, water-soluble vitamins, calcium, iron, and iodine (Johnson & Everitt, 2007; Wang and Zhao, 2010; and Kay *et al*, 2011).

1.1.3.3d. Pinocytosis. This is nonspecific in the substances that it transports and is used primarily for the absorption of extracellular fluids (ECF) (Johnson, & Everitt, 2007; Wang and Zhao, 2010; and Kay *et al*, 2011).

1.1.3.3e. Endocytosis and exocytosis. These include the globulins, phospholipids, lipoproteins, antibodies and viruses (Johnson, & Everitt, 2007; Wang and Zhao, 2010; and Kay *et al*, 2011).

1.1.3.3f. Bulk flow and 'solvent drag,' for example, are water and electrolytes. Capillary breaks, for example, are the intact blood cells. However, the maternal leukocytes - organisms such as *Treponema pallidum* - are known for independent movement (Johnson, & Everitt, 2007; Wang and Zhao, 2010; and Kay *et al*, 2011).

The foetus communicates with the mother via the placenta. Several factors, including maternal health, smoking, hypoxia, and nutritional status, may influence the maternal pregnancy (Johnson, & Everitt, 2007; Wang and Zhao, 2010; and Kay *et al*, 2011). Hormones produced by the placenta alter maternal metabolism and behavior, nutrient intake, and uterine artery blood flow. These changes are necessary to promote placental development and growth (Johnson, & Everitt, 2007; Wang and Zhao, 2010; and Kay *et al*, 2011).

1.2. Synthesis and secretion of placental hormones and growth factors.

A wide variety of hormones are produced that shift control of many regulatory functions away from the mother to the foetus. This is essential for optimal control of the gestation (Kay *et al*, (2011).

1.2.1. Placental hormones.

Most placental hormones are synthesised and secreted from the cytotrophoblast, syncytiotrophoblast, or both during pregnancy. The villous stromal cells and macrophages i.e. Hofbauer cells, are another source of hormones and growth factors (Kay *et al*, (2011). By 10 days post-conception cytotrophoblast and syncytiotrophoblast are present. Cytotrophoblast stains positive for expression of hypothalamic-like protein hormones: gonadotropin releasing hormone (GnRH), corticotrophin releasing hormone (CRH), and thyrotropin releasing hormone (TRH). Syncytiotrophoblast stain for the corresponding pituitary-like peptide hormones for example (hCG) analogous to pituitary (LH), (ACTH) and (hCT).

The villous syncytiotrophoblast is a major source of placental hormones such as *Human chorionic gonadotropin (hCG)*, *Oestrogens and progesterone*, *Human placental lactogen (hPL)*, *Human placental growth hormone*, Insulin growth factors and endothelial growth factor. Autocrine, paracrine and endocrine hormones from the cytotrophoblasts and syncytiotrophoblast regulate placental development and function. These hormones also modulate maternal physiological changes that occur in pregnancy and promote foetal growth. The EVT's synthesize human placental lactogen (hPL) and markers of migrating cells within the endometrium and myometrium (Knobil *et al*, 1994; Wang & Zhao, 2010; and Kay *et al*, 2011). The lytic activity of the syncytiotrophoblast cells causes the rupture of both maternal arterial and venous blood vessels with a resultant flow of maternal blood from the arteries into the lacunar spaces and back into the maternal system via the veins (Wang & Zhao, 2010; and Kay *et al*, 2011).

The syncytiotrophoblast is the main endocrine component of the placenta and forms the epithelial covering of the villous tree (Wang & Zhao, 2010; Williams, 2010; and Kay *et al*, 2011).

The major placental hormones for successful pregnancy outcome are listed below (Knobil *et al*, 1994; Johnson & Everitt, 2007; Wang and Zhao, 2010; and Kay *et al*, 2011).

1.2.1.1. *Human chorionic gonadotropin (hCG)*. Structurally, this glycoprotein resembles luteinizing hormone (LH) and is synthesized by syncytiotrophoblast before implantation. It is responsible for maintaining the maternal corpus luteum that secretes progesterone and oestrogens essential for pregnancy maintenance. Thus, (*hCG*) stimulates (LH) receptor in the foetus (Kay *et al*, 2011) and is the basis for early pregnancy tests. Production peaks at eight weeks and then gradually declines. By the end of the first trimester, the placenta produces enough of these steroids to maintain the pregnancy and the corpus luteum is no longer needed (Kay *et al*, 2011).

1.2.1.2. *Oestrogens and progesterone*. The placenta can produce progesterone independently from cholesterol precursors and oestrogen together with the foetal adrenal gland, as it does not contain all the necessary enzymes itself. Oestrogen stimulates uterine blood flow and contributes to maternal weight gain. However, progesterone's primary function is endometrial decidualisation, myometrial quiescence, and decreases insulin sensitivity (Kay *et al*, 2011).

1.2.1.3. *Human placental lactogen (hPL)*. Human placental lactogen (hPL) is also called human chorionic somatomammotropin (hCS): it is similar in structure to growth hormone and influences growth, maternal mammary duct proliferation and lipid and carbohydrate metabolism. This hormone is also called human chorionic somatomammotropin (hCS): It is similar in structure to growth hormone and influences growth, maternal mammary duct proliferation, and lipid and carbohydrate metabolism (Kay *et al*, 2011).

1.2.1.4. *Human placental growth hormone*. This differs from pituitary growth hormone by 13 amino acids and replaces maternal pituitary growth hormone from 15 weeks until the end of pregnancy. Its function is the regulation of maternal blood glucose levels for the foetus with adequate nutrient supply. Its secretion is stimulated by insulin production, i.e. low maternal blood glucose levels. It promotes weight gain (Kay *et al*, 2011). It stimulates gluconeogenesis in the maternal liver.

1.2.1.5. *Other hormones.* Human placental growth hormones function is the regulation of maternal blood glucose levels for the foetus with adequate nutrient supply. It is the Insulin-like growth factors (IGFs) produced in large quantities by cytotrophoblasts that regulate foetal and placental growth. IGFs stimulate proliferation, and differentiation (Kay *et al*, 2011). Matrix metalloproteinases (MMPs) are proteolytic enzymes that break down the extracellular matrix within the deciduas. IGFs and MMPs are both believed to be important for placental development (Hills *et al*, 2004). It is from the 4 to 5-week-old placenta that the endothelial growth factor is produced and stimulates proliferation of the trophoblast.

In the placenta, both Human chorionic thyrotropin (hCT) and Human chorionic adrenocorticotropin (hACTH) are produced in small amounts. Their functions are similar to pituitary thyrotropin and pituitary adrenocorticotropic hormone (ACTH). It is the decidua cells that produce relaxin that softens the cervix and pelvic ligaments in preparation for childbirth. Also, produced are the growth hormone variant (HGH-V), parathyroid hormone-related protein (PTH-rP), calcitonin, relaxin, leptin, neuropeptideY, inhibins, activins, and atrial natriuretic peptide. In addition there are hypothalamic –like releasing and inhibiting hormones namely thyrotrophin-releasing hormone (TRH), gonadotrophin-releasing hormone (GnRH), corticotrophin-releasing hormone (CRH), somatostain and growth hormone-releasing hormone (GHRH) (Wang & Zhao, 2010; Williams, 2010; Kay *et al*, 2011).

1.2.1.6. Embryonic and placental development.

The process of fertilization is fusion of spermatozoon and an ovum in the ampulla of the fallopian tube (Johnson, & Everitt, 2007; Wang & Zhao, 2010; Williams, 2010; Kay *et al*, 2011). This is followed by differentiation during embryonic development. The formation of the placenta begins with the outer cell layer trophoctoderm, which consists of a simple layer of specialized epithelial cells termed trophoblast. Trophoblastic cells are formed during the process embryogenesis and have a critical role at the foetal-maternal interface until term. The trophoblast exhibits the most variable structure, function and developmental pattern of all placental components. The inner layer embryoblast consists of undifferentiated pluripotent embryonic stem cells that develop into the embryo. These differentiate at the morula stage and form

the embryonic part of the placenta. Several days of slow cleavage division occurs for the first 3 to 4 days, as the blastomeres divides continually without growth to produce the morula. This enters the uterine cavity about 3 to 4 days after fertilization. It is the gradual accumulation of fluid between the cells of the morula (Johnson, & Everitt, 2007; Wang & Zhao, 2010; Williams, 2010; and Kay *et al*, 2011).

1.2.1.7. Implantation and the Blastocyst.

Implantation of the embryo into the uterine wall takes place 6 or 7 days after fertilization. This process involves three phases: the initial adhesion of the blastocyst to the uterine wall is known as apposition and adhesion brings an increased physical contact between the blastocyst and the uterine epithelium. The final phase is trophoblast invasion, an essential process for embryo implantation and placental formation (Kay *et al*, 2011). The main structural and functional units of the placenta are chorionic villi. Key cells inside chorionic villi are cytotrophoblast. They proliferate, invade, migrate and differentiate to syncytiotrophoblast (Kay *et al*, 2011; and Williams, 2010). It's the extravillous trophoblast (EVT) invasion that migrates into the maternal decidualized endometrium, invades the myometrium, penetrates the maternal uterine vasculature and remodels the uterine spiral arteries supplying oxygen and nutrients during early placentation (Kay *et al*, 2011; Williams, 2010). The trophoblast cells derived from the outer cell layer of the blastocyst (trophectoderm) mediate implantation. This is the process by which the early embryo establishes a physical connection with the mother. This is called placentation. The placenta begins to develop after implantation approximately eight days after fertilisation. However, the precursor cells to the placenta are present at the first differentiation stage of the blastocyst as shown in Figure 2. In the first stage of implantation day 6 there is a change in structure of the trophoblast cells with a reduction in number of apical microvilli allowing a closer association of the trophoblast cells and the uterus (Williams, 2010). The embryonic stem cells that develop into the embryo during implantation actively secrete hCG, which is detected in maternal serum on the 8th day after ovulation (Wang & Zhao, 2010; and Williams, 2010).

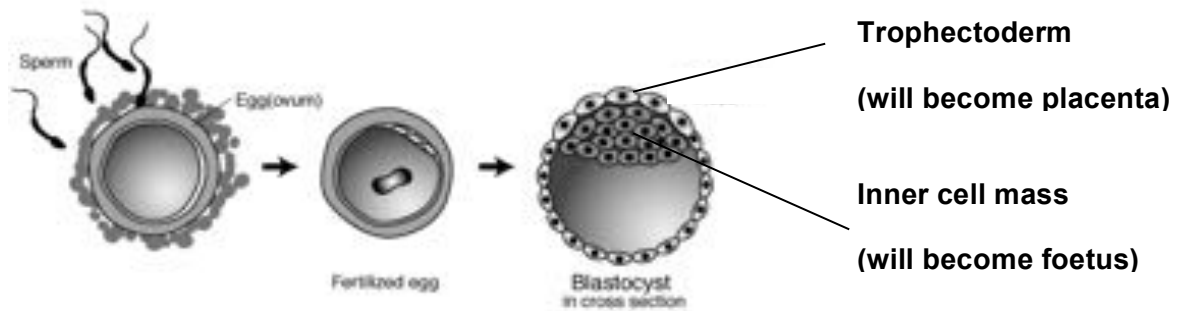
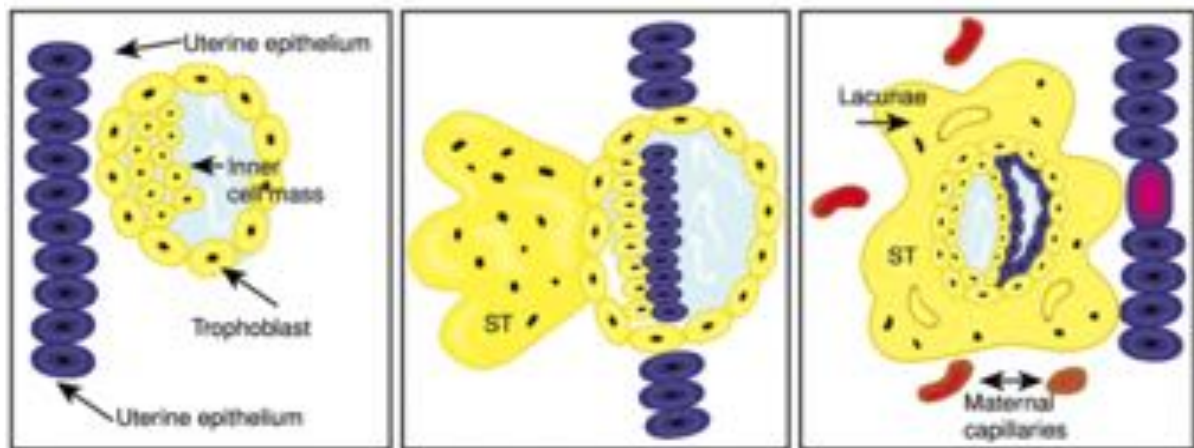


Figure 2. The blastocyst development and differentiation between placental trophoblast and foetal inner cell mass (from Carlson, 2011).

Implantation is accomplished through the invasive activities of syncytiotrophoblast that are derived from the cytotrophoblast (Johnson, & Everitt, 2007; Wang & Zhao, 2010; Williams, 2010; and Kay *et al*, 2011). This is then followed by interdigitation of the uterine microvilli and the trophoblast cell membrane. At this stage the trophoblast differentiates into two layers, the cytotrophoblast and the syncytiotrophoblast, which is made from fused cytotrophoblast formed into multinucleated cells. The inner cytotrophoblast layer is composed of individual and rapidly dividing cells.



A)

B)

C)

Figure 3A represents blastocyst implantation and differentiation. Figure 3B represents the blastocyst and cytotrophoblast proliferation during invasion. Figure 3C represents the invasive multinucleated syncytiotrophoblast. Adapted from Kay *et al*, (2011).

The first step in implantation of the blastocyst is known as apposition, as shown in Figure 3A. The outer layer is trophoblast that is comprised of trophoblast cells (yellow). The trophoblast cells form the multiple cytotrophoblasts. The adhesion process occurs within the inner cell mass near the decidua in the blastocyst. An invasive multinucleated syncytiotrophoblast (ST) is formed. The bilaminar disk of embryoblast and amnioblast arise because of inner cell mass differentiation, as shown in Figure 3B. The syncytiotrophoblast vacuoles coalesce to form the lacunae following the process of invasion in Figure 3C. The maternal blood at a high volume and low pressure with erosion of maternal capillaries fills in the intervillous space of lacunae. The syncytiotrophoblast (ST) cells have highly invasive properties during implantation and penetrate the uterine epithelium into the underlying stroma. As the syncytiotrophoblast continue to proliferate and delineate extracytoplasmic cavities. This process results in formation of lacunae lined with syncytiotrophoblast cells, as shown in Figure 3C above. Primary villi formed by the trophoblast cells are invaded by mesenchyme cells and transformed into secondary villi. Syncytiotrophoblast lines the foetal side of the intervillous space opposite the decidualized endometrium of the maternal side (Kay *et al*, 2011).

Syncytiotrophoblast cells are exposed to the maternal bloodstream and separated from the foetal circulation, as shown in Figure 6.

Therefore, most placental hormones are protein hormones for example PAPP, hCG, ADAM12, PP-13 are for the first trimester, and hCG, Inhibin A, uE3, AFP for second trimester prenatal diagnosis (Kay *et al*, 2011). These are mainly secreted into the maternal circulation in concentrations much higher than in the foetus. Syncytiotrophoblasts interact with the maternal blood supply to secrete placental hormones directly into the circulation. Decidua lines the maternal surface of the intervillous space and secretes protein hormones (Williams, 2010). Decidual hormones include epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) and leukaemia inhibitory factor (LIF) (Lyall and Belfort, 2007; and Kay *et al*, 2011).

At this stage, the embryo develops inside the uterus and there is very little maternal blood supply to the embryo.

1.2.1.8. Placental oxygenation.

Normal early placental villous development occurs in a low oxygen environment (PO_2 ~3%), which increases to 8% oxygen during 10-12 weeks of pregnancy (Rodesch *et al*, 1992). The higher level then persists for the remainder of a successful pregnancy. This change in placental oxygen supply for the first 10 and 12 weeks of gestation is a critical time for establishing the maternal-placenta circulation. The cytotrophoblast plugs in maternal spiral arteries limit the access of maternal arterial blood flow to the placenta intervillous space. During weeks 10-12 of pregnancy the cytotrophoblast plugs are lost from the maternal spiral arteries, which leads directly to increased maternal blood supply and oxygen to the placenta (Wang & Zhao 2010). Crucially, this change is linked to increased expression of enzymes in the placenta that protect against oxidative stress (Jauniaux *et al*, 2000). It is suggested that cytotrophoblast plugs give rise to intermittent placental blood flow, hypoxia/reperfusion and oxidative stress (Wang & Zhao 2010; Burton *et al*, 2003; and Hubel, 1999). This results in extensive cellular damage, which is believed to be a key factor in the pathogenesis of pre-eclampsia (Burton and Jauniaux 2004). However, the maternal blood supply is transformed from around eight weeks gestation following a secondary wave of invasion.

1.2.1.9. Trophoblast differentiation and different subtypes.

The trophoblast has a critical role at the foetal-maternal interface until term. The trophoblast exhibits the most variable structure, function and developmental pattern of all placental components. The trophoblast lineage develops first at the blastocyst stage. After blastocyst implantation is complete, further differentiation occurs, as generations of the syncytiotrophoblast subsequently form two main trophoblast types of placental villi known as villous cytotrophoblast and villous syncytiotrophoblast (Benirschke *et al*, 2006; Kay *et al*, 2010; and Williams, 2010). Those trophoblast cells that start to invade the maternal uterine tissues are termed extravillous trophoblast with the respective subtypes (Modified from Benirschke *et al*, 2006; Kay *et al*, 2010; Wang & Zhou, 2010; and Williams, 2010) as shown in Figure 4. Eventually, these extravillous trophoblast cells migrate as interstitial trophoblast first enter the decidua, then subsequently the endometrial stroma. The interstitial trophoblasts penetrates

further into the wall of spiral arteries known as intermural trophoblast, that reach the vessels are called endovascular trophoblast. There are some interstitial trophoblast that both fuse and cause generation of multinucleated trophoblast giant cells between the endometrium and myometrium.

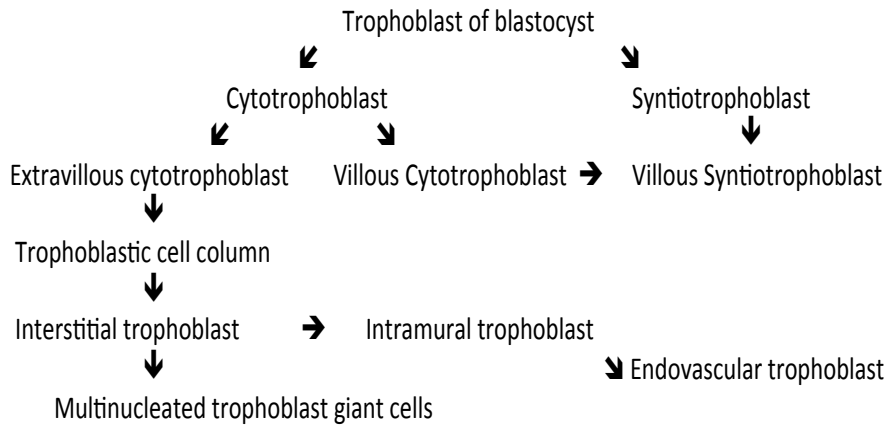
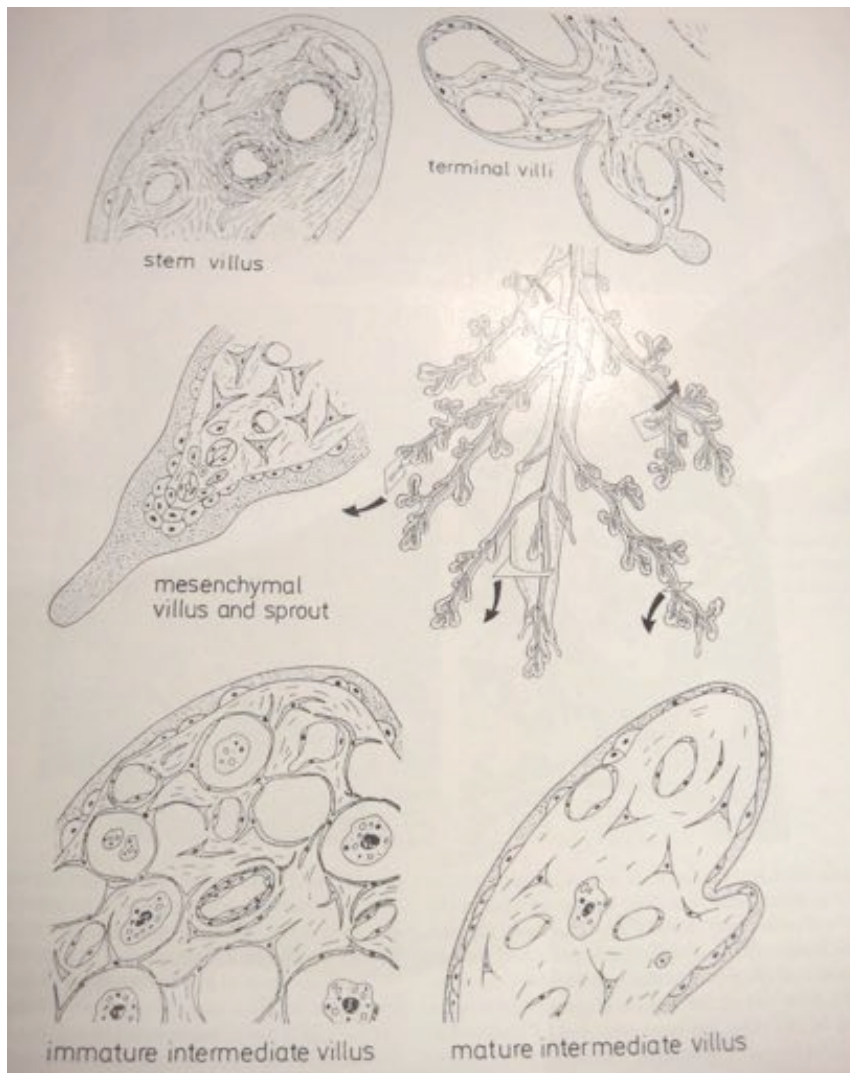
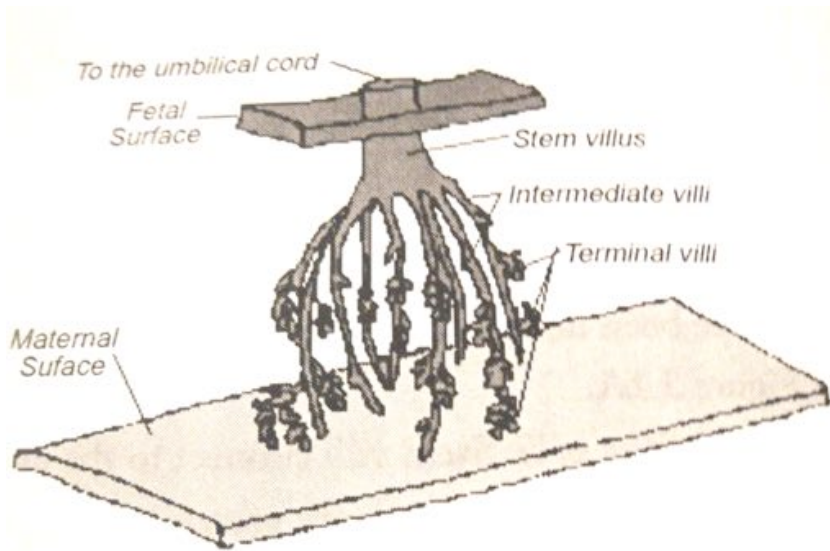


Figure 4. represents the differentiation of trophoblast lineage and subtypes (modified from Benirschke *et al*, 2006; Kay *et al*, 2010; Wang & Zhou, 2010; and Williams, 2010).

1.2.2. Placental villous tree.

An outer layer of multinucleated syncytiotrophoblast and a subjacent layer of mononucleated cytotrophoblasts form a placental villous trees structure in Figure 5a. (Modified from Benirschke *et al*, 2006), in Figure 5b. represents cross section of different types of villi and vessel branches of a cotyledon (Modified from Benirschke *et al*, 2006), with the villi projections are shown in Figure 6 (Kay *et al*, 2010; and Williams, 2010).

a.



b.

Figure 5a. represents a human placental villous tree. Figure 5b represents cross section of different types of villi and vessel branches of a cotyledon (Modified from Benirschke *et al*, 2006).

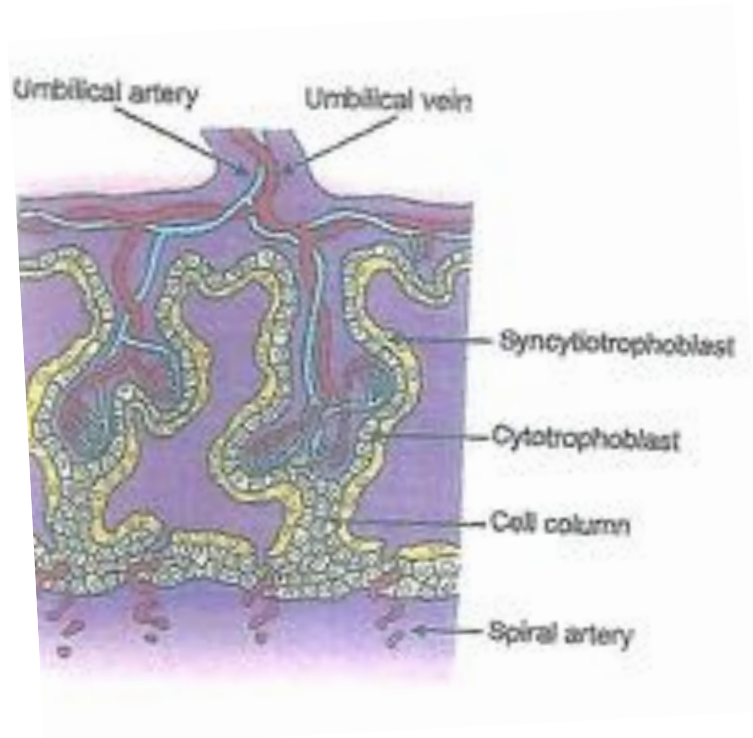


Figure 6. represents villi with in a cotyledon of placental circulation (modified from Kay *et al*, 2010).

1.2.2.1. Types of placental villi.

Placental villous detailed structure as in the cross section in Figure 7. Modified from Human Embryology (Law, 1999). It's development starts with the mesenchymal villi from 5 weeks' p.c. (post conception), differentiate into four villus types: Immature intermediate villi, stem villi, terminal villi and mature intermediate villi that are the tertiary villi prominent beyond 20 weeks of gestation (Benirschke *et al*, 2006; Kay *et al*, 2010; and Williams, 2010). These different types of placental villi are shown in Figure 5b (Modified from Benirschke *et al*, 2006). The primary chorionic villi are formed by the syncytiotrophoblast by about 20 days of p.c., and intermediate trophoblast between the lacunae that are invaded by columns of cytotrophoblastic cells (Kay *et al*, 2010; and Williams, 2010). These form the cytotrophoblast shell from the growth to the periphery and the interface between the trophoblast and endometrium. Secondary chorionic villi develop from the mesenchymal core as extraembryonic mesoderm invades the primary villi. As the foetal vessels are vascularised, the mesenchymal villus becomes the tertiary villus with both syncytiotrophoblasts and cytotrophoblasts that are the outer layer of the trophoblasts

(Kay *et al*, 2010; Williams, 2010). At the end of 4 months essentially, placenta is established as it grows in diameter, thus villous tree expansion occurs, complementing growth in size of the uterus throughout pregnancy (Kay *et al*, 2010; and Williams, 2010).

1.2.2.2. Placental villous.

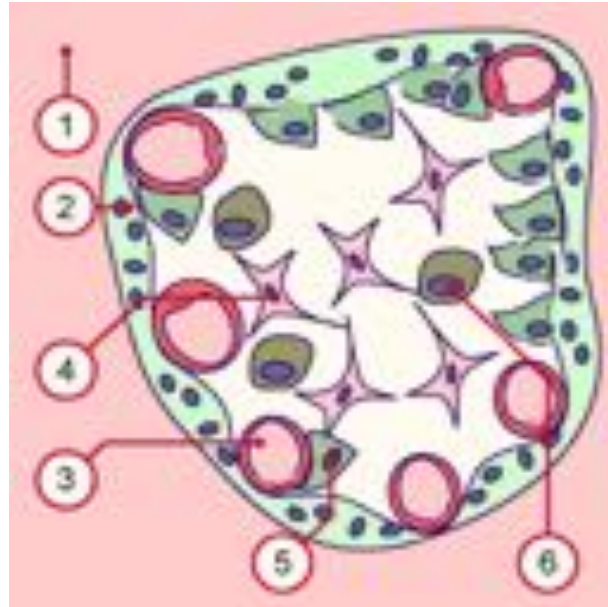


Figure 7. Placental villous detailed structure as in the cross section above. 1. Intervillous space, 2. Syncytiotrophoblast, 3. Foetal capillaries, 4. Extraembryonic mesenchyme, 5. Cytotrophoblast, 6. Hofbauer macrophages. Modified from Human Embryology (Law, 1999).

1.2.2.3. Placental development in pregnancy.

In early pregnancy, the villi are distributed over the entire periphery of the chorionic membrane. The progression of human blastocyst implantation requires cytotrophoblast cells fusion and formation of non-proliferative multinucleated syncytiotrophoblast (Benirschke *et al*, 2006; Kay *et al*, 2010; and Williams, 2010). Syncytiotrophoblast's vital role in foetal maternal exchanges and endocrine activity to release hormones that are involved in homeostasis of pregnancy for example polypeptide hormones as chorionic gonadotrophin (CG) and placental lactogen (PL) and steroid hormones as progesterone and oestrogens (Benirschke *et al*, 2006; Kay *et al*, 2010; and Williams, 2010). Soon after implantation, around day 14 cytotrophoblast cells and the syncytiotrophoblast layer give rise to extravillous trophoblast (EVT) cells (Benirschke *et al*, 2006; Kay *et al*, 2010; and Williams, 2010). Thus, EVTs invade the maternal endometrium and migrate into the decidua. Then, one pole extends outward toward the endometrial cavity and the opposite pole forms the placenta from villous trophoblast and anchoring cytotrophoblasts. The chorion grows fairly uniformly around the whole periphery of the vesicle during 2 months of embryological development (Benirschke *et al*, 2006; Kay *et al*, 2010; and Williams, 2010).

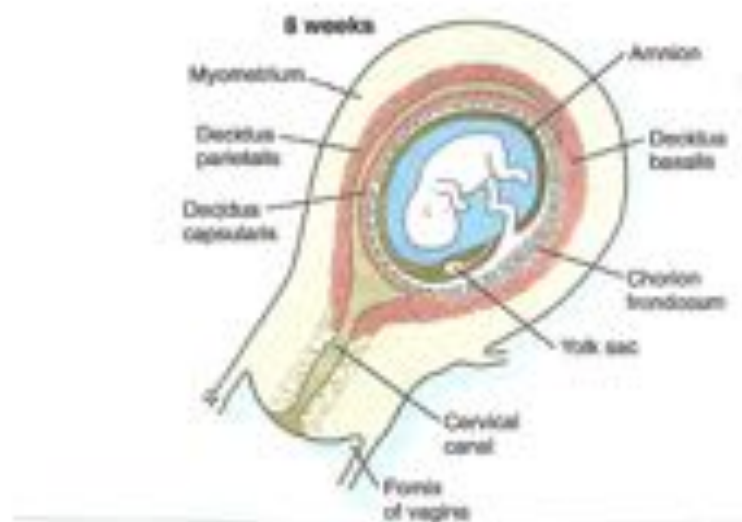


Figure 8. Represents the relationship between foetus and maternal decidua from 8 weeks of pregnancy (modified from Carlson, 2009).

However, 3 months later the chorion in contact with the decidua basalis develops extensive frond-like villous outgrowths into the decidua as the chorion frondosum, as shown in Figure 8.

Chorionic villi proliferate to form the chorion frondosum, the foetal component of the placenta. Then, the remainder of the chorion becomes smooth chorion laeve. The chorion frondosum and decidua capsularis develop into the flattened placenta and the vessels connecting the chorion to the embryonic circulation becomes the umbilical cord (Benirschke *et al*, 2006; Kay *et al*, 2010; Williams, 2010). With continued growth of the embryo to foetus, the decidua capsularis merges with the parietalis. Eventually, the EVT's invade the myometrium and penetrate into the uterine vasculature (Benirschke *et al*, 2006; Kay *et al*, 2010; and Williams, 2010).

1.2.2.4. Placental functions for foetal growth and development depends on trophoblast invasion, as shown in Figure 9.

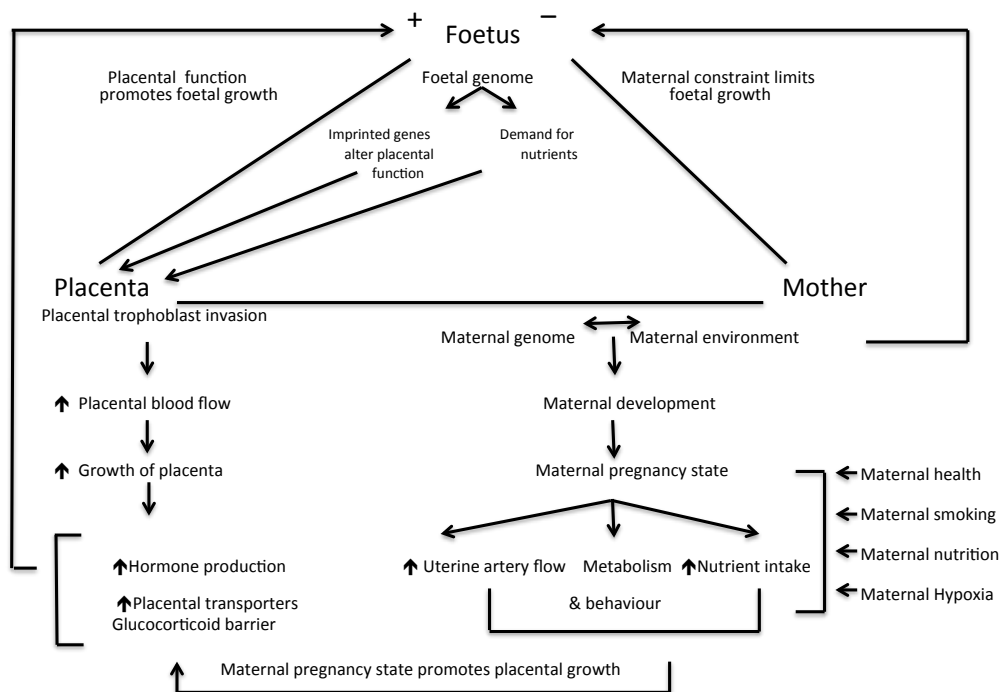


Figure 9. represents the interactions between the foetus, placenta and mother during human pregnancy (modified from Murphy *et al*, 2006).

During the first trimester an increase in blood flow in the placenta is necessary for placental growth which in turn increases hormone production in order for the placenta to carry out functions discussed earlier and transporters to transfer nutrients and waste exchange between the mother and foetus. Adequate placental function promotes foetal growth, which is influenced by the foetal genome and maternal constraint this refers to the limited capacity of the uterus to support foetal growth and is important to limit foetal overgrowth to ensure the mother's capacity for future successful pregnancies. A defect in any of these processes can lead to alterations in foetal growth, which has adverse consequences both in the short term and long term (Murphy *et al*, 2006; and Kay *et al*, 2011).

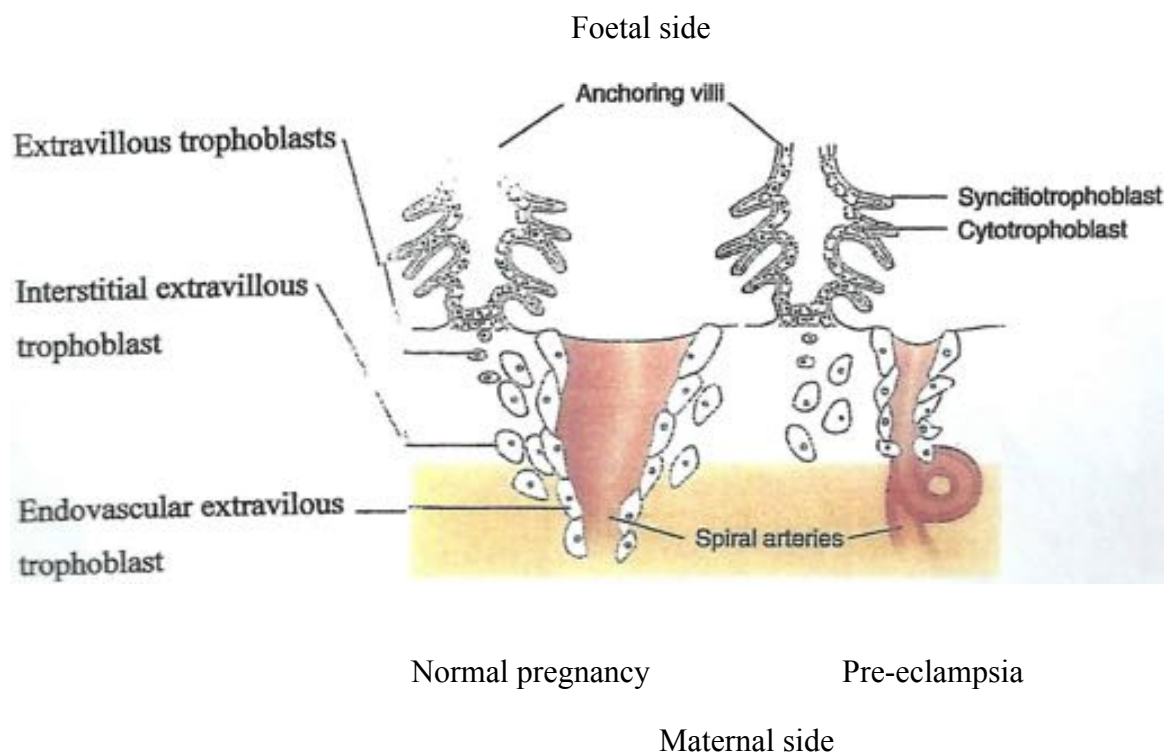


Figure 10. represents the trophoblast invasion and placental implantation in normal pregnancy and pre-eclampsia (modified from Williams, 2010).

In Figure 10. trophoblast invasion and placental implantation shows proliferation of extravillous trophoblast cells (EVTs) from the anchoring villous. The trophoblastic lineage gives rise to the three main cell types in the human placenta: syncytiotrophoblast, villous cytotrophoblast and extravillous trophoblast cells. Trophoblastic invasion in the myometrium and the decidua precedes endovascular invasion. The extravillous trophoblast cells (EVTs) proliferate to form the cell columns. The EVT invades the decidua and eventually penetrates the myometrium to

form trophoblast giant cells. This trophoblastic also surrounds the spiral arteries (Lyall and Belfort, 2007; Kay *et al*, 2010; and Williams, 2010; Kingdom *et al*, 2011). The EVT are interstitial trophoblast and endovascular trophoblast as in Figure 11 that invade and transform uterine spiral arteries during extensive remodelling for pregnancy to create low-resistance blood flow that is characteristic of the human placenta.

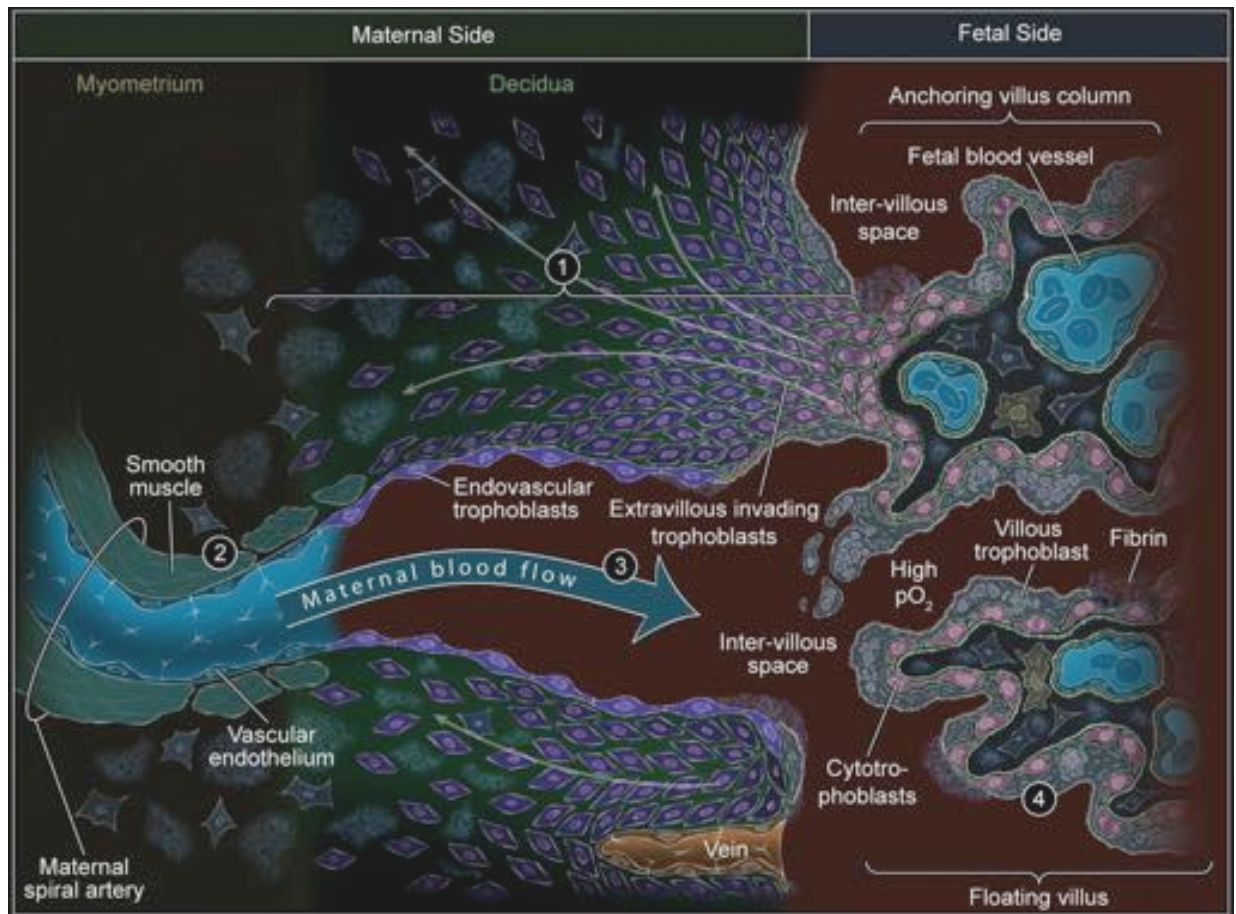


Figure 11. represents the normal human placental development where EVT cytotrophoblasts proliferate in anchoring columns and invade the decidua. 1) Transformation of uterine spiral arteries. 2) Changes arbitrate high volume at low pressure into the intervillous space. 3) Placental villi covered by villous trophoblast compartment. 4) Cytotrophoblasts proliferate to generate the outer syncytiotrophoblast directly with the maternal blood (modified from Kingdom *et al*, 2011).

1.2.2.5. Invasion and spiral artery remodelling.

Vascular remodelling is a characteristic of pregnancy comprising a complex sequence of key events that begins after implantation. There are many growth factors that have been described to be important in the process of invasion and involved in angiogenesis, for example: VEGF, FGF2, PLGF, HB-EGF, PDGF, HGF, PTN, MK, CD105 and Eng a cell surface co-receptor of TGF- β 1 and (TGF)- β 3 (Pijnenborg *et al*, 2010). Trophoblast has an important role in the complete physiological change of the spiral arteries. It is during the first 10-12 weeks of gestation, extravillous trophoblasts 'plugging' the spiral arteries are blocked, thus preventing the maternal blood flow from entering the villous space (Kay *et al*, 2010; Wang & Zhao 2010; Williams, 2010). An environment of physiological hypoxia is created for early placental villous and foetal development (James *et al*, 2006). This study of (James *et al*, 2006) implied that the maternal-placenta circulation is partially established at the end of the first trimester between 10 and 12 weeks of gestation. Blood flow into the extravillous space is established by the 11th week. Spiral artery remodelling is detected after 18 weeks (Wood, 2014). The trophoblastic invasion process begins early in pregnancy and continues until 20th week of gestation. As trophoblastic differentiates into villous trophoblast and extravillous trophoblast. These two types of extravillous trophoblast are found outside the villous, endovascular and interstitial trophoblast (Kay *et al*, 2010; Wang & Zhao 2010; and Williams, 2010). It is the villous trophoblasts that give rise to the chorionic villi, which primarily transport oxygen and nutrients between foetus and mother (Kay *et al*, 2010; Wang & Zhao 2010; Williams, 2010). The villous cytotrophoblast cells proliferate throughout pregnancy and fuse to generate the syncytiotrophoblast (Kay *et al*, 2010; Wang & Zhao 2010; Williams, 2010).

Remodelling creates a dilated high-flow, low-resistance vessel and endovascular extravillous trophoblast (EnEVT) arises as groups of trophoblastic cells that detach from the trophoblastic shell. The endovascular trophoblast cells express the adhesion molecule CD56, which is thought to function in the formation of the endovascular trophoblast plugs (Kay *et al*, 2010; Williams, 2010). These trophoblast plugs disappear as the extensive remodelling of the spiral arteries within the decidual and myometrium takes place (Kay *et al*, 2010; and Williams, 2010). Then, EVTs enlarge the vessel diameter by replacing the maternal vascular endothelial and muscular lining

(Kay *et al*, 2010; and Williams, 2010). Fibrinoid necrosis of the vessel media and a loss of endothelium occurs, converting muscular vessels into flaccid sinusoidal sacs. This vascular transformation is necessary to accommodate the huge increase in blood flow (3.5-fold) to the intervillous space required to support the developing foetus (Kay *et al*, 2010; and Williams, 2010). Thus, EVT's are non-proliferative and separate, and invade the maternal endometrium, migrate into the decidua, myometrium and penetrate into the uterine vasculature, as shown in the Figure 11.

During early placentation, human extravillous cytotrophoblasts invade the maternal uterine spiral arteries of the decidua and myometrium in the haemochorial arrangement (Lyall and Belfort, 2007; Kay *et al*, 2010; Pijnenborg *et al*, 2010; Wang & Zhao 2010; and Williams, 2010; and Kingdom *et al*, 2011). Maternal uterine spiral arteries remodelling is an important event that takes place in the tunica media. This is unique to human pregnancy. Tunica media provides the mechanical strength of the blood vessel because of its circular layer of the smooth muscle (Lyall and Belfort, 2007; Kay *et al*, 2010; Pijnenborg *et al*, 2010; Wang & Zhao 2010; and Williams, 2010; and Kingdom *et al*, 2011). This contains elastin, collagen and is innervated by sympathetic nerve fibers. EVT provides steady perfusion of the placental sinusoids. These placental sinusoids are a type of vessel with large capacity low resistance, but lack contractibility with maternal blood. They are unimpeded by the influence of vasoactive substances (Lyall and Belfort, 2007; Kay *et al*, 2010; Pijnenborg *et al*, 2010; Wang & Zhao 2010; and Williams, 2010; and Kingdom *et al*, 2011). The conversion of these spiral arteries is associated with the loss of their vascular smooth muscle cells (VSMCs) and most of their endothelial cells by apoptosis or migration out of the vessel wall (Lyall and Belfort, 2007; Kay *et al*, 2010; Pijnenborg *et al*, 2010; Wang & Zhao 2010; and Williams, 2010; and Kingdom *et al*, 2011). This vascular transformation causes the muscular, tightly coiled decidua spiral arteries into dilated sinusoids, allowing an increase in uterine blood flow to perfuse the placenta (Lyall and Belfort, 2007; Kay *et al*, 2010; Pijnenborg *et al*, 2010; Wang & Zhao 2010; and Williams, 2010; and Kingdom *et al*, 2011).

1.2.2.6. There are many factors that influence the growth and development of the placenta as shown in the Figure 12.

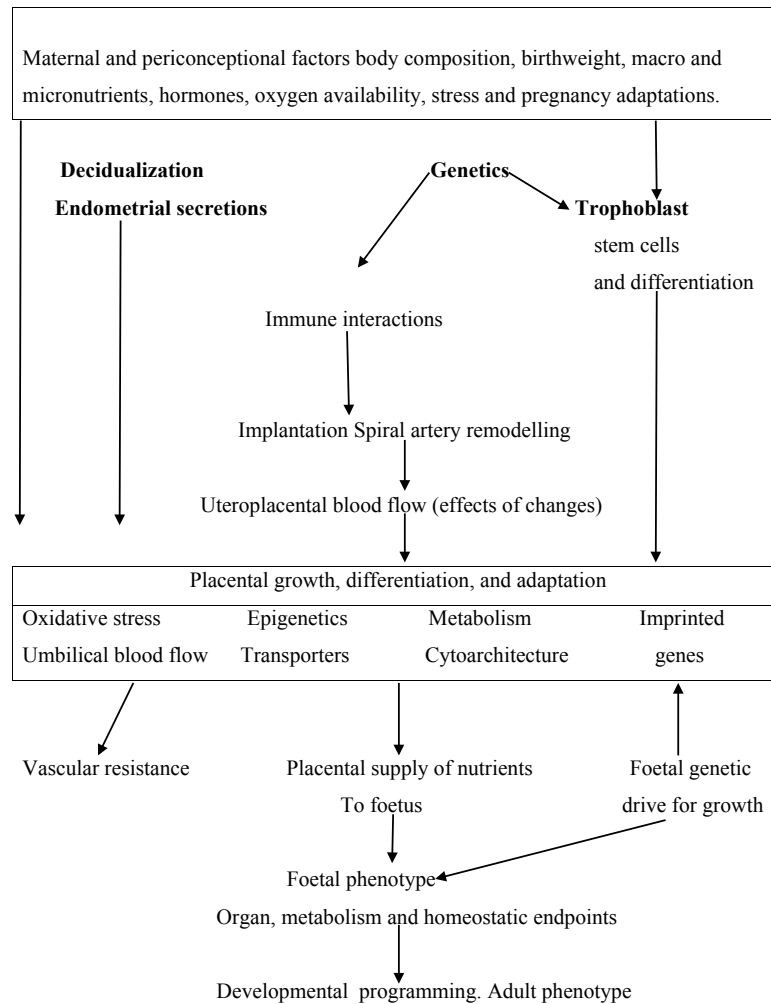


Figure 12. Representation of the major factors affecting placental development and function, and the ways in which these may affect the developmental programming of the foetus modified from (Burton *et al*, 2011).

1.2.2.7. In normal pregnancies, the transformation of spiral arteries into utero-placental arteries is around mid-gestation.

After reaching and remodelling the spiral arteries, the EVT then gradually extends laterally, reaching the periphery of the placenta around mid-gestation (Pijnenborg *et al*, 1980), as shown in Figure 13.

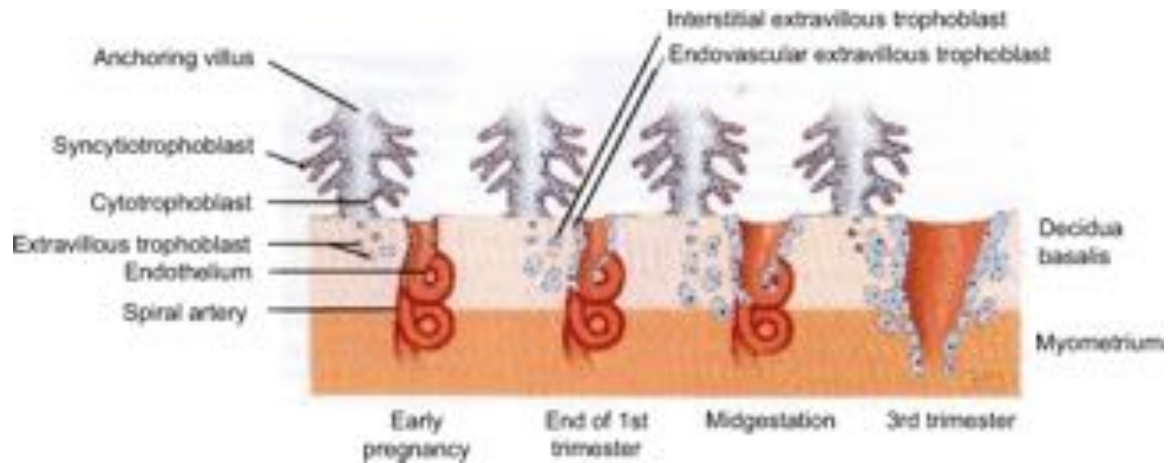


Figure 13. During pregnancy the spiral artery remodels (adapted from Williams Obstetrics; Wang & Zhao 2010).

Depth-wise changes normally extend as far as the inner third of the uterine myometrium within the central region of the placental bed, but the extent of invasion gets progressively shallower towards the periphery (Pijnenborg *et al*, 1980), as shown in Figure 13.

The main aim of these vascular changes is to optimize the distribution of maternal blood into a low-resistance uterine vascular network and ultimately inside the placental intervillous chamber. At term, the utero-placental circulation carries approximately 600 ml of maternal blood per minute (Ramsey and Donner, 1980; and Lyall and Belfort, 2007). During this process trophoblastic cells aggregate within the vessels lumen and plug the distal segments of the spiral arteries. Thus, before 10 weeks of gestation, the intervillous space contains mostly glandular secretion products together with the plasma filtrate that is free of maternal blood cells (Williams, 2010; Wang & Zhao, 2010; and Kay *et al*, 2011). The remodelling of the maternal uterine spiral arteries involves a series of steps (Lyall and Belfort, 2007), which are critical for the normal growth and development of the foetus. The term ‘physiological

change' in spiral arteries was originally used to describe the disappearance of the normal muscular and elastic structures of arteries and their replacement by fibrinoid material in which trophoblastic cells are embedded. Thus, a healthy utero-placental homeostasis depends on highly regulated extravillous trophoblastic cell functions, such as proliferation, migration and invasion. This process is impaired in pre-eclampsia, foetal growth restriction, and invasion (Kay *et al*, 2010; Wang & Zhao 2010; and Williams, 2010).

Angiogenesis and vascular remodelling mechanisms in the foetomaternal unit function are poorly understood, but are crucial for normal foetal development. There are a series of growth factors and angiogenic growth factors that are important in the process of invasion.

1.2.2.8. Angiogenesis.

Angiogenesis is a physiological process involving the formation of new blood vessels from pre-existing vessels. This is a critical process for normal tissue growth and development, as well as the formation of arteries, veins and capillaries in an embryo. Angiogenesis is important and necessary for repair of wounds or regeneration of tissue during wound healing and is promoted by growth factors. It is essential for the formation of the placenta during pregnancy (Kingdom *et al*, 2000; and Kay *et al*, 2011). Angiogenic factors that are produced locally have been identified in the human placenta. These promote vasculogenesis and angiogenesis in the placenta and the most potent include the VEGF, FGF2 and PLGF family. There are many other growth factors for example heparin-binding growth factor (HB-EGF), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), pleiotrophin (PTN), midkine (MK), cluster differentiation (CD105) and Endoglin (Eng) a cell surface co-receptor of transforming growth factor (TGF)- β 1 and (TGF)- β 3 (Pijnenborg *et al*, 2010). Placental vascular growth begins at 21st day p.c. and continues throughout gestation (Wang *et al*, 2010). During vasculogenesis and angiogenesis, many angiogenic factors produced by the placenta cells play important roles in regulating the development of placental vasculature, for example trophoblast, Hofbauer cells, pericytes and endothelial cells.

Angiogenesis is an important process in the normal development of the placenta, as it's responsible for many growth factors. Vascular endothelial growth factor (VEGF) is a potent angiogenic growth factor that initiates vasculogenesis and stimulates angiogenesis whilst modulating trophoblastic survival and function (Kay *et al*, 2011; and Pijnenborg *et al*, 2010). Extensive angiogenesis and invasion of the maternal decidua by trophoblast are essential for the development and function of the placenta. Knockout studies in mice demonstrate the crucial role of vascular endothelial growth factor (VEGF) in embryonic development (Ferrara *et al*, 1996; and Camelié *et al*, 1996). Vascular endothelial growth factors (VEGF), placenta growth factor (PLGF) and their receptors VEGFR-1/Flt-1, VEGFR-2/KDR and VEGFR-3 have important roles in vasculogenesis and angiogenesis (Kay *et al*, 2011; and Pijnenborg *et al*, 2010).

1.2.2.9. *Key factors involved in trophoblast invasion.*

In addition to placental hormones, development of placenta is believed to involve a series of growth factors. There are many growth factors that have been described to be important in the process of invasion. Key factors are thought to include a series of autocrine growth factors, such as angiogenic factors that are produced locally and have been identified in the human placenta, the most potent of which include the vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF2) and placental growth factor (PLGF) family. There are many other growth factors, for example heparin-binding growth factor (HB-EGF), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), pleiotrophin (PTN), midkine (MK), cluster differentiation (CD105) and Endoglin (Eng), a cell surface co-receptor of transforming growth factor (TGF)- β 1 and (TGF)- β 3 (Pijnenborg *et al*, 2010).

1.2.3. The vascular endothelial growth factor (VEGF) family.

Vascular endothelial growth factor (VEGF) is synthesised by cells that stimulate new blood vessel formation. In addition, it is a mitogen for vascular endothelial cells. The VEGF family has at least 7 members, VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, PLGF and snake venom-derived (SV) VEGFs. All these members, except VEGF-E and vs. VEGF are encoded in the mammalian genome. VEGF is a

polypeptide structurally related to platelet-derived growth factor (PDGF). VEGF regulates the growth and differentiation of multiple components of the vascular system, especially blood and lymph vessels. VEGF and their receptors interactions and signalling are shown in Figure 14.

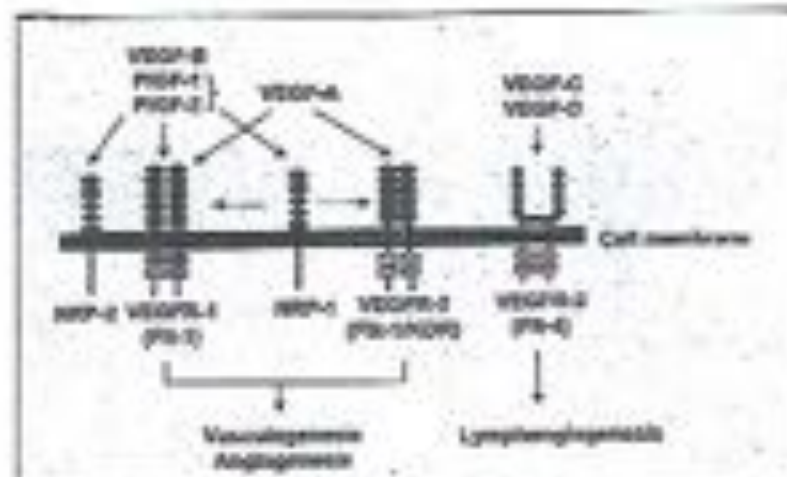


Figure 14. The Vascular endothelial growth factors (VEGF) and their receptors' interactions and signalling. Abbreviations: Vascular endothelial growth factors (VEGF), vascular endothelial growth factors receptors (VEGFR1, VEGFR2, and VEGFR3) and neuropilin (NRP-1 and -2) (modified from Wang and Zhao, 2010).

1.2.3.1. *The vascular endothelial growth factor (VEGF) receptors.*

VEGF receptors are present in the human placenta and bind VEGF to initiate important interactions. The vascular endothelial growth factor receptor family consists of three members, VEGF receptor-1 (VEGFR-1, Flt-1), VEGF receptor-2 (VEGFR-2, Flk-1/KDR) and VEGF receptor-3 (VEGFR-3), and they are structurally distantly related to the PDGFR family, as shown in Figure 1. These participate in vasculogenesis and angiogenesis during placental development. VEGFR-1 has a very high affinity for VEGF-A, but under physiological conditions, VEGFR-1 only weakly stimulates endothelial proliferation. It is expressed not only on vascular endothelial cells, but also in monocyte/macrophage-lineage cells, and stimulates their migration (Shibuya, 2008). The VEGFR-1 gene expresses two types of mRNA, one for the full-length VEGFR-1 receptor, and another for the ligand-binding region alone as a secreted soluble protein (sFlt-1), an important mediator in pre-eclampsia. The level of sFlt-1 protein was reported to be abnormally high in the serum of preeclampsia

patients, and recent studies indicated that abnormal trapping of natural VEGF-A with a large amount of sFlt-1 results in hypertension and proteinuria, the major symptoms on the maternal side of pre-eclampsia. Thus, sFlt-1 is an important target in the treatment of pre-eclampsia.

The ability of VEGFR-2 to bind with VEGF-A is about one order of magnitude weaker than that of VEGFR-1. VEGFR-2 generates a variety of angiogenic signals not only for endothelial proliferation, but also for cell migration/morphogenesis including tubular formation. The 951-tyrosine was shown to regulate cell migration by binding with an adaptor, TSAd (Shibuya, 2008).

1.2.3.2. Actions of vascular endothelial growth factor (VEGF).

The VEGF family is important in many cellular functions including proliferation, apoptosis, mitogenesis and permeability. The angiogenic activity of PLGF and VEGF-B is usually about 10-fold weaker than that of VEGF-A. VEGF-A, and VEGF-E induce angiogenesis with very low side effects, such as oedema, cytokine production and inflammatory reactions, commonly observed both with VEGF-A treatment and in VEGF-A transgenic mice. These unique characteristics of VEGF-E indicate humanized VEGF-Es to be good candidates for proangiogenic agents (Shibuya, 2008).

1.2.3.3. Placental Growth Factor (PLGF) and their receptors.

Persico discovered in (1991) that PLGF is an angiogenic factor of the VEGF family. There are four isoforms of mature PLGF proteins in humans, PLGF-1, PLGF-2, PLGF-3 and PLGF-4. Hauser and Weich (1993) showed that PLGF-1 and PLGF-2 are the dominant forms and differ by an insertion of a highly basic 21 amino acid at the carboxyl terminal sequence. This is an important region that contains a heparin-binding motif allowing the PLGF-2 to bind to heparin (Hauser and Weich, 1993). Although both PLGF-1, and PLGF-2 bind to the VEGFR-1, Ribatti, (2008) showed that PLGF-2 binds to the (neuropilin-1 and neuropilin-2) and to the heparin sulphate proteoglycans (HSPG). PLGF functions are important for angiogenesis and inflammation. Vuorela *et al*, (1997) demonstrated that immunostaining of PLGF is localized on the trophoblast and the villous core of the endothelial cells. Ribatti *et al*, (2008) proposed these mechanisms for angiogenic function of PLGF. First, in the

endothelial cells PLGF binds to VEGFR-1 and facilitates VEGF binding that activates VEGFR-2 (Ribatti *et al*, (2008). Secondly, it promotes the vessel growth and the recruitment of monocytes and macrophages (Ribatti *et al*, (2008). Thirdly, it mobilises the hematopoietic progenitor cells from the bone marrow (Ribatti *et al*, (2008). PLGF is a multitasking cytokine affecting various cellular activities. The pleiotropic actions of PLGF, includes effects on survival, migration, proliferation, metabolism and activation effects on vascular endothelial cells, pericytes and smooth muscle cells. Nonvascular cells are macrophages, fibroblasts and epithelial cells (Ribatti *et al*, (2008).

Carmeliet *et al*, (2001) showed a PLGF/VEGF heterodimer that is highly potent endothelial mitogen. As shown in Figure 12b, (Luttun and Carmeliet, 2003) in pre-eclampsia the hypoxia promotes VEGF and VEGFR-1 (sFlt-1) expression, but down-regulates PLGF production. Altered, Flt-1 affects the villous development and releases excess amount of soluble sFlt-1 into the maternal circulation. This excess sFlt-1 in the bloodstream is associated with a lower free VEGF and PLGF in the systemic vasculature. An increased production of sFlt-1 by trophoblast is a critical pathogenic factor in pre-eclampsia (Wang and Zhao, 2010). PLGF expression is a promising PE marker to be used during the first trimester of pregnancy.

Carmeliat *et al*, (2001) suggested that VEGF and PLGF contribute to both physiological angiogenic processes. There are several mechanisms that are involved in angiogenic functions of PLGF, it binds to VEGFR-1 in the endothelial cells facilitating VEGF binding and activation of VEGFR-2 (Ribatti *et al*, 2008) and interacts with VEGFR-2 that differentiates this growth factor from VEGF- mediated angiogenesis (Kay *et al*, 2011). Although numerous factors have been implicated in angiogenesis, recent observations, including gene knockout studies in mice, have led to the identification of the major factors regulating the angiogenic process, including those that occur during placental vascularisation.

1.2.3.4. Fibroblast growth factor 2 (FGF2) and their receptors.

The FGFs major functions are potent angiogenic factors that promote endothelial cell proliferation and vascularisation (Kay *et al*, 2011). Gospodarowicz *et al*, (1985) study showed that the placenta is a good source of FGFs. FGFs are multifunctional proteins and stimulate a variety of biological effects including pluripotency and angiogenesis. The localization of PLGF is expressed strongly by the villous trophoblast cells. These regulate the vascularization process in both decidua and in the placenta (Pijnenborg *et al*, 2010). The fibroblast growth factor (FGF) family consists of 18 members: FGFs 1–10 and FGFs 16–23 that are structurally related multifunctional polypeptide growth factors (Beenken & Mohammadi, 2009). FGFs are important in many cellular processes including proliferation, differentiation, migration and cell survival throughout prenatal and postnatal life. FGFs are major growth factors of the placenta with FGF2 as the dominant form (Arany and Hill, 1998; Borowicz *et al*, 2007). The study of Yamaguchi & Rossant (1995) suggests that FGFs are important regulators of multiple developmental processes. FGF1 is known as the “acidic” and FGF2 is the “basic” form. They function as potent angiogenic factors and promote endothelial cell proliferation and vasculogenesis. Some FGFs exert their effects by interacting with four different receptors (FGFR1–4) identified in humans as activating signal transduction pathways, such as the MAPK cascade, and stimulate mitogenesis, differentiation and cell migration. FGFR receptors FGFR1 and FGFR3 are expressed only within the villous stroma, whereas FGFR2 and FGFR4 are expressed both within the villous stroma and in the trophoblast (Anteby *et al*, 2005), suggesting that these receptors may mediate the responsiveness of trophoblast to the growth-promoting effects of FGFs. Their expression is controlled at the levels of transcription, mRNA stability and translation (Szebenyl & Fallon, 1999). FGF signalling is important as the regulator of embryonic development, homeostasis and regenerative processes. FGFs biological actions are through the binding, dimerizing and activating cell surface FGF receptors (FGFRs). In Figure 15. FGF/FGFR (FGF receptor) signalling for heparan sulphate proteoglycans (HSPG)-binding proteins, as both the growth factor and receptor bind to heparan sulphate (HS), and HS or heparin (Harmer, 2006; Wang and Zhao, 2010). Brown *et al*, (2014), study confirmed the crystal structure of fibroblast growth factor 18 (FGF18) being a glycoprotein. Zhong *et al's*, (2006) study suggests that, FGF18 were present in the trophoblast stem (TS) cells and in pre-implantation

embryos. FGF18 was expressed in both the human and mouse placenta is important for implantation and early placental function (Zhong *et al*, 2006). *In vitro* and *in vivo* studies of mice by (Hague *et al*, 2007) suggested that FGF18 has a prominent role in chondrogenesis, osteogenesis, for skeletal development and growth, embryogenesis and post-natal development.

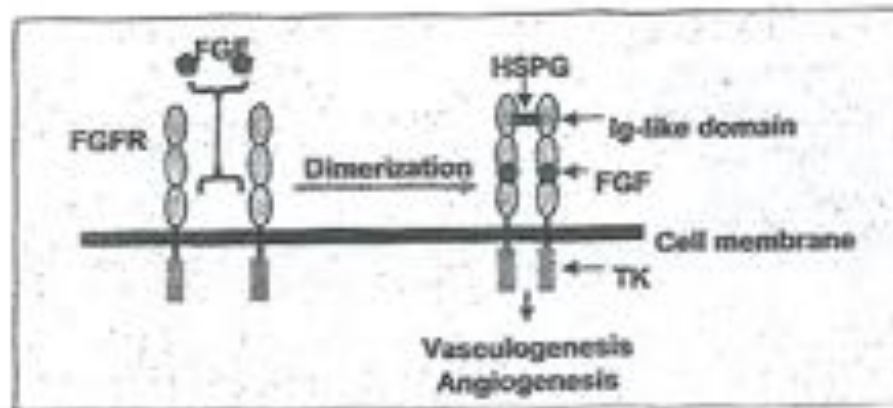


Figure 15. Fibroblast growth factor 2 and its receptor (FGF2/FGF2R) signalling is presented and modified from Wang and Zhao, (2010).

1.2.3.5. Hepatocyte growth factor (HGF).

Hepatocyte growth factor (HGF) is a potent mitogen. It has been shown to stimulate dissociation and mobility of epithelial cells. HGF is a secreted heparin-binding glycoprotein originally identified in rat platelets because of its ability to stimulate mitogenesis in hepatocytes (Strain *et al*, 1982). Uehara *et al*, (1995) demonstrated HGF importance in mammalian pregnancy using the HGF knock out mice. Somerset *et al's* (1998), immunocytochemical study localised HGF protein throughout placental villi across gestation. The c-met (HGF receptor) protein was localized only in the perivillous trophoblast and vascular endothelium. They suggested that HGF, derived from the perivascular tissue of the stem villous arteries, might be important in controlling normal villous development. They showed reduced expression of HGF within IUGR placenta does not provide a causative link with abnormal villous development. The study of Somerset *et al's*, (2000) suggested that a circulating serine protease known as HGF-activator (HGF-A) is responsible for activation of HGF. This study throughout gestation demonstrated the anti-HGF-A/zymogen antibody

immunostaining in placental villi and membranes (Somerset *et al*, 2000). The active HGF is an important regulator of trophoblast growth (Somerset *et al*, 2000). In placenta, HGF promotes trophoblast migration and invasion, but does not affect cellular proliferation (Somerset *et al*, 2000).

1.2.3.6. Platelet-derived Growth factor (PDGF).

The platelet-derived growth factor (PDGF) family induces a variety of cell processes including proliferation, survival, movement, deposition of extracellular matrix (ECM) and tissue remodelling. The PDGF family is composed of two classical growth factors, PDGF-A and PDGF-B. These factors interact with two receptors, PDGFR alpha (PDGFR α) and PDGFR beta (PDGFR β), with cell and specific affinities and specificities. The PDGFs A–C and their receptors PDGFR α and PDGFR β have been shown to promote cellular responses such as proliferation, survival and migration (Hoch & Soriano 2003). In the human placenta, PDGFR α/β is expressed within the syncytiotrophoblast and the villous cytotrophoblast (Gurski *et al*, 1990) this localization together with reduced expression in FGR placentas suggests that signalling via PDGFR α may regulate trophoblast proliferation in the human placenta.

The classical PDGFs can bind directly to their surface receptors. PDGF-A and PDGFR α are associated with embryogenesis, CNS and organ development whereas; PDGF-B and PDGFR β are involved with the development of vasculature, especially with vascular smooth muscle cells. PDGF-BB/PDGFR-BB is important in angiogenesis at the level of cell development, vascular smooth muscle cells (VSMC) and pericytes. The active PDGFs are homodimers, PDGF-AA, PDGF-BB, PDGF-CC, PDGF-DD and the heterodimer PDGF-AB. PDGF induces mitogenic and migratory responses in a wide variety of cells by activating specific receptor tyrosine kinases in both PDGF α - and β receptors. The different isoforms of PDGF bind to glycosaminoglycans. The study by Rolny *et al*, (2002) showed that exogenous heparin amplifies a homodimer PDFG-BB that is induced by the PDGF α -receptor phosphorylation and signalling, for cellular responsiveness.

1.2.3.7. Midkine (MK).

This is a heparin-binding growth factor with a molecular weight of 13kDa (Iwaski *et al*, 1997; Muramatsu, 1993; Muramatsu, 2010). MK and pleiotrophin (PTN) share 10 conserved cysteine residues and show 50% identity in the amino acid sequence. MK has many functions including cell proliferation, migration, survival, differentiation and angiogenesis. MK is highly expressed during mid-to-late gestation and studies suggest its role in embryonic and foetal development (Iwaski *et al*, 1997; and Muramatsu, 1993; Muramatsu, 2010). Qi-Wen Fan *et al*, (2000) showed that among the placental tissues, MK was detected in the chorion, the fetal component of the placenta, whereas PTN was found in the decidua basalis and the placenta (Qi-Wen Fan *et al*, 2000). Thus, MK and PTN distinctive expression suggests their differential role in early development of a mouse. MK has been most extensively studied in neural tissue (Qi-Wen Fan *et al*, 2000).

1.2.3.8. Pleiotrophin (PTN).

This is a 136 amino acid secreted heparin-binding growth factor with a lower molecular weight than MK (Schulte *et al*, 1996; Ball *et al*, 2009). PTN has many functions and is involved in cell growth, migration and differentiation mediated through multiple receptors (Schulte *et al*, 1996; and Ball *et al*, 2009). PTN, its receptor syndecan-1 (SDC-1) and receptor protein tyrosine phosphatase beta (RPTP β) are expressed in the placenta (Schulte *et al*, 1996; Ball *et al*, 2009). They are important for invasion of trophoblast cells and vascular endothelial injury (Schulte *et al*, 1996; and Ball *et al*, 2009). PTN/RPTP β can regulate the metabolism of catecholamines and participates in the oxidative stress response (Schulte *et al*, 1996; Ball *et al*, 2009). Syndecan-1 and syndecan-3 (SDC1 and SDC3) are important for the developmental processes in first-trimester human placentation. Expression of PTN and its receptors in the trophoblast is found exclusively in humans (Schulte *et al*, 1996; and Ball *et al*, 2009). PTN blocks apoptosis in hepatoma cell lines and inhibits apoptosis of lung cancer cells (Schamberger *et al*, 2004).

1.2.3.9. Endoglin (CD105).

CD105 is a cell-surface glycoprotein most recently identified as an optimal indicator of proliferation of human endothelial cells. The finding that CD105 is over-expressed on vascular endothelium in angiogenesis tissues has prompted several pre-clinical studies designed to gain a deeper understanding of the role of CD105 in angiogenesis, and to evaluate the most appropriate clinical settings to utilize CD105 as a therapeutic target.

CD105 is a component of the receptor complex of transforming growth factor (TGF)- β - a pleiotropic cytokine involved in cellular proliferation, differentiation and migration. The binding of TGF- β 1 to CD105 reduces the levels of CD105 phosphorylation and the levels of CD105 expression modulate the effects of TGF- β 1 (Duff *et al*, 2003). In this respect, it is of interest that the inhibition of CD105 expression enhanced the ability of TGF- β 1 to suppress growth, migration and capacity to form capillary tubes of cultured endothelial cells. In the absence of TGF- β 1, CD105 shows an anti-apoptotic effect in endothelial cells under hypoxic stress, suggesting a protective role of CD105 against pro-apoptotic factors. In addition, the discovery that levels of CD105 regulate the expression of different components of the extracellular matrix including fibronectin, collagen, PAI-1 and lumican, is also suggestive of a crucial role of CD105 in cellular transmigration. The complex experimental findings indicate that CD105 is a powerful marker of angiogenesis, and that it might play a critical role in the pathogenesis of vascular disease and tumour progression (Fonsatti *et al*, 2001).

In summary, important maternal growth factors and the signalling cascades in the placenta are transforming growth factor (TGF)- β 1, insulin-like growth factors (IGF1) and (IGF2), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factors (FGF)-2 and FGF4, that bind to a variety of receptors. All of these are increased within the maternal circulation, and their elevated levels are sustained throughout gestation. They all have important roles in promoting the growth of the developing foetus. However, all of these growth factors exert their effects via intracellular cascades that utilize signalling molecules, many of which are dysregulated in foetal growth disorders. These growth factors induce receptor autophosphorylation and bind various scaffolding proteins that activate the PI3K and

MAPK (ERK) pathways via a sequence of phosphorylation resulting in transcription of target genes. These are involved in regulating cellular events such as proliferation and survival (Forbes and Westwood, 2010).

1.2.4. The heparin-binding epidermal growth factors (HB-EGF).

Studies by Lash *et al*, (2003) and Hills *et al*, (2006) showed that the effects of HBEGF, VEGF and PLGF and interferon gamma IFN γ are modified in placenta by heparin. Their studies found levels of HB-EGF mRNA were low in pregnant endometrium and high in placental tissues at an early stage of development. Recently there has been much interest in the effects of heparin-binding epidermal growth factor (HB-EGF) as a possible cytoprotective factor in human placenta. HB-EGF may be induced by low oxygen tension and is expressed in both villous and extravillous trophoblast at high levels in the first trimester and throughout gestation (Yoo *et al*, 1997). HB-EGF has a strong cytoprotective activity and is an important signalling protein that regulates trophoblast invasion during early placentation. Within the human placenta HB-EGF expression is down-regulated following hypoxia/reperfusion (Leach *et al*, 2008). In other tissue systems, both HB-EGF and amphiregulin are increased following oxidative stress (Hurbin *et al*, 2005). Since these factors are also expressed in placental tissue, it seems likely that they are also important survival factors for trophoblast.

Imudia *et al*, (2008) and Leach *et al*, (2004) suggested that within the human placenta HB-EGF stimulates trophoblast invasion and acts as a survival factor and blocks apoptosis induced by hypoxia. Imudia *et al's*, (2008) study suggests that HB-EGF induces trophoblast extravillous differentiation and prevents apoptosis. Deficient HB-EGF signalling during placental development could impair trophoblast survival, differentiation and invasion, leading to poor placental perfusion and hypertension as in pre-eclampsia because HB-EGF is down-regulated in placentas delivered by women with pre-eclampsia. It is suggested that the effects of heparin are mediated through binding to HB-EGF and enhancement of HB-EGF activity (Bose *et al*, 2005; and Hills *et al*, 2006).

1.2.4.1. Human placental abnormalities related to pre-eclampsia.

Pre-eclampsia is an obstetrical disorder of placental function. Trophoblast proliferation, angiogenesis and invasion are important and are decreased in pre-eclampsia (Benirschke *et al*, 2006; Crocker, 2007; Lyall and Belfort, 2007; Kay *et al*, 2010; Kingdom *et al*, 2011; Wang & Zhau, 2010; and Williams, 2010). Humans are predisposed to pre-eclampsia because of individual or combination of these features, for example: the depth of extravillous trophoblast invasion, maternal uterine artery remodelling, complex structure of the chorionic villous tree, and surface area of transport and diffusion in the villous trophoblast (Benirschke *et al*, 2006; Crocker, 2007; Lyall and Belfort, 2007; Kay *et al*, 2010; Kingdom *et al*, 2011; Wang & Zhou, 2010; and Williams, 2010).

The novel hypothesis for pre-eclampsia is that it evolves in two stages as shown in Figure 15. (Redman and Sargent, 2000; and Lyall and Belfort, 2007). The prevailing theory is that pre-eclampsia is an obstetrical syndrome that starts in the placenta and ends in the maternal endothelium (Lyall and Belfort, 2007; Kay *et al*, 2010; Kingdom *et al*, 2011; Wang & Zhou, 2010; and Williams, 2010).

Defective placentation is initiated in the first stage, resulting in inadequate formation of extravillous trophoblastic invasion, incomplete remodelling of the spiral arteries in the decidua, maintenance of vascular activity and resistance in the myometrium (Brosens *et al*, 1972; Lyall and Belfort, 2007; Kay *et al*, 2010; Kingdom *et al*, 2011; Wang & Zhau, 2010; and Williams, 2010). As a consequence, placental ischaemia, reperfusion, hypoxia, and oxidative stress and formation of reactive oxygen species (ROS) follow. The second stage of the disease is characterized by the clinical obstetrical symptoms, for example hypertension, proteinuria, and oedema, caused by release of placental derived factors maternal vascular endothelial cell dysfunction, maternal systemic inflammation and organ failure (Lyall and Belfort, 2007; Kay *et al*, 2010; Kingdom *et al*, 2011; Wang & Zhau, 2010; and Williams, 2010). It was suggested that the vascular endothelial damage is caused by placental-derived material or anti-angiogenic growth factors (Lyall and Belfort, 2007; Kay *et al*, 2010; Kingdom *et al*, 2011; Wang & Zhau, 2010; and Williams, 2010).

1.2.4.2. Pre-eclampsia is presented as a two-stage model.

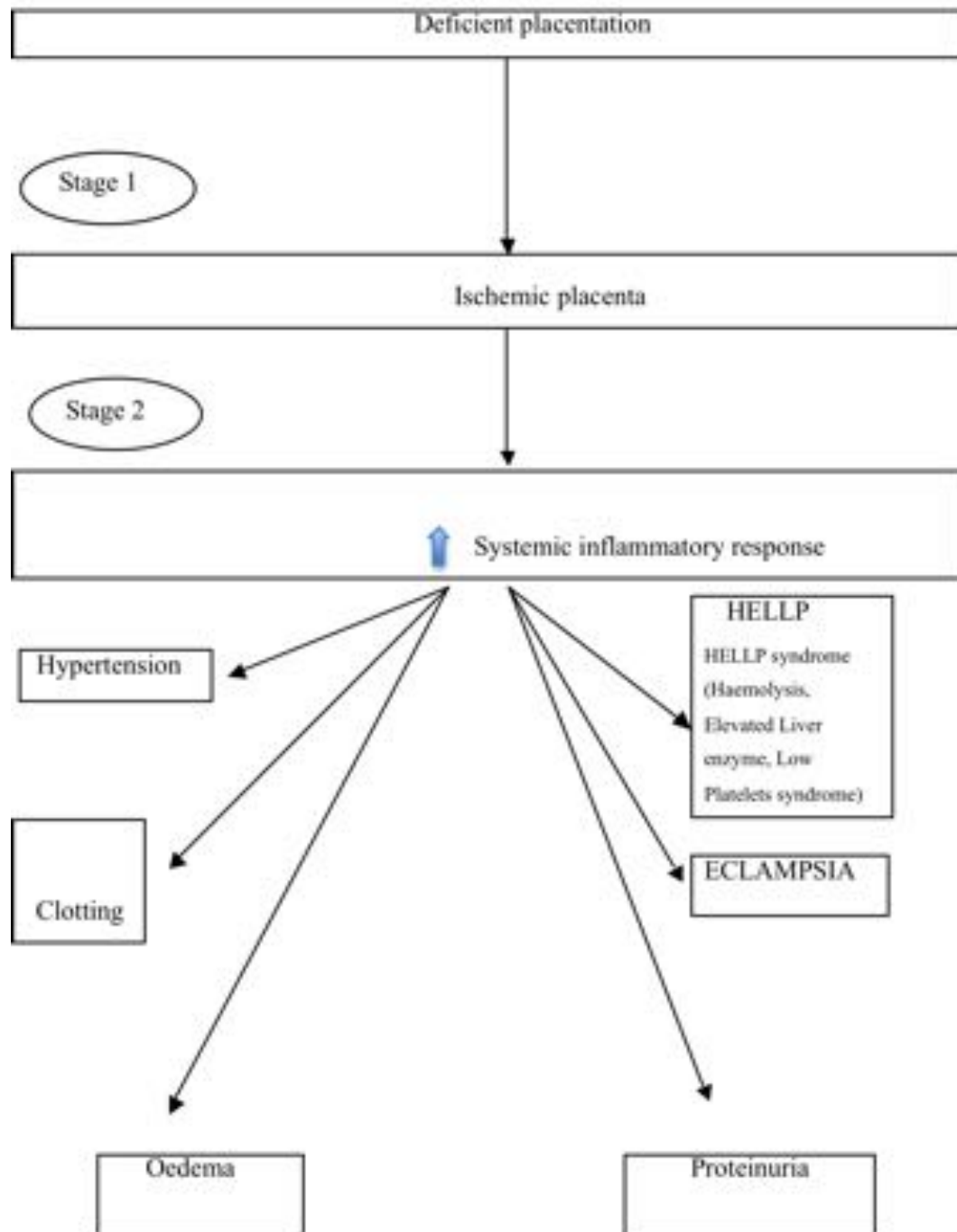


Figure 16. Pre-eclampsia presented as two stages of evolution. Modified from (Redman and Sargent, 2000; and Lyall and Belfort, 2007). Other disorders associated with placental insufficiency include pre-term labour and foetal growth restriction.

As in Figure 10. the decidua vessels become lined with EVT's because of the shallow invasion (Benirschke, 2006; Lyall and Belfort, 2007; Kay *et al*, 2010; Kingdom *et al*, 2011; Wang & Zhau, 2010; and Williams, 2010).

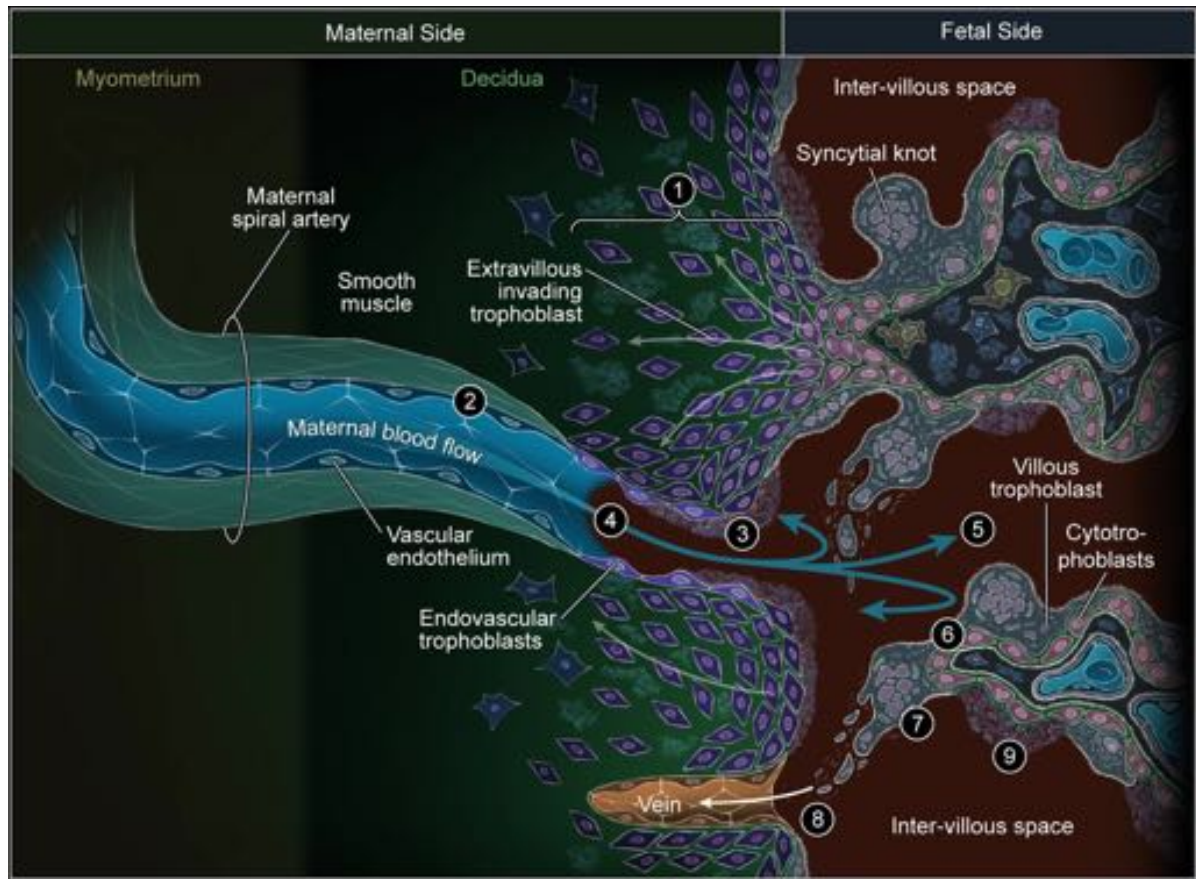


Figure 17. represents Uteroplacental vascular insufficiency. 1) EVT cytotrophoblast are less and may be removed by the maternal immune system. 2) Distal spiral arteries are narrower. 3) Diseased followed by atherosclerosis or local fibrin deposition. 4) Reduced Endovascular invasion. 5) Hypoxia or hypoxia-re-oxygenation injury in the placenta. 6) Direct effects on villous trophoblast compartments. 7) May trigger the formation of syncytial knots. 8) Accumulation, with fragmentation and shedding may occur into the maternal blood. 9) Areas that are deficient in syncytial fusion may exhibit focal necrosis. (Adapted from Kingdom *et al*, 2011).

Brosens *et al*, (1972), *in situ* study confirmed inadequate shallow cytotrophoblast invasion in placentas from pregnancies that are complicated by pre-eclampsia. In PE, trophoblastic invasion of the spiral arteries appears to be incomplete, as shown in

Figure 10. and 17, and the vessels remain responsive to vasoconstrictors. Vasospasm, increased peripheral vascular resistance, and thus reduced organ perfusion characterize the disease. The syndrome is polymorphic in that virtually every organ system can be affected (Lyall and Belfort, 2007; and Williams, 2010). In pre-eclamptic women defective physiological changes were restricted to the decidual segments of the uterine spiral arteries (Brosens *et al*, 1972). Some studies have also shown evidence of endothelial dysfunction in women with PE. There is a reduction in secretion of endothelial vasodilator substances, but increase in concentration of potent vasoconstrictor endothelium (Lyall and Belfort, 2007; and Williams, 2010). The effect of all these changes is the reduction in placental blood flow (Lyall and Belfort, 2007; and Williams, 2010). PE adversely affects the mother, by vascular dysfunction, and is frequently associated with intrauterine growth restriction (IUGR) of the foetus, presumably as a result of the reduction in nutrient supply (Lyall and Belfort, 2007; and Williams, 2010; and Kingdom *et al*, 2011).

Studies of women with abnormal placental syndromes have shown that they are at risk of developing cardiovascular diseases and metabolic disorders such as hypertension, ischaemic heart disease, stroke, obesity, and diabetes mellitus after pregnancy. Abnormal placental function impacts on the foetus because of the increased vascular resistance and improper nutrient transport. The mother and foetus interact via endocrine signals from the placenta, which control the complex process of foetal growth together with environmental factors. Over the last decade significant research progress has been made in the field of placental vascular biology because of advanced cellular and molecular technologies (Knobil & Neil, 2006; Pijnenborg *et al*, 2010; Wang & Zhao 2010; Williams, 2010; and Kay *et al*, 2011).

1.2.4.3. *Angiogenic growth factors in pre-eclampsia.*

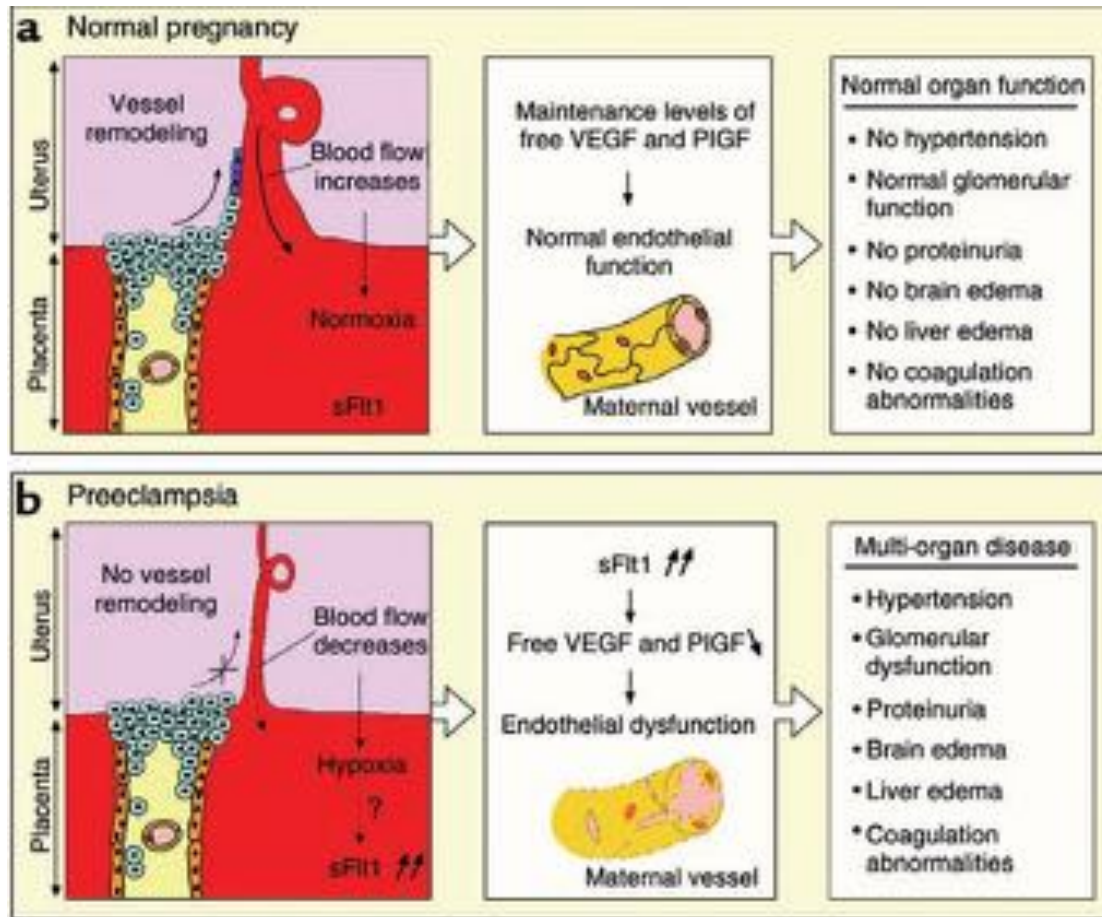
There were several varying hypotheses and theories for the placenta in the aetiology of pre-eclampsia. Proangiogenic and anti-angiogenic growth factors are released during a normal healthy pregnancy (Kingdom *et al*, 2011; and Lyall and Belfort, 2007). An appropriate placental expression of angiogenic factors may therefore be an important cause of infertility and foetal growth retardation (Williams, 2010). Recent

clinical work on regulation of placental angiogenesis could become a novel and powerful method for ensuring positive outcomes for most pregnancies. Several studies on expression of anti-angiogenic growth factors, soluble fms-like tyrosine kinase 1 (sFlt-1), transforming growth factor β (TGF- β) and endoglin are upregulated in pregnancies affected with PE. However, pro-angiogenic growth factors such as VEGF, and PLGF are downregulated in these pregnancies inadequate cytotrophoblast invasion, endothelial dysfunction and impaired placental angiogenesis in PE women (Ahmad and Ahmed, 2004; Ahmed and Ramma, 2014; Maryland *et al*, 2003; and Young *et al*, 2010).

There is evidence that the VEGF family of growth factors and receptors are important during placental development. Geva *et al*'s, (2002) study suggested that high levels of VEGF, VEGFR-1, and sVEGFR-1 are seen in pre-eclampsia, which is related to the hypoxic status of the placenta. Current knowledge has advanced beyond early hypothesis that were suggested 12 years ago and at that time data was relevant for example a possible mechanism was outlined in Figure 18a,b (Luttun & Carmeliet, 2003). During normal pregnancies (18a) the blood flow and supply of nutrients with oxygen to the foetus increases by vascular remodelling. The VEGFR Flt-1 acts as a potent VEGF antagonist by binding VEGF, reducing free circulating concentrations of VEGF and may contribute to the pathogenesis of pre-eclampsia opposing vasodilation and contributing to the development of hypertension (18b).

Another hypothesis was suggested 16 years ago by (Lash *et al*, 1999), that extravillous trophoblast invasion and motility is mediated by VEGF. VEGF may limit the degree to which trophoblast cells invade. Once again, at that time data was relevant. Akercan *et al*, (2008) suggested that the placental villi show branching angiogenesis, as is the case with anaemic pregnancy, pregnancy at high altitude, and in pre-eclampsia. VEGF is up-regulated in response to placental hypoxia. Tsatsaris *et al*'s (2003) study suggests VEGFs are involved in placental vascular development. Increased levels of VEGF-A and VEGF-1 mRNAs in compromised pregnancies may reflect the hypoxic status of the placenta. Kingdom & Kaufmann, (1999), and Khaliq *et al*, (1999) suggest VEGF-A is one of the most important growth factors for endothelium and is involved in regulating vasculogenesis, as it induces endothelial cell proliferation as well as angiogenesis. VEGF-A transcription is activated by

oncogenes in response to hypoxia. The results are conflicting in several studies aiming to analyze the expression of VEGF-A in pathological conditions during pregnancy. Lyall *et al*, (1997) measured free VEGF using maternal serum samples. The free VEGF decreased in pre-eclampsia, compared with normal pregnancies. Some authors report reduced levels of VEGF-A in maternal circulation and others show increased levels of VEGF-A in maternal circulation compared with normal pregnancies.



In Figure 18a and 18b presents functions of VEGF, sFlt1, and PLGF in normal pregnancy and pre-eclampsia (Luttun & Carmeliet, 2003).

Recent evidence also confirms a role for VEGF in foetal and placental angiogenesis, as during early pregnancy expression of VEGF mRNA is greater in foetal placenta compared with maternal placental (endometrial) tissues. Gene knockout studies have provided convincing evidence for a central role of VEGF in foetal and placental angiogenesis. In mice, homozygous knockouts of the genes for VEGFRs (VEGFR-1

[also known as flt-1] or VEGFR-2 [KDR or flk-1] led to defects in foetal and placental vasculogenesis (the initial formation of the vasculature) and angiogenesis resulting in embryonic death by about Day 8. of pregnancy (length of pregnancy \approx 20 days) (Fong *et al*, 1995; and Shalabi *et al*, 1995). Similarly, homozygous gene knockouts for VEGF itself were lethal by about Day 11. of pregnancy, and these embryos exhibited dramatic cardiovascular defects, such as delayed or abnormal development of the heart, aorta, major vessels and extraembryonic vasculature, including the yolk sac and placenta (Carmeliet *et al*, 1996). Foetal and placental angiogenesis are absolutely dependent on VEGF, but threshold levels of VEGF must be achieved for normal vascular development to occur.

1.2.4.4. Pathogenesis of pre-eclampsia.

PE has been the subject of extensive research, but the pathophysiology of the disease and precise causes are unknown. Failure of placental oxidative stress and placental development as a result of deficient spiral artery remodelling are believed to be important in the development of pre-eclampsia (Lyll and Belfort, 2007). It stems from a defect of placentation in early trophoblast invasion of the spiral arteries as depicted in Figure 1. Many investigators have confirmed observations of restricted physiological conversion of spiral arteries in pre-eclampsia over several years (Frusca *et al*, 1989; Gerretsen *et al*, 1981; Hanssens *et al*, 1998; Hustin *et al*, 1983; Khong *et al*, 1986; Lyll *et al*, 2001b; Meekins *et al*, 1994a; Moodley and Ramsaroop, 1989; Pijnenborg *et al*, 1991; and Sheppard and Bonnar, 1981).

Presently, there is no accepted treatment for these conditions. Although, the causes of pre-eclampsia are unclear, it is accepted that a failure of placental development is an important predisposing factor and that the resulting alterations in placental oxygen levels may be crucial in the development of this disorder (Hung *et al*, 2002, and Rajmakers *et al*, 2004). Abnormal first-trimester trophoblast differentiation is associated with pregnancies complicated by pre-eclampsia and/or intrauterine growth restriction (IUGR). A key histopathologic correlation is shallow invasion and aberrant remodelling of maternal spiral arteries, which leads to decreased uteroplacental perfusion and the resulting alterations in placental oxygen levels may be crucial in the development of this disorder (Hung *et al*, 2002; and Rajmakers *et al*, 2004).

Experimental hypoxia is correlated with pre-eclampsia features, inducing trophoblast cell death, release of proinflammatory cytokines and oxidative stress (Hung *et al*, 2002; Hung *et al*, 2004; Benyo *et al*, 1997; and Benyo *et al*, 2001). The putative effect of reduced oxygenation on global gene expression changes in placental tissues from pre-eclamptic patients remains unclear. Failure of the oxygen-associated developmental events contributes to placental disease (Hung *et al*, 2004; Genbacev *et al*, 1996; and Caniggia *et al*, 2000). Flow patterns can also be obtained from individual spiral arteries. Matijevic *et al*, (1995) evaluated blood flow through central and lateral spiral arteries at 17–20 weeks, revealing lower resistance and pulsatility indices in the central arteries known to undergo physiological change at that period of gestation (Pijnenborg *et al.*, 1983). In the third trimester, women with pre-eclampsia show significantly higher impedance to flow in the spiral arteries than normotensive controls (Matijevic and Johnston, 1999). These findings, therefore, provide a strong support for the original hypothesis that defects in the uterine vasculature underlie impaired maternal placental blood flow.

It is suggested that intermittent placental blood flow, hypoxia/reperfusion, oxidative stress (Hubel *et al*, 1999; and Burton *et al*, 2003) and the appearance of free radicals occur. This results in extensive cellular damage, lipid peroxidation and apoptosis within the placenta. The resulting tissue damage is believed to be a key factor in the pathogenesis of pre-eclampsia (Burton and Jauniaux, 2004). Louwen *et al*, (2013) demonstrated increased expression of a key oncogene in B lymphoma genesis known as B-cell Lymphoma 6 in pre-eclamptic placentas. It was localised in the nucleus of villous cytotrophoblasts. Alterations in B-cell Lymphoma 6 may be involving pathogenesis in pre-eclampsia.

1.2.4.5. *Treatment and prevention of pre-eclampsia.*

In severe pre-eclampsia, there is greater risk of serious complications to both mother and baby. The only complete cure for pre-eclampsia women is the delivery of the baby. Prevention or treatments of PE options available are limited. The studies by Kingdom *et al*, (2011), and Lyall and Belfort, (2007) investigated the effects of heparin on placental function by a pilot randomized trial using unfractionated heparin (UFH) in preventing recurrent PE. Proteoglycans (PG) are abundantly expressed in the placenta and a reduction in Glycosaminoglycans (GAGs) may contribute a

possible mechanism. Recently, pharmacological use of GAGs during pregnancy is popular with randomised controlled trials suggesting that patients that were treated throughout pregnancy with heparin had decreased risk of developing PE. Hills *et al*, (2006) study suggests that unfractionated heparin inhibits apoptosis, which was induced by different signals in the human 1st trimester cytotrophoblast cells. Clinical administration of heparin during pregnancy could be influenced via the actions of HB-EGF because of heparin sulphate is a co-factor of HB-EGF. The use of heparins to prevent PE appears promising. Therefore, dysregulation of heparin-binding growth factors could have a crucial role in the aetiology of PE. The mechanisms by which heparin act within the placenta requires further research to maximise the efficacy of pharmacologic GAGs and minimise their side effects.

1.2.4.6. *Animal models of pre-eclampsia.*

Animal models have failed to provide definitive insights into the pathogenesis of pre-eclampsia because of their limited applicability to the human form of the disease. Despite these limitations, several theoretical mechanisms have been proposed that reconcile foeto-placental abnormalities and clinical features of the maternal syndrome. These include apoptosis, oxidative stress and hypoxia.

1.2.4.7. *Mechanisms of placental insufficiency.*

The conditions described above are believed to have a common aetiology in pre-eclampsia that involves several common pathological pathways such as apoptosis, hypoxia and oxidative stress.

1.2.4.8. *Apoptosis.*

Apoptosis is programmed cell death (Kingdom *et al*, 2000) and has been observed in pregnancies complicated by pre-eclampsia or intrauterine growth retardation (IUGR) (Allaire *et al*, 2000). It is important for normal placental development, but it may also be involved in the pathophysiology of pregnancy-related diseases. It differs from necrosis in that the former is an active form of cell death dependent on the internal machinery of the cell and the latter is an accidental death caused by factors outside the

cell. Trophoblast apoptosis increases in normal placentas as gestation proceeds. Depending on the stimuli, apoptosis may be initiated intrinsically by the mitochondrial pathway or extrinsically by either the death receptor-mediated pathway or in response to exogenous stimuli such as cytokines. These factors, such as superoxide anion radical, O₂⁻, and peroxynitrite anion induce a variety of forms of cellular damage including endothelial dysfunction, abnormal platelet activation and apoptosis (Lyall *et al*, 2007). It is unknown whether the regulators of apoptosis are differentially expressed placentas of pre-eclamptic women (Sakuragi *et al*, 1994; and Kim *et al*, 1995).

1.2.4.9. Hypoxia and oxidative stress.

Hypoxia and oxidative stress promote the excess release of placental tumour necrosis factor (TNF α) (Kingdom *et al*, 2000) into the maternal circulation by the hypoxic placenta that might promote endothelial dysfunction in pre-eclampsia (Benyo *et al*, 1997). Amplification of injurious effects of placental TNF α by increased maternal free fatty acids is then possible. Free fatty acids are highly inflammatory (Toboreket *et al*, 1996). Placental Lipid peroxidation produced in pregnancy may mediate disturbance of the maternal vascular endothelium. It is not clear whether this mechanism results in impaired placental development or if the impaired development results in hypoxia (Hills *et al*, 2006).

Hypoxia as in Figure 17. is a potent stimulus for endothelial dysfunction during pregnancy. Placento-foetal hypoxia may provide a link for pre-eclampsia. Another mechanism via which placento-foetal hypoxia may affect several circulations is through the activation of oxidative stress (Lyall and Belfort, 2007; and Kay *et al*, 2011).

1.2.5. Oxidative stress.

Oxidative stress arises in the presence of ROS and excess oxygen. Both high and low levels of oxygen can cause tissue damage including the low non-physiological oxygen levels (hypoxia) (Kay *et al*, 2011; Pijenburg *et al*, 2010; Wang & Zhao 2010; and Williams, 2010; and Hansson *et al*, 2015). Oxidative stress may be the point at which multiple factors converge resulting in endothelial cell dysfunction and the consequent clinical manifestations of pre-eclampsia. ROS production seems increased in pre-

eclamptic placentae, as evidenced by increased peroxynitrite formation. Additionally, the concentration of ascorbic acid is decreased in the maternal circulation in pre-eclamptic women, which could be indicative of reduced anti-oxidant potential. Increased ROS and potentially decreased anti-oxidant capacity will propagate lipid peroxidation leading to leukocyte activation, platelet adhesion and vasoconstriction. Increased placental oxidative stress in pre-eclampsia promotes formation of lipid peroxides, which alter cell membranes by increasing incorporation of cholesterol, oxidised free fatty acids and low density lipoprotein. There is evidence that an imbalanced angiogenic factors such as VEGF/sflt-1, PLGF are early markers to the pathogenesis of pre-eclampsia and oxidative stress is induced in placenta as a result. There is evidence of oxidative stress in pre-eclampsia, in both the maternal circulation and in the placenta (Kay *et al*, 2011; Pijnenborg *et al*, 2010; Wang & Zhao 2010; and Williams, 2010; and Hansson *et al*, 2015).

There appears to be an increase in ROS generation in the placenta of pre-eclamptic women (Kay *et al*, 2011; Pijnenborg *et al*, 2010; Wang & Zhao 2010; and Williams, 2010). There is evidence for increased nitrotyrosine formation in the preeclampsia placenta suggestive of ONOO⁻ production, perhaps arising from local nitric oxide (NO) production coupled with increased xanthine oxidase generation of O₂⁻ and either regionally decreased or inadequate SOD. Whether this could lead to oxidative stress and/or endothelial dysfunction in the systemic circulation is uncertain. Beneficial/compensatory effects of ONOO⁻ are also plausible. For example, ONOO⁻ can lessen leukocyte rolling and adhesion to endothelial cells and inhibit platelet aggregation (Lyll and Belfort, 2007).

The genesis of pre-eclampsia is clearly related to deficient trophoblast invasion and failure of uterine artery remodelling (Kay *et al*, 2011; Pijnenborg *et al*, 2010; Wang & Zhao 2010; Williams, 2010). In normal pregnancy the spiral arteries feeding the intervillous space of the placenta increase greatly in diameter and become refractory to vasomotor agents. This involves replacement of endothelium by invading trophoblast (the trophoblast cells assuming an endothelial cell adhesion molecule phenotype) and replacement of the internal elastic lamina and smooth muscle by trophoblast and fibrinoid matrix (Lyll and Belfort, 2007; Wang & Zhao 2010). This transformation is complete by 20 weeks of gestation. Along with venous distension, this accounts for the increased blood supply to the intervillous space necessary to

meet the demands of the rapidly growing fetoplacental unit during the later stages of gestation (Lyall and Belfort, 2007; and Wang & Zhao 2010).

1.2.5.1. Placental Lipid peroxidation.

Lipid peroxidation products are candidate factors that may mediate disturbance of the maternal vascular endothelium. *In vitro* production of lipid hydroperoxides and thromboxane are reportedly increased in both trophoblast cell and villous tissues from women with pre-eclampsia (Kay *et al*, 2011; Lyall and Belfort, 2007; Wang & Zhao 2010; and Williams, 2010). Glutathione peroxidase, an enzyme that removes hydrogen peroxide and converts lipid hydroperoxides to less reactive alcohols, may be deficient in placental tissue from pre-eclamptic women. This is observed in conjunction with increased *in vitro* placental production. The altered prostaglandin ratio might provoke vasospasm with exacerbation of placental ischemia, increased cell damage and increased lipid peroxidation (amplification of oxidative stress). There are scores of reports that lipid peroxidation products, primarily measured as thiobarbituric acid-reactive substances (which include malondialdehyde), are increased in plasma/sera of women with pre-eclampsia (Lyall and Belfort, 2007; and Wang & Zhao 2010).

1.2.5.2. Placental Nitrotyrosine, Xanthine Oxidase, and superoxide dismutase SOD.

Peroxynitrite anion (ONOO^-) is capable of nitrating proteins and inducing lipid peroxidation. The nitration of tyrosine can occur by other free radical processes (Wang & Zhao 2010). Superoxide-independent pathways have been reported, such as interaction of nitrogen dioxide ($\text{NO}_2\cdot$) with tyrosyl radicals generated by myeloperoxidase during oxidative stress. Changes in xanthine oxidase in the pre-eclampsia placenta further suggest ischemic or inflammatory injury. However, a recent reappraisal of xanthine oxidase in human tissues has suggested that both D and O isoforms can generate reactive oxygen species during posthypoxic reperfusion. There appears to be an increase in ROS generation in the placenta of pre-eclamptic women. There is evidence for increased nitrotyrosine formation in the preeclampsia placenta suggestive of ONOO^- production, perhaps arising from local $\text{NO}\cdot$ production coupled with increased xanthine oxidase generation of $\text{O}_2^{\cdot-}$ and either regionally

decreased or inadequate SOD. Whether this could lead to oxidative stress and/or endothelial dysfunction in the systemic circulation is uncertain. Beneficial/compensatory effects of ONOO⁻ are also plausible. For example, ONOO⁻ can lessen leukocyte rolling and adhesion to endothelial cells and inhibit platelet aggregation (Lyall and Belfort, 2007; and Wang & Zhao 2010).

1.2.5.3. Ascorbate Oxidative Consumption.

During its antioxidant action, ascorbate undergoes two consecutive one-electron oxidations to dehydroascorbic acid with intermediate formation of the ascorbate radical (Lyall and Belfort, 2007; and Wang & Zhao 2010). Ascorbate radical is detectable by electron paramagnetic resonance (EPR) spectroscopy whereas ascorbate and dehydroascorbate are EPR-silent. The initial signal amplitude of ascorbate radical is directly proportional to the overall rate of ascorbate oxidation, whereas the signal duration is inversely proportional. Ascorbate radical thus serves as a gauge of ongoing oxidative stress in plasma (Lyall and Belfort, 2007; and Wang & Zhao 2010). If stable peroxidation metabolites are produced during placental oxidative stress and enter the maternal circulation, these could contribute to widespread endothelial dysfunction (Lyall and Belfort, 2007; and Wang & Zhao 2010).

1.2.5.4. Placental syncytiotrophoblast microvesicles (STBM) and pre-eclampsia.

Placental cytokines and anti-angiogenic factors produce excessive maternal inflammatory response in pre-eclampsia (Kay *et al*, 2010; and Reddy *et al*, 2008). Placental debris comprises from trophoblasts cells, cellular fragments as shown in Figure 16. and multinucleated syncytial knots (Kay *et al*, 2010; Kingdom *et al*, 2011; and Reddy *et al*, 2008). Human placental microparticles are membrane-bound structures that derive from syncytiotrophoblast are known as placental syncytiotrophoblast microvesicles (STBM) (Kay *et al*, 2010; and Reddy *et al*, 2008). These are generated from trophoblast cell activation, apoptosis or necrosis. The STBM's are shed into the maternal circulation in normal pregnancy and are significantly increased in early-onset pre-eclampsitic women. *In vitro* studies suggest that microparticles modulate maternal vascular endothelial dysfunction. The plasma from pre-eclamptic women contains placental microparticles as soluble fms-like-tyrosine kinase (sFlt-1) and influences angiogenesis (Kay *et al*, 2010; and Reddy *et al*, 2008).

Some recent studies have hypothesized the possible role of STBM in the new aetiology of pre-eclampsia. Reddy *et al*, (2008) hypothesized that labour and placental separation leads to an increase in shedding of placental debris (STBM, cell-free foetal and maternal DNA and cell-free placental mRNA) into maternal circulation. Another, hypothesis that placental STBM proteins are differentially expressed in pre-eclampsia when compared to normal pregnancies was reported by (Baig *et al*, 2014). They suggested that differential placental protein expression of STBM pathophysiological importance in pre-eclampsia.

Dusse *et al*, (2012) study suggested endothelium activation is important because there was a higher number of endothelial cell-derived Microparticles (MPs) in women with severe pre-eclampsia, as erythrocyte-derived MPs levels were increased in women of pre-eclampsia. Cronqvist *et al*, (2014) study showed that haemoglobin Hb perfusion alters of two placenta specific micro-RNAs; mir-517a and mir-517b content of release placental syncytiotrophoblast vesicles (STBMs). STBMs may function as carriers of Hb into the maternal circulation.

1.2.5.5. Disorders associated with placental insufficiency.

Pregnancy complications such as preterm labour (PTL), pre-eclampsia (PE), and intrauterine growth retardation (IUGR) affect a considerable number of pregnancies and account for significant perinatal morbidity and mortality. These diseases consists of an abnormal development and function of the placenta, that is, placental insufficiency (Lyall and Belfort, 2007; Williams, 2010). The most common adverse pregnancy outcomes in women are pre-eclampsia, pre-term labour, and intrauterine growth restriction (Williams, 2010; Pijenburg *et al*, 2010; and Kay *et al*, 2011).

1.2.5.6. *Preterm labour.*

The WHO defines preterm or premature as delivery of an infant before 37 completed weeks of gestation (Williams, 2010). Clinically, deliveries below 34 weeks' gestation may be a more relevant definition. There is no set lower limit to this definition, but 23–24 weeks' gestation is widely accepted, which approximates to an average foetal weight of 500g. Preterm birth occurs in about 5–10% of all births in resource-rich countries, but in recent years the incidence seems to have increased in some countries, particularly the USA, where the rate reached 12.7% in 2005. Williams, (2000) found little reliable evidence for incidence in resource-poor countries.

Preterm birth is the leading cause of neonatal death and infant mortality, often as a result of respiratory distress syndrome due to immature lung development. Children who survive are also at high risk of neurological disability. Observational studies have found that one preterm birth significantly raises the risk of another in a subsequent pregnancy (Williams, 2010).

1.2.5.7. *Foetal growth restriction.*

The clinical term “small for gestational age” (SGA) is used for foetuses whose birth weight falls below the 10th percentile of weight for their gestational age. Foetal growth restriction (FGR) is a condition where infants have failed to achieve their growth potential and are small, because baby's growth slows and have experienced foetal growth restriction (Williams, 2010). Imprinting in the placenta is an important means of regulating placenta and foetal growth and disturbances result in disorders. Genomic imprinting is an example of regulating foetal growth. IUGR is a characteristic of the imprinting disorder. Mouse knockout studies have also demonstrated that altered expression of imprinted genes can cause changes in the ability of the placenta to exchange nutrients by altering the thickness and surface area of exchange in placental tissues (Murphy *et al*, 2006).

1.2.5.8. Heparin-binding growth factors and their receptors in the human placenta.

There is an increasing list of growth factors whose activity is modified following binding to heparin. Furthermore, cells within the human placenta as shown in table 1. express a large number of these heparin-binding growth factors. In the following sections these factors are described in detail.

Factor	Placental expression	Reference	Regulates placental function	Reference	Modified by heparin	Reference
VEGF	Y	Tseng <i>et al</i> , 2006	Y	Anteby <i>et al</i> , 2004	Y	Lash <i>et al</i> 2003
PLGF	Y	Clark <i>et al</i> , 1998	Y	Dessai <i>et al</i> , 1995	Y	Lash <i>et al</i> 2003
HB-EGF	Y	Leach <i>et al</i> , 2002	Y	Hills <i>et al</i> , 2006	Y	Hills 2006
IFN γ	Y	Bulmer <i>et al</i> , 1990	Y	Karmakar <i>et al</i> , 2004	Y	Hills <i>et al</i> 2006
bFGF	Y	Zhong <i>et al</i> , 2006	Y	Anteby <i>et al</i> , 2004	-	-
PDGF	Y	Gurski <i>et al</i> , 1990	Y	Lash <i>et al</i> , 2003	-	-
HGF	Y	Kilby <i>et al</i> , 1996	Y	Dash <i>et al</i> , 2005	-	-
TGF β	Y	Selick <i>et al</i> , 1994	Y	Cannagia <i>et al</i> , 1999	-	-
IL-8	Y	Abrahams <i>et al</i> , 2005	Y	Abrahams <i>et al</i> , 2005	-	-
AR	Y	Lyssiak <i>et al</i> , 1995	Y	Lyssiak <i>et al</i> , 1995	-	-
PTN	Y	Shulte <i>et al</i> , 1996	-	-	-	-
MK	Y	Milner <i>et al</i> , 1992	-	-	-	-
AT	Y	Corfman <i>et al</i> , 1987	-	-	-	-

Table 1. Heparin-binding growth factors present in placental tissues.

Abbreviations: vascular endothelial growth factor (VEGF), placental growth factor (PLGF), heparin-binding epidermal growth factor (HB-EGF), interferon gamma (IFN γ), fibroblast growth factor 2(FGF2), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), transforming growth factor beta (TGF β), interleukin-8 (IL-8), amphiregulin (AR), pleiotrophin (PTN), midkine (MK), Amphoterin (AT) and – not tested.

These include the vascular endothelial growth factor (VEGFs), placental Growth Factor (PLGF), transforming growth factor (TGF) and hepatocyte growth factor (HGF) (Selick *et al*, 1994; Kilby *et al*, 1996; Clark *et al*, 1998; Leach *et al*, 2002; and Tseng *et al*, 2006). As can be seen in the Table 2. many of these factors have been shown to modify placental function *in vitro*. These include, but may not be limited to promotion of invasion, motility, proliferation and cytoprotection. In addition to these

anti-inflammatory and immunomodulatory effects, heparin also enhances the ability of several growth factors, including hepatocyte growth factor, epidermal growth factor (EGF), heparin-binding EGF (HB-EGF) and fibroblast growth factors (FGFs), to activate their cognate cell surface receptors (Folkman and Shing, 1992; and Li *et al*, 1999). Whereas the angiogenic effects of VEGF are known, there is increasing evidence that these factors are important in blocking the effects of oxidative stress. For example, MK blocks apoptosis induced by hypoxia reperfusion in cardiomyocytes (Horiba *et al*, 2006). PTN blocks apoptosis in heptoma cell lines and inhibits apoptosis of lung cancer cells (Park *et al*, 2008). Basic FGF blocks apoptosis of rat embryonic cells (Schamberger *et al*, 2004).

The nine growth factors studied in this study are summarised in Table 2. In summary, various growth factors are involved in placental development. Many of these bind to glycosaminoglycans (GAGs) affecting activity.

Growth Factors and their receptors	Established function	Reference
VEGF VEGF (VEGF-A) VEGF-C VEGF-D VEGF-E (viral factor)	Motility, Invasion Angiogenesis Vascular maintenance Lymphangiogenesis Lymphangiogenesis Angiogenesis	Tseng <i>et al</i> , 2006 Fonsatti <i>et al</i> , 2001
PLGF	Cytoprotection Angiogenesis, Inflammation effects on survival, migration, proliferation, metabolism, and activation effects on vascular endothelial cells, pericytes and smooth muscle cells. Nonvascular cells are macrophages, fibroblasts and epithelial cells.	Clark <i>et al</i> , 1998 Fonsatti <i>et al</i> , 2001
HB-EGF	Cytoprotection	Leach <i>et al</i> , 2002
FGF2	Mitogenesis many cellular processes including proliferation, differentiation, migration and cell survival throughout prenatal and postnatal life	Zhong <i>et al</i> , 2006 (Beenken & Mohammadi, 2009). Arany and Hill, (1998); Borowicz <i>et al</i> , (2007).
PDGF PDGFs A-C and their receptors PDGFR α and PDGFR β	Motility variety of cell processes including proliferation, survival, movement, deposition of extracellular matrix (ECM) and tissue remodelling. angiogenesis at the level of cell development, vascular smooth muscle cells (VSMC) and pericytes. promote cellular responses such as proliferation, survival, and migration	Gurski <i>et al</i> , 1990 (Hoch & Soriano 2003).
HGF	Cytoprotection promotes placental villous trophoblast migration and invasion but does not affect cellular proliferation.	Kilby <i>et al</i> , 1996 Somerset <i>et al</i> 's, (2000)
PTN syndecan-1 (SDC-1) and receptor protein tyrosine phosphatase beta (RPTP β)	Proliferation invasion of placental villous trophoblast cells and vascular endothelial injury. Invasion of trophoblast cells and vascular endothelial injury.	Shulte <i>et al</i> , 1996 Ball <i>et al</i> , 2009
MK	Cell proliferation, migration, survival growth/differentiation Angiogenesis	Milner <i>et al</i> , 1992
CD105	Angiogenesis, Proliferation	Fonsatti <i>et al</i> , 2001

Table 2. Some of the heparin-binding growth factors present in placental tissues. Abbreviations: vascular endothelial growth factor (VEGF), placental growth factor (PLGF), heparin binding epidermal growth factor (HB-EGF), fibroblast growth factor 2 (FGF2), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), pleiotrophin (PTN), midkine (MK) and cluster differentiation (CD105).

As mentioned earlier, there is evidence that the activities of many of these growth factors are regulated by a series of sugar polymers consisting of chains of monosaccharide's or disaccharide units known as glycosaminoglycans, for example heparin.

1.2.5.9. Heparin.

A number of growth factors described bind to a series of carbohydrate polymers called glycosaminoglycans. Heparin is a well-known soluble glycosaminoglycan (GAG) with a molecular weight ranging from 6,000 to 40,000 Daltons (Da). Low molecular weight heparin (LMWH) is made by partial hydrolysis or enzymatic degradation of unfractionated heparin (UFH; Shriver *et al*, 2000). Heparin is a polymeric chain composed of repeating disaccharide units of D-glucosamine and uronic acid linked by 1→4 interglycosidic bond. The uronic acid residue can be either D-glucuronic acid or L-iduronic acid (Mulloy *et al*, 1993; Said *et al*, 2013). The linear structure of Heparin is shown in Figure 19. A single disaccharide unit of heparin is composed of a 2-O-sulphated α -L-iduronic acid and 6-O-sulphated, N-sulphated glucosamine.

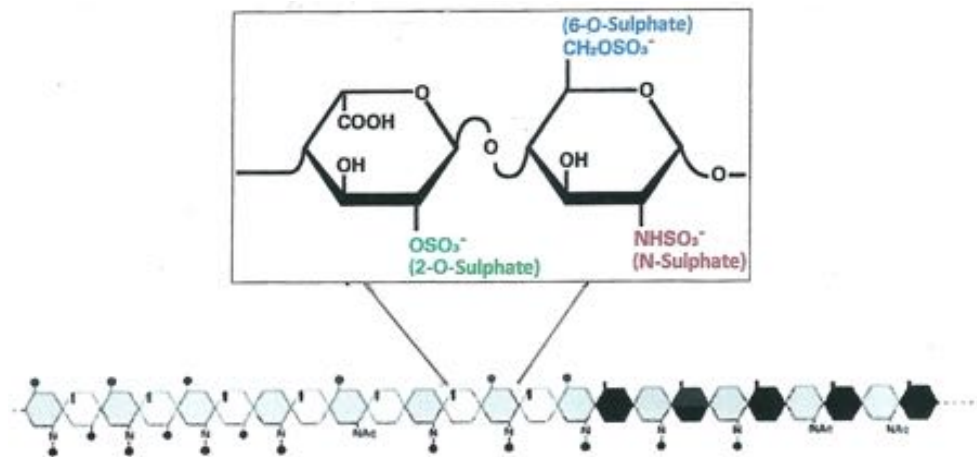
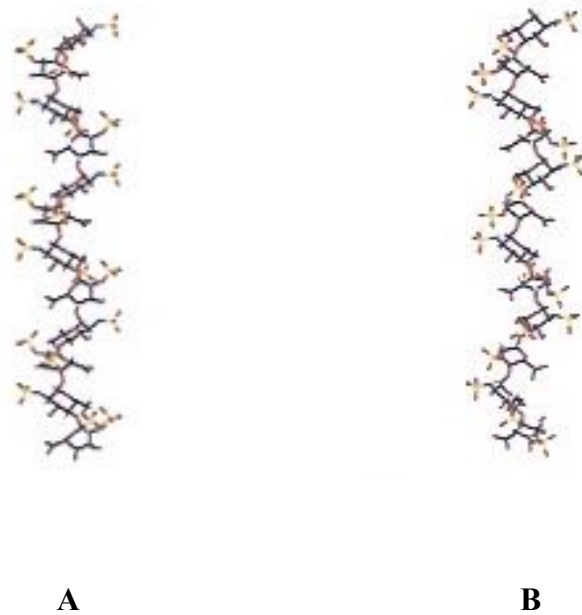


Figure 19. Heparin structure modified from Mulloy *et al*, (1993).

The three-dimensional structure of heparin is complicated and the conformational equilibrium is influenced by sulphation state of adjacent glucosamine sugars. These are essential for determining the critical functional groups involved in the recognition

and activation of different proteins. Heparan sulphate (HS) carboxyl groups, give it a very strong negative charge. HS are produced in living cells by a complex multi-step enzymatic biosynthetic process.

A heparin dodecasaccharide is composed of 6-O-sulphated, N-sulphated glucosamine and 2-O-sulphated α -L-iduronic acid (GlcNS(6S)-IdoA(2S) repeat units. The two models in Figure 20. show: one in which all IdoA two sulphur (2S) = (2-O-sulpho- α -L-iduronic acid) are in the 2S_0 conformation (**A**), and one in which they are in the carbon (1C_4) conformation (**B**).



- **A** = 1HPN (all IdoA (2S) residues in 2S_0 conformation)
- **B** = 1HPN (all IdoA (2S) residues in 1C_4 conformation)

Figure 20. Three-dimensional chemical structure of heparin polysaccharide. Modified from Ferro *et al*, (1990).

Heparin has a helical conformation, the rotation of which places clusters of sulphate groups on either side of the helical axis. The colour key as carbon is white, oxygen is red, nitrogen is blue and sulphate is yellow.

Heparin is also effective in lowering the recurrence of pre-eclampsia (Mello *et al*, 2005; Rai *et al*'s, 1997) and other disorders associated with placental development and oxidative stress. Heparin acts as an anticoagulant, preventing the formation of

clots and extension of existing clots within the blood. In normal pregnancy there is an increase in procoagulants and impaired fibrinolysis. The beneficial effects of heparin on pregnancy outcome in women with prothrombotic disorders are mediated by its anticoagulant properties, but intravascular or intervillous blood clots are rarely found. There is evidence that the underlying mechanism is not through its anticoagulant effects. To determine the rationale for this treatment a series of studies have been carried out. Bose *et al*, (2005); Hills *et al*, (2006), and Mello *et al*, (2004) showed that heparin has direct effects on placental and trophoblast cells, in addition to its vascular effects. The *in vitro* studies of Hills *et al*, (2006) demonstrated that heparin itself inhibits trophoblast apoptosis from a variety of stimuli. Results suggest that heparin may be useful in the management of at-risk patients, even in the absence of an identifiable thrombophilic disorder. Said *et al*, (2013) suggested that heparin therapy might reduce the development of pre-eclampsia, as observed in recent clinical studies.

The mechanism that mediates these effects may be brought about through the interactions with heparin-binding growth factors (HBGFs). Signal transduction by the epidermal growth factor (EGF) receptor tyrosine kinase, as evidenced by receptor autophosphorylation, is induced by HB-EGF only in the presence of heparin, in contrast to EGF-induced receptor autophosphorylation, which is independent of heparin. In summary, it has been demonstrated to us Hills *et al*, (2006) that heparin activates multiple anti-apoptotic pathways in human trophoblast.

Proteins involved in signalling and adhesion at the cell surface recognize and bind HS chains (Said *et al*, 2013). There is evidence that some actions are mediated via a series of growth factors (Hills *et al*, 2006). These are called heparin-binding growth factors (HBGFs) (Hills *et al*, 2006; and Said *et al*, 2013).

1.2.6. Other glycosaminoglycans.

Glycosaminoglycans (GAGs) are large linear polysaccharides constructed of repeating disaccharide units either of two modified sugars *N*-acetylgalactosamine (GalNAc) or *N*-acetylglucosamine (GlcNAc) with the primary configurations containing an amino sugar (either GlcNAc or GalNAc) and an uronic acid (either glucuronic acid and/or iduronic acid).

1.2.6.1. Actions of proteoglycans.

The majority of GAGs are linked to core proteins involving a specific trisaccharide composed of two galactose residues and a xylose residue, forming proteoglycans as shown in Figure 21. The protein cores of proteoglycans allow multiple GAG attachments.

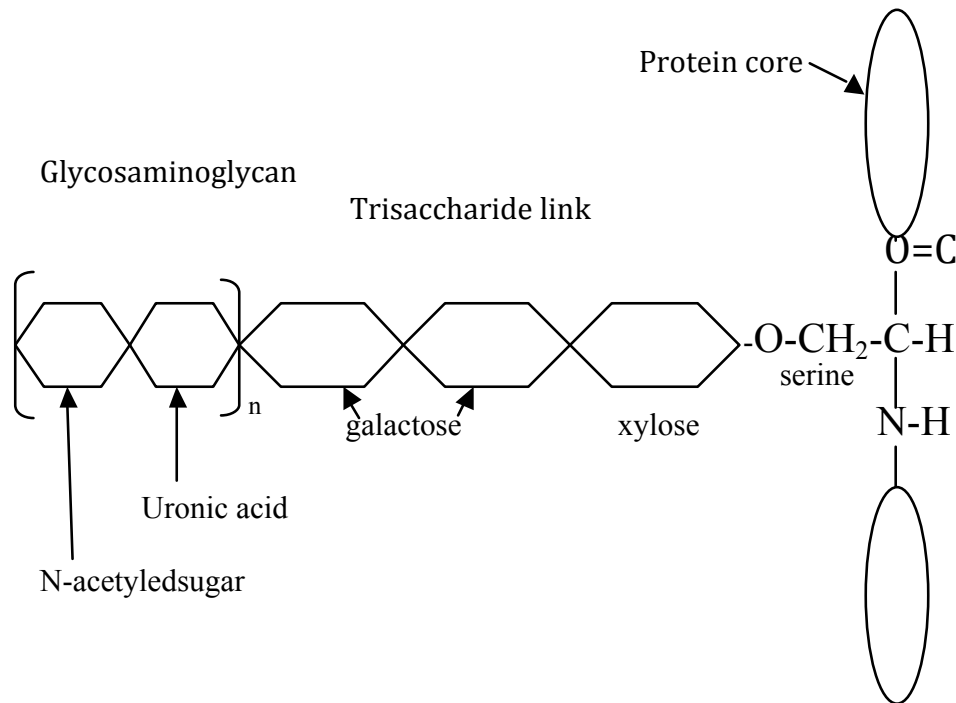


Figure 21. The structure of GAG linkage to protein in proteoglycans is presented. Modified from King, (2013).

The specific GAGs of physiological significance are five identified glycosaminoglycan chains: Hyaluronan (also called hyaluronic acid), dermatan sulphate, chondroitin sulphate, heparin, heparan sulphate, and keratan sulphate (King, 2013). Hyaluronate are large polymers and are synthesized in the plasma membrane. Chondroitin sulphates are the most abundant GAG and are associated with protein to form proteoglycans. They are the major component of the extracellular matrix (ECM). Heparan sulphate is associated with protein forming heparan sulphate proteoglycans (HSPG) and are major HSPG forms are the syndecans and glypicans (Said *et al*, 2013). Sydecans are membrane-bound protein glycosaminoglycans. Glypicans are heparan sulphate protein glycosaminoglycans linked to the plasma membrane. They are comprised of polysaccharide chain of heparan-sulphate covalently linked to proteins (proteoglycans). These differ according to the

extent of sulphation of the saccharides. For example a syndecan HS proteoglycan involves a single transmembrane α -helix and a glypican HS proteoglycan attaches to the outer surface of the plasma membrane via covalent linkage to a phosphatidylinositol lipid. Perlecan is expressed in the basement membrane and extracellular matrix and are components of the extracellular space in tissues. Dermatan sulphate may function in wound repair and infection. Keratan sulphates are associated with protein forming proteoglycans.

1.2.6.2. Heparin-binding growth factors (HBGFs) and glycosaminoglycans (GAGs) interactions.

The biological activity of HBGFs is brought about through different mechanisms. The HSPGs stabilizes the growth factor and protects it from proteolytic degradation. Heparan-sulphate proteoglycans (HSPGs) may activate an intracellular transduction signal by interacting directly with growth receptors through three different mechanisms, as indicated in Figure 22.

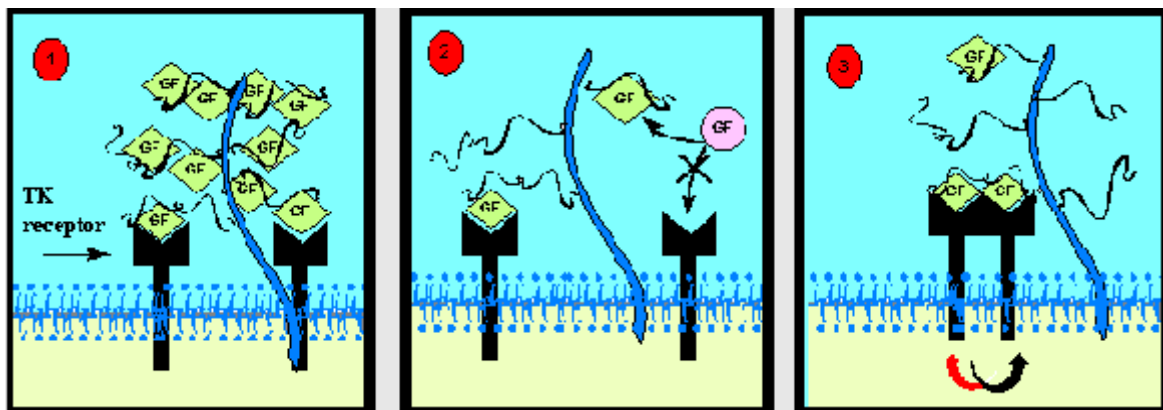


Figure 22. Modified from (Ruoslahti and Yamaguchi, 1991).

HSPGs increase the local concentration of the growth factor (GF) to the tyrosine-kinase (TK) receptor. HSPGs induce a conformational change of the growth factor for its interaction with TK receptor. HSPGs induce growth factor oligomerization with TK receptor dimerization and activation. HBGFs all have a heparin-binding motif.

Hills *et al*, (2006) demonstrated that heparin abrogates apoptosis of primary first trimester villous trophoblast in response to treatment with the pro-inflammatory cytokines interferon (IFN)- γ and tumour necrosis factor (TNF)- α . Heparin suppresses

natural killer cell cytotoxicity (Yamamoto *et al*, 1985; and Johann *et al*, 1995), prevents leukocyte adhesion/influx (Christopherson *et al*, 2002; Manduteanu *et al*, 2002; and Wan *et al*, 2002), antagonizes interferon (IFN)- γ signalling (Fritchley *et al*, 2000) and inhibits complement activation (Girardi *et al*, 2004). The ability of heparin to antagonize cell death induced by such diverse apoptotic signals suggested that it acts as a survival factor for human trophoblast. It has been demonstrated in the study of Hills *et al*, (2006) that heparin, like epidermal growth factor (EGF) and heparin-binding EGF (HB-EGF), elicit phosphorylation of the EGF receptor and activation of the phosphatidyl inositol 3-kinase (PI3K)-, the extracellular signal-related kinase 1/2 (ERK1/2)- and the c-Jun NH2 terminal kinase (JNK)-signal transduction pathways in primary villous trophoblast.

1.2.7. Aim of the Project.

In summary, various growth factors are involved in placental development and many of these bind glycosaminoglycans (GAGs) and this binding affects activity. Several aims in this study were therefore to test the main hypothesis that pre-eclampsia is associated with dysregulation of heparin-binding growth factors.

These aims were:

1. To determine whether heparin and heparin-binding growth factors have a role in regulating normal placental development and disorder as pre-eclampsia.
2. To determine whether midkine and pleiotrophin's expression may have a role in placental development.
3. To identify if heparin-binding growth factors and their receptors could be detected in placental tissue.
4. To determine heparin-binding growth factors' role for the villous trophoblast invasion and migration *in vitro* with in presence and absence of heparin.
5. To determine the amount of total quantitative mRNA production of placental expression in normal placenta and in pre-eclampsia with primer assays of heparin-binding growth factors using quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR).

I investigated the expression of heparin-binding growth factors VEGF, PLGF, FGF2, HGF, PDGF- BB, HB-EGF, MK, PTN and CD105 in normal pregnancy and pre-eclampsia. This study used immunohistochemical staining procedures in human placenta samples of patients with normal pregnancy and pre-eclampsia. I examined the role of these heparin-binding growth factors on trophoblast invasion using *in vitro* studies in cultures of human fibrosarcoma HT1080 invasive cells (positive control); NIH 3T3 mouse embryonic fibroblast non-invasive cells (negative control) and immortalized human primary villous trophoblastic cell-line TCL-1. Since immunohistochemistry can only give semiquantitative information, this study was extended to quantify mRNA expression with HBGFs using real time PCR and the identification of HBGF receptors.

Chapter 2

Materials and Reagents

2.1 Equipment

2.1.2. General equipment

Pipettes	Fisher Laboratories (Loughborough, UK)
Pipette controller	Fisher Laboratories (Loughborough, UK)
Pipettors	Fisher Laboratories (Loughborough, UK)
Sterile graduated pipettes	Fisher Laboratories (Loughborough, UK)
Manual pipettes and sterile filter tips	Fisher Laboratories (Loughborough, UK)
5ml Bijou tubes	Fisher Laboratories (Loughborough, UK)
Eppendorf tube	Fisher Laboratories (Loughborough, UK)
Ceti inverted microscope	Jencons (Milton Keynes, UK)
Vortex mixer	Fisher Laboratories (Loughborough, UK)
Vortex mixer Vortex-2-genie	Scientific industries Inc (USA)
Lens cleaning tissue	Whatman International Ltd (Maidstone, UK)
Balancer Acculab	Jencons (CO, UK)
ALC multispeed refrigerated centrifuge	PK131R (DJBLab care Co, UK)
Tissue Tek embedding centre	Leica EG1150H(Milton Keynes UK)

2.1.3. Disposables

Sterile disposable serological graduated pipettes	Fisher Laboratories (Loughborough, UK)
15/50ml Sterile, polypropylene disposable centrifuge tubes	Fisher Laboratories (Loughborough, UK)
1-20µl, 20-200 µl, and 200-1000 µl pipette tips	Fisher Scientific Laboratories (Loughborough, UK)
Powder free nitrile Gloves	Fisher Laboratories (Loughborough, UK)

2.1.4. Immunohistochemistry (IHC)

Pipettes	Fisher Laboratories (Loughborough, UK)
Glass cover slips	Fisher Laboratories (Loughborough, UK)
Superfrost poly-L lysine glass slides	Fisher Laboratories (Loughborough, UK)
Ceti microscope	Nikon, and Ceti (Kingston, UK)
Camera	Nikon, and Ceti (Kingston, UK)
Diaphot microscope and	Nikon, and Ceti (Kingston, UK)
Digital camera DXM1200F	Digital camera DXM1200F Labmade (Leighton Buzzard, UK)

Lucia Digital camera software	Lucia imaging (Drahou, Czech Republic)
Fluormeter 96 well plate reader	Leica (Milton Keynes, UK)
Tissue Tek embedding centre	Leica (Milton Keynes, UK)
Microtome RM2235	Leica (Milton Keynes UK)
Tissue Floatation Bath	Medite (Milton Keynes, UK)
Microwave	Jencons (CO, UK)

2.1.5. Cell Culture plus invasion and migration studies

Corning cell culture flask canted neck, sterile, pyrogenic and polystyrene.	(Corning Incorporated, USA)
1/2ml pastette	Fisher Laboratories (Loughborough, UK)
100ml Sterile flask or plate	Fisher Laboratories (Loughborough, UK)
BD Falcon FluoroBlok 96-Multiwell insert system	Fisher Laboratories (Loughborough, UK)
Fluorimeter 96 well plate reader	BMG Labtech, Aylesbury, UK
Haemocytometer	Thermo Fisher Scientific (Loughborough, UK)
Super frost plus coverslip	Fisher Laboratories (Loughborough, UK)
Manzel- Glaser cover glasses	Fisher Laboratories (Loughborough, UK)
Cell counter	Fisher Laboratories (Loughborough, UK)
Sterile Cryogenic vials Nalgene	Thermo Fisher Scientific Laboratories (Loughborough, UK, Rochester, USA)
Sterile Eppendorf tubes Nalgene	Thermo Fisher Scientific Laboratories (Loughborough, UK, fAG-Germany)
Incubator water bath	(JB Aqua26 Grant, UK)
37°C CO ₂ incubator	Lab mode, Milton Keynes (UK)
Timer	Fisher Laboratories (Loughborough, UK)
Light microscope	GXD202 Model XD 202 Fisher Laboratories (Loughborough, UK)
BD Bio coat Invasion System-20°C	BD Biosciences (Discovery Lab ware Bedford, USA)
BD Bio coat Migration System	BD Biosciences (Discovery Lab ware Bedford, USA)
Microchip Electrophoresis System for DNA/RNA Analysis	MCE®-202 MultiNA Shimadzu Laboratories (Loughborough, UK)

PCR Tech TC- 512	Jencons-PLS Fisher Laboratories (Loughborough, UK)
PCR Tech TC-3000G	Jencons-PLS Fisher Laboratories (Loughborough, UK)
Autochemi system UVP	Jencons-PLS Fisher Laboratories (Loughborough, UK)
UV –visible Spectrophotometer 1650 PC	Fisher Laboratories (Loughborough, UK)
UV –Trans illuminator PC with camera	Fisher Laboratories (Loughborough, UK)
UV – Trans illuminator portable	Fisher Laboratories (Loughborough, UK)
Microwave Proline Fast set 600	Fisher Laboratories (Loughborough, UK)

2.1.6. *Materials and Reagents*

Immunohistochemistry (IHC)

Antibodies to the following:

VEGF vascular endothelial growth factor	Dako cytomation (Ely, UK)
CD105 cluster differentiation	Dako cytomation (Ely, UK)
PLGF placental growth factor	Abcam, (Cambridge, UK)
FGF 2 basic fibroblast growth factor	(RDsystems ,UK)
PDGF- BB platelet-derived growth factor	Abcam, (Cambridge, UK)
HGF hepatocyte growth factor	(Sigma-Aldrich, UK)
HB-EGF heparin-binding epidermal growth factor	(RDsystems ,UK)
MK midkine	(RDsystems, UK)
PTN pleiotrophin	(Sigma-Aldrich, UK)
Vascular endothelial growth factor receptor-1 FLT-1	(Sigma-Aldrich, UK)
VEGFR-2	(Sigma-Aldrich, UK)
Fibroblast growth factor receptor-1 FGFR-1	(Sigma-Aldrich, UK)
Phosphate buffered sterile saline – PBS	Vector Labs (Peterborough, UK)
Secondary antibodies and conjugate	Vector Labs (Peterborough, UK)
Protein blocking reagent	Vector Labs (Peterborough, UK)
Normal Goat/Rabbit/Horse Serum	Vector Labs (Peterborough, UK)
Triton	VWR (Lutterworth, UK)
Vector ABC Universal kit	Vector Labs (Peterborough, UK)
Vector EliteABC kit (PK-6100)	Vector Labs (Peterborough, UK)
Hydrogen peroxide	Fisher Laboratories (Loughborough, UK)
Citric acid	VWR Ltd (Lutterworth UK)
Ethanol	VWR Ltd (Lutterworth UK)
Xylene	VWR Ltd (Lutterworth UK)

DAB (3,3'-Diaminobenzidene) substrate (SK-4100)

Novacostra (Milton Keynes, UK)

PBS (Phosphate buffered saline, (20 mM sodium phosphate, 150 mM NaCl, pH 8.0)

Mayer's Haematoxylin

Fisher Laboratories (Loughborough, UK)

2.1.8. Cell Culture

Cells lines

HT-1080 cells, NIH/3T3(ATCC), JEG-3

ATCC

TCL-1.

Imperial College

Heparin

(Sigma-Aldrich, UK)

Presept (hypochlorite) Tablets Johnson and Johnson

(Medical Company Ltd, UK)

Foetal bovine serum -20°C

(Bios era, UK)

Antibiotic (penicillin, 50X) solution

(Gibco invitrogen™ Paisely, UK)

L-glutamine 2-4mM -20°C

(Sigma-Aldrich company, UK)

Tryple express

(Gibco invitrogen™ Paisely, UK)

Tissue culture growth media

Serum free medium with antibiotic (penicillin/ streptomycin) solution

RMPI media 1640/ DMEM

(Gibco invitrogen™ Paisely, UK)

Serum free medium with antibiotic, L-glutamine and foetal bovine serum (10%)

RMPI media 1640/ DMEM

(Gibco invitrogen™ Paisely, UK)

RMPI1640

(Gibco invitrogen™ Paisely, UK)

DMEM Dulbecco's Modified Eagle Medium

(Gibco Life Technologies Corporation, UK)

Complete medium Fisher Laboratories

(Loughborough, UK)

Sterile PBS

Fisher Laboratories

(Loughborough, UK)

Distilled or deionized water

(Middlesex University, Hendon, UK)

Cell wash buffer

(Sigma-Aldrich company, UK)

Calcein AM

(Sigma-Aldrich company, UK)

Chemoattractant:

5% foetal bovine serum in DMEM.

(Bios era, Gibco Life Technologies Corporation, UK)

2.1.9. Polymerase chain reaction (PCR)

Mortar and pestle	Fisher Laboratories (Loughborough, UK)
Liquid nitrogen	Fisher Laboratories (Loughborough, UK)
Stabilization Reagent	Qiagen Ltd (Manchester, UK)
QIA Shredder	Qiagen Ltd (Manchester, UK)
RNA Later RNA Stabilization Reagent	Qiagen Ltd (Manchester, UK)
Reverse transcription reaction	Qiagen Ltd (Manchester, UK)
Cyber Green kit	Qiagen Ltd (Manchester, UK)
FGF2 Primer assay	Qiagen Ltd (Manchester, UK)
PGF Primer assay	Qiagen Ltd (Manchester, UK)
Succinate dehydrogenase complex subunit A (SDHA) Primer assay	Qiagen Ltd (Manchester, UK)
MK Primer assay	Qiagen Ltd (Manchester, UK)
PTN Primer assay	Qiagen Ltd (Manchester, UK)
RNase-free water	Qiagen Ltd (Manchester, UK)
Ambion TBE Buffer	Invitrogen Life Technologies Ltd (Paisley, UK)
Blue juice gel loading buffer	Invitrogen Life Technologies Ltd (Paisley, UK)
Ultra Pure Agarose gel	Invitrogen Life Technologies Ltd (Paisley, UK)
RNase Away	Qiagen Ltd (Manchester, UK)
DNA ladder	Invitrogen Life Technologies Ltd (Paisley, UK)
SYBER safe DNA gel stain	Invitrogen Life Technologies Ltd (Paisley, UK)
β -mercaptoethanol (β -ME)	Fisher Laboratories (Loughborough, UK)
Gel electrophoresis camera	Fisher Laboratories (Loughborough, UK)
Gel electrophoresis plate tray	Fisher Laboratories (Loughborough, UK)

2.2. Tissue Samples:

Tissues used in this study comprised of placenta samples collected from 87 women (n=87) attending Queen Charlotte, Chelsea Hospitals and Chase Farm. The group comprised first trimester prior to labour (n=4), term placenta (after 36 weeks gestation) not in labour (n=26), preterm labour (before 36 weeks gestation, n=17), term following labour onset (n=21), and (after 20 weeks of gestation) pre-eclampsia (n=19).

The VEGFR-1, (sflt), VEGFR2 and FGF2 receptor study group comprised term placenta (after 36 weeks gestation) not in labour (n=22), preterm labour (before 36 weeks gestation, n=6), term following labour onset (n=21), and pre-eclampsia (n=12).

Protocol A was performed for fibroblast growth factor 2 (FGF2) on placental specimen (n=109) in different dilutions (1 in 100, n=15), (1 in 200, n=56), and (1 in 400, n=10), control (n=17), and a negative control IgG (n=11) were optimised in the absence of citrate buffer. These slides (n=56) in dilution 1 in 200 were incubated overnight at 2-8°C. However, in protocol B the procedure was performed on placental specimen in the presence of citrate buffer for the following heparin-binding growth factors VEGF (n=87), PLGF (n=87), FGF2 (n=87), HGF (n=87), PDGF-BB (n=87), and CD105 (n=87) except for HB-EGF (n=42), MK (n=35), and PTN (n=30), control using placental specimens in different dilutions, control (n=51), and a negative control IgG (n=10).

2.3. Ethical considerations

There are ethical considerations involving the Human Tissue Act (HTA) 2004 (Herrings, 2006). The Hammersmith, Queen Charlotte and Chelsea Hospitals, NHS Trust Research Ethics Committee, and Barnet, Haringey and Enfield local Research Ethics Committee approved study. Informed written consent was obtained from each patient before obtaining the placenta samples. The Department of Natural Sciences Research Ethics subcommittee (Montgomery, 2003) of Middlesex University approved this study.

Following collection, tissues were numerically coded and anonymised so that no information could be traced back to an individual. All tissues are stored within a controlled laboratory area with restricted access.

2.4. Methods

2.4.1. Principle of immunohistochemistry.

Immunohistochemistry (IHC) was first described in the 1930s, but it was not until 1942 that the first immunohistochemistry study was reported. The term immunohistochemistry is often used interchangeably with immunocytochemistry and immunostaining (Crowther, 1995; Law, 1996). However, immunohistochemistry specifically refers to a technique applied to intact tissue, whereas immunocytochemistry refers to a technique applied to isolated cells.

Immunohistochemical staining is used for detecting specific antigens in placenta tissues. It combines anatomical, immunological and biochemical techniques for the identification of specific tissue components by means of a specific antigen/antibody reaction tagged with a visible label. This makes it possible to visualize the distribution and localization of specific cellular components within a cell or tissue. Improvements have been made in protein conjugation, tissue fixation methods, detection labels and microscopes, making immunohistochemistry a routine and essential tool in many laboratories. A primary antibody is used that is designed to specifically bind to a molecule of interest on the tissue. An enzyme-conjugated secondary antibody was then applied. These are designed to specifically bind to the primary antibody, which is itself bound to the molecule of interest. The secondary antibody labelled with biotin was visualized after adding the enzyme-specific substrate. A complex of avidin, labelled with horseradish peroxidase, and biotin (ABC) is applied to identify levels of molecule of interest protein within the placenta tissue. Avidin, a large glycoprotein, has a very high affinity for biotin. Biotin, a low molecular weight vitamin, can be conjugated to a variety of biological molecules including antibodies. The presence of the peroxidase labelled avidin biotin complex is visualised by the addition of 3, 3'-Diaminobenzidine (DAB) and hydrogen peroxide. The DAB reaction deposits a brown chromagen substrate where there are peroxide enzymes present as shown in Figure 23.

In the current study, this technique has been applied to identify the expression of a series of heparin binding growth factors; namely VEGF, PLGF, FGF2, HGF, PDGF-BB, HB-EGF, MK, CD105, and PTN in placental tissue.

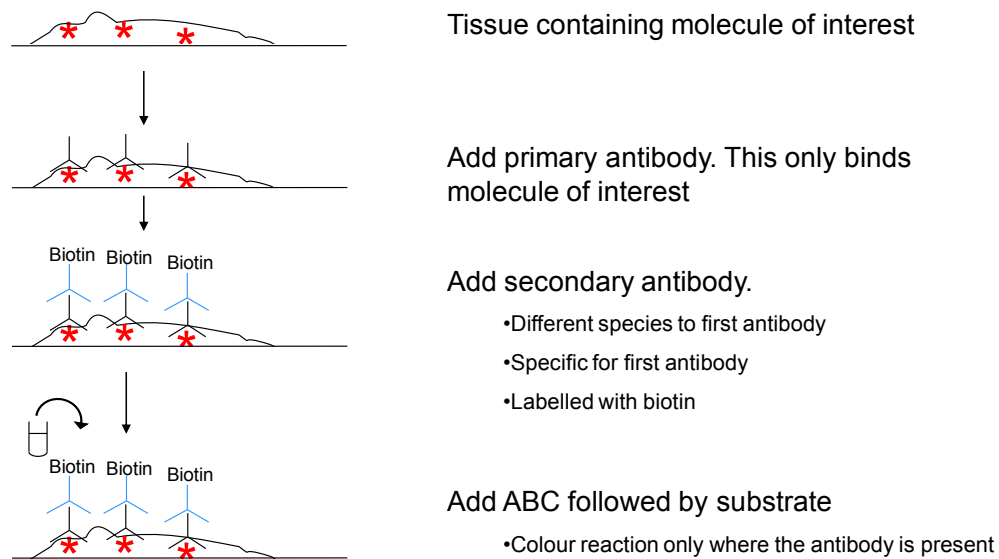


Figure 23. The principle of immunohistochemistry.

Key * Molecule of interest, Y Primary antibody, Y secondary antibody.

Epitope retrieval- Unmasking of Antigen and blocking of peroxidase activity

In the current study, including an epitope retrieval step for antigens requiring “unmasking” according to the primary antibody specifications refined the immunohistochemistry technique.

Several endogenous substances may interfere with IHC results, such as endogenous peroxidase, endogenous fluorescence, endogenous antibody binding capability (Fc

receptors), endogenous biotin. Therefore, blocking the background staining is crucial in IHC to avoid acquiring false positive staining. The blocking of endogenous enzymes, such as alkaline phosphatase, acidic phosphatase and esterase's can be achieved with destroying their activity by boiling, even for a short time at 100°C. This boiling prevents blocking of peroxidase and this may be achieved by preincubating the slides in 3% H₂O₂ in 1% sodium azide PBS for 30 minutes followed by several washes. The heat retrieval method used here involved the use of a citric acid solution maintained at pH of 6.0 and the container being placed with the slide in a microwave oven. The temperature was set to 100°C and left for 20 minutes. The tissue sections were incubated and then rinsed with PBS. The primary antibody was applied to the slide covering the placental specimen with different dilutions to allow optimisation (Abcam, 2010).

2.4.2. Photomicrographs of Immunohistochemical staining.

Photomicrographs were taken in Cell Culture Laboratory using the diaphot microscope, digital Nikon (Kingston, UK) camera DXM1200F, and LUCIA digital camera software, Lucia imaging (Drahou, Czech Republic) from (2010 to 2011), Ceti microscope Nikon, and Ceti (Kingston, UK) from (2012 to 2014).

2.4.3. Immunocytochemistry protocol.

2.4.5. Tissue fixing and processing.

The placenta tissue samples were fixed as a standard procedure using neutral buffered formalin fixative solution incubated overnight to preserve the chemical structure of the tissue. Tissues were then stored in 70% ethanol prior to processing. For tissue processing the water in the tissues was gradually replaced by using a graded solvent series of dehydration agents namely ethanol and finally xylene. The tissue was immersed in 50% ethanol for three minutes. The secondary solvent fixation was with 70% ethanol for 3 minutes, 95% for 3 minutes, and 100% for 3 minutes. Following this, using xylene cleared the placental tissue samples.

2.4.6 Tissue embedding.

The Tissue embedding process was carried out using our automated Tissue Tek. Metal moulds and labelled plastic cassettes were used to form embedding tissue specimens in molten paraffin wax as 'block', from which sections may be cut. The tissue specimen was picked with warm forceps and placed in the centre of the metal mould on the hotplate. The metal mould was placed under the heated lamp to allow the molten paraffin to be poured over the tissue specimen. The labelled plastic cassette was placed over the metal mould with the tissue specimen in the centre and more paraffin wax was added to cover the cassette. The tissue specimen metal was placed on a cold plate for rapid cooling of the paraffin wax. When the paraffin wax had solidified and was stable, the metal mould and excess wax from the plastic cassette were removed. The tissue specimen was then mounted in the clamp of a microtome for thin microscopic sectioning.

2.4.7. Slide preparation.

A microtome was used to cut 5µm sections of each tissue. Sections were placed onto a tissue flotation bath at 37°C. This allowed the tissue to stretch and soften so that it would easily be mounted onto the poly L-lysine coated super frost glass slides.

2.4.8. Protocol A and B for VEGF, PLGF, FGF2, HGF, PDGF- BB, HB-EGF, MK, CD105 and PTN Immunohistochemistry.

Protocol A was performed for fibroblast growth factor 2 (FGF2) the primary antibody was applied to the slide covering the placental specimen in different dilutions (1 in 100), (1 in 200), and (1 in 400) control, and a negative control IgG were optimised in the absence of citrate buffer. These slides in dilution 1 in 200 were incubated overnight at 2-8°C. However, in protocol B the procedure was performed in the presence of citrate buffer for the following heparin-binding growth factors VEGF, PLGF, FGF2, HGF, PDGF-BB, and CD105 except for HB-EGF, MK, and PTN, using placental specimens in different dilutions, control, and a negative control IgG were optimised.

The primary antibody was applied to the slide covering the placental specimen (n=87) in the presence of citrate buffer with different dilutions and optimised for VEGF primary antibody (goat, 1 in 50 dilution), HGF primary antibody (goat, 1 in 100 dilution) and PDGF-BB primary antibody (rabbit, 1 in 100 dilution) and control. After preparation of slides, the sections were deparaffinised by 2 changes of xylene, of 5 minutes each, then re-hydrated in 2 changes of absolute alcohol, for 3 minutes each. The re-hydrating procedure was continued by incubation with 90% alcohol for 3 minutes and then 70% alcohol for 3 minutes. The slide was blocked with (50% in PBS) diluted normal serum (horse serum) in PBS for 5 minutes and incubated with VEGF primary antibody (goat, 1 in 50 dilution); HGF primary antibody (goat, 1 in 100 dilution) and PDGF-BB primary antibody (rabbit, 1 in 100 dilution) for 30 minutes at room temperature. The sections were incubated in preheated citrate buffer with 1M Tris to at pH 8.0 with HCL, and 0.5M EDTA for 10 minutes in a microwave and boiled in a microwave for 4 minutes. The slides were left to cool in heated buffer for 20 minutes. Rinses and washes in PBS were carried out in between the following steps. The next steps of this protocol are described below.

The procedure was identical to that used for VEGF, HGF and PDGF-BB except after the sections were deparaffinised the slides for FGF2, PLGF, CD105, HB-EGF, IgG, MK and PTN were quenched by the endogenous peroxidase activity, and incubated with a blocking reagent 3% H₂O₂ in PBS for 1 minute. The slides were then washed in running tap water for 1 minute. The sections were incubated in preheated citrate buffer with 1M Tris to at pH 8.0 with HCL, and 0.5M EDTA, for 10 minutes in a microwave and boiled in a microwave for 4 minutes. The slides were left to cool in heated buffer for 20 minutes. Rinses and washes in PBS were carried out in between the following steps. Normal horse serum (50% in PBS) was used to non-specific binding of secondary antibody and blocked for 5 minutes. The FGF2 primary antibody was raised in a final dilution of (goat, 1 in 200 and 1 in 2000), control and IgG (a negative control) was applied to the slide covering the placental specimen with different dilutions and optimistised. The slide was incubated with primary antibody (rabbit, 1 in 1000) dilution for PLGF, and CD105, primary antibody (goat, 1 in 50 dilution) for HB-EGF, primary antibody (rabbit, 1 in 200 dilution) for MK and primary antibody (goat, 1 in 100 dilution) for PTN and control incubated overnight at 2-8°C. The next steps of this protocol are described below.

2.5.3. The VEGFR-1 (sflt-1)/ PLGF, VEGFR2 and FGFR-1 receptors.

The primary antibody was applied to the slide covering the placental specimen in the presence of citrate buffer with different dilutions and optimised. These slides were all prepared the same day for VEGFR-1 (sflt-1)/ PLGF receptor primary antibody (goat, 1 in 200 dilution), VEGFR-2 primary antibody (goat, 1 in 400 dilution) and FGFR-1 primary antibody (goat, 1 in 200 dilution) and control. After preparation of slides the sections were deparaffinised with 2 changes of xylene of 5 minutes each. In 100% alcohol for 3 minutes, 90% alcohol for 3 minutes, and 70% alcohol for 3 minutes. Then, the slide was blocked with 50% in PBS normal serum for 5 minutes. The slide was incubated with primary antibody rabbit for VEGFR-1 (sflt-1)/ PLGF (1 in 200 dilution), primary antibody VEGFR2 (1 in 400 dilution) and primary antibody goat FGF2 (1 in 200 dilution) and control for 2 hours. Then, secondary antibody was applied for 30 minutes for VEGFR-1 (sflt-1)/ PLGF and VEGFR-2 at room temperature. For FGFR-1 the secondary antibody was applied for 45 minutes at room temperature. The sections were incubated in preheated citrate buffer with 1M Tris to at pH 8.0 with HCL, and 0.5M EDTA for 10 minutes in a microwave and boiled in a microwave for 4 minutes. The slides were left to cool in heated buffer for 20 minutes. Rinses and washes in PBS were carried out in between the following steps. The next steps of this protocol are described below.

The diluted secondary biotinylated antibody (anti-rabbit Ig G (H+L) or anti-goat Ig G (H+L), 1 in 100 dilution) was applied for 30 minutes at room temperature. The section was incubated with a complex of avidin, labelled with horseradish peroxidase, and biotin (ABC) was applied for 20 minutes at room temperature. The slide was developed with 3, 3'-Diaminobenzidine (DAB) and hydrogen peroxide for 5 minutes followed by a counter staining in Mayer's Haematoxylin solution for 10 minutes. Next the slide was differentiated in 1% acid alcohol for a few seconds. The slides were washed in running tap water for 5 minutes and followed the dehydration procedure. The slides were incubated in 70% alcohol for 2 minutes, 90% alcohol for 2 minutes, and 100% alcohol for 2 minutes, and in 2 changes of absolute alcohol for 3 minutes each. Finally, the slide was cleared in 2 changes of xylene, 3 minutes each. The slide was mounted in xylene -based mounting medium. The slide was then ready for examination under the Ceti microscope.

2.5.4. Scoring and staining.

Staining was scored according to intensity (0 none, 1= weak, 2= moderate, 3= moderate to strong and 4= strong) and coverage (0 = negative, 1-20%= 1, 21- 40= 2, 41-60=3, 61-80=4, 81-100=5). The overall score was given as the product of intensity and coverage.

2.5.5. Statistical Analysis.

Since the tissue scoring represented did not show a normal distribution, all analysis was carried out using non-parametric statistical tests on computer software Stats Direct. All the values were expressed as medians. Comparison between staining for each set of placental tissues versus all other groups combined, comparisons between the four individual groups and comparison of labour versus non-labour was carried out using the Kruskal-Wallis ANOVA test, and the Mann-Whitney *U* test. Correlations between variables non-grouped were performed using the Spearman rank correlation test. Differences were considered significant when the probability of events occurring by chance alone was < 1 in 20 (p-value of < 0.05) was considered statistically significant. The Rho represents Spearman's rank correlation coefficient. The p-value of < 0.05 and p-value of < 0.01 is considered statistically significant.

2.5.6. Photomicrographs of IHC.

Photomicrographs were taken for the immunohistochemistry in the Biomedical Cell Culture Laboratory using the camera Nikon and Ceti (Kingston, UK).

2.5.6. Cell Tissue Culture.

The cell lines analysed were TCL-1, a human_villous trophoblast cell line, NIH/3T3 as negative control, a non-invasive mouse fibroblast cell line, and HT-1080 cells, a highly invasive human fibrosarcoma cell line as a positive control. All were grown in cell culture prior to being used for invasion assay. The protocol used for the following invasion assay applied thawing cells, trypsinising cells and counting cells in the absence of BD FluoroBlok 96-Multiwell insert plate uniformly coated with Matrigel Matrix.

A one hundred millilitre bottle of complete medium (with antibiotic-100IU/ml penicillin and 100µg/ml streptomycin), 2mM L-glutamine and (10%) foetal bovine serum was warmed in a water bath at 37°C. A vial of cells was removed from the liquid nitrogen carefully using cryogloves. Warm medium was added to thaw cells. The thawed contents were transferred to a sterile fifteen ml centrifuge tube containing 10ml complete medium. The tube was centrifuged at 1500rpm for five minutes at room temperature. The cell pellet was checked and was visible at the bottom of the tube. Carefully, the medium was poured off into waste container leaving cell pellet behind. An appropriate volume of fresh complete medium (3ml for a 25cm² flask and 15ml for a 75 cm² flask) was added. The cells were resuspended in the medium by vortex mixing. The cell suspension was transferred to a sterile flask, then to incubator.

The cells were examined using a phase contrast microscope. The medium was removed from the cells and replaced with ten ml serum-free medium. Then, the serum-free medium was removed, and replaced with 10ml fresh serum-free medium. 1-2 ml trypsin solution was added to cells. The flask of cells was placed in standard CO₂ incubator until the cells began to detach from the flask surface. The time for this to happen varied with each cell type, but usually took about five minutes. A phase contrast microscope was used to check the cells had detached. 10ml fresh complete medium was added to flask to neutralise trypsin activity. The cell suspension was transferred to a 15ml centrifuge tube, and then centrifuged at 1500rpm for three minutes at room temperature. The cell pellet was then checked and was visible at the bottom of the tube. The medium was carefully poured off into waste container leaving cell pellet behind. An appropriate volume of fresh complete medium (3ml for a 25cm² flask and 15 ml for a 75 cm² flask) was added. The cells were resuspended in medium

by vortex mixing of the centrifuge tube. The cell suspension was transferred to a sterile flask.

Follow the trypsinizing protocol, the cells were thoroughly resuspended in the appropriate volume of complete medium. This volume was dependent on how many cells were present in the cell pellet. The haemocytometer (and cover slip) was aligned on the phase contrast microscope under two hundred magnification. Twenty microliters (μl) of the cell suspension were taken and placed on the haemocytometer close to (but not on) the cover slip. The cells were counted in the large square that takes up almost the whole field of view. The cells were counted in two more grids of the same size. The average value was calculated and the mean multiplied by ten thousand. This was number of cells in one millilitre.

2.5.7. Principles of invasion and migration for cellular analysis: Cell Invasion Assay.

Cell invasion is the intrusion on and destruction of adjacent tissues, particularly with respect to cancer cells. Cell migration is the movement of cells from one area to another, generally in response to chemical signal, and is important in diverse physiological and pathological processes including embryonic development, cell differentiation, wound healing, immune response, inflammation, angiogenesis and metastasis. Cell invasion is related to, and encompasses, cell migration.

2.5.8. Cell Invasion assay.

The invasion chamber consists of two chambers separated by a filter coated with ECM. The upper surface of the insert membrane is coated with a uniform layer BD Matrigel Matrix which serves as a reconstituted dried basement membrane *in vitro* providing a true barrier to non-invasive cells while presenting an appropriate protein structure to study invasion. Invasive cells are able to degrade the matrix proteins in the layer. Invasive cells move through the extracellular matrix (ECM), which is a complex structure that surrounds and supports the cells within living systems. These then pass through the pores of the polycarbonate membrane. Finally, the cells are removed from the top of the membrane and the invaded cells are stained and quantified. The detection of cell invasion is quantified using Calcein AM. Calcein Am

is a non-fluorescent, hydrophobic compound that easily permeates intact, live cells. Cells internalize it, and intracellular esterases cleave the acetomethylester (AM) moiety to generate free calcium. This cleavage makes Calcein AM fluorescent. It may be used to quantitate the number of cells that have migrated or invaded. Thus, the invasive capacity of a given cell sample is determined by measuring Calcein AM measured by fluorescence at ex 490nm, em 520nm. The cell invasion assay was performed using a BD Falcon FluoroBlok 96-Multiwell insert plate with an 8-micron pore size polyethylene terephthalate (PET) membrane that has been uniformly coated with Matrigel Matrix. The coating process occludes the pores of the membrane, blocking non-invasive cells from migrating through the membrane. The invasive cells are able to detach themselves from and migrate through the coated membrane (Partridge & Flaherty, 2009). The BD Bio Coat invasion assay system provides cells with conditions that allow assessment of their invasive property *in vitro*.

Quantitation of cell invasion was achieved by post-cell invasion labelling with Calcein AM and measuring the fluorescence of invading cells. Since the BD Falcon FluoroBlok membrane effectively blocks the passage of light from 490-700nm at > 99% efficiency, a bottom-reading fluorescence plate reader does not detect fluorescently labelled cells that have not invaded. However, cells that have invaded to the underside of the membrane are no longer shielded from the light source and are detected with the respective plate reader (Partridge & Flaherty, 2009).

The cell suspension was placed in serum-free medium at the top (apical) chamber on an artificial extra cellular matrix (ECM) within insert. Then the cells are incubated overnight at 37°C, standard culture conditions in the presence of test media containing specific chemoattractant (5% foetal calf serum FCS) in the bottom (basal) chamber. Heparin binding growth factor (HBGF) was added to cells in apical chamber. Cells migrate from the top chamber through the coated to the bottom of the filter. Cells were pre-incubated with calcein AM and 4µM was added. Cell dissociation/Calcein AM solution is placed in the bottom chamber to dissociate the invading cells from the filter. Calcein fluorescence in live cells was observed as shown in Figure 24.

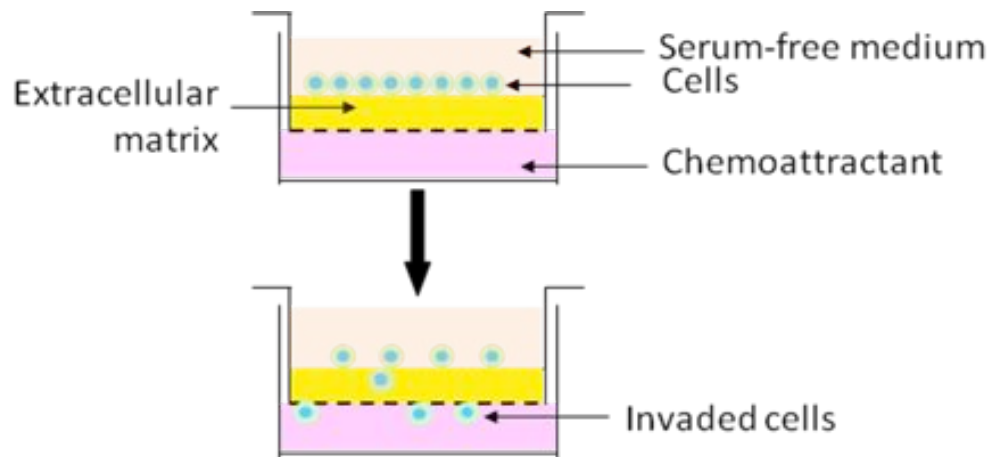


Figure 24. Illustration of a Cell Invasion assay.

2.5.9. An *in vitro* cell invasion assay in the absence of BD Falcon fluoroblok.

A total of 40,000 cells are required per insert and 200,000 cells for standard curve (about 500,000 cells in total).

Preparing inserts: The extracellular matrix (ECM) gel aliquot at 4°C was thawed out for about 30 minutes along with small tips and about 15ml serum free medium (sfm). These items were placed in flow hood on ice along with a 24-well plate with 8µm pore size inserts in 6 of the wells. The ECM 1 in 10 with SFM (100µl + 900µl) was diluted and mixed by flushing with the cold pipette tip.

A one hundred microliter (µl) diluted ECM was transferred into each of the inserts. It was incubated for 4-24 hours at 37°C.

Seeding cells: as this protocol uses different cell types as the human fibrosarcoma HT1080 invasive cells (positive control), and human villous trophoblast cell-line TCL-1. The cells were trypsinised and washed with warmed medium. Then, cells were counted and diluted to a concentration of 20,000 cells/100µl. Then, 500µl of warmed medium with 10% FCS was added to each well under the insert. Then, 200µl cell suspension was added to each insert (40,000 cells in total). Then, factors to test to the cell suspension (pre-incubate) were added. Then, these were incubated overnight at 37°C under standard conditions.

Staining: a two micromolar (μM) calcein solution (stock $400\mu\text{M}$, 1 in 200, $25\mu\text{l}$ in 5ml PBS) was prepared. Then, ten percent (%) acetic acid (1ml glacial acetic acid plus 9ml PBS) was prepared. Then, $200\mu\text{l}$ of $2\mu\text{M}$ calcein was transferred to 6 fresh wells of 96-well plates. The medium was removed from the inserts and place inserts in wells with Calcein. Then, incubated for 1 hour at 37°C in incubator. Then, $200\mu\text{l}$ of the 10% acetic acid was transferred to 6 other fresh wells of 96-well plates. The inserts were placed into wells with 10% acetic acid and gently agitated for 5-7 minutes. The inserts were removed and transferred acetic acid to 96-well plates. The fluorescence of each well was read in plate reader 492nm excitation, 520nm emission.

Standard curve

The cells were trypsinised and washed with warmed medium. Then, cells were counted and diluted to a concentration of $20,000$ cells/ $100\mu\text{l}$ in medium containing 10% foetal calf serum (FCS). The cell suspension and medium (containing 10% FCS) was added to a 96-well plate as shown in Table 3.

Well	Volume cell suspension (μl)	Volume medium (μl)	Cell number
1	200	0	40,000
2	200	0	40,000
3	100	100	20,000
4	100	100	20,000
5	50	150	10,000
6	50	150	10,000
7	25	175	5000
8	25	175	5000
9	10	190	2000
10	10	190	2000
11	5	195	1000
12	5	195	1000
13	0	200	0
14	0	200	0

Table 3. The different cell types, cell suspension and medium (containing 10% FCS) added to a 96-well plate.

Then incubated at 37°C under standard culture conditions until cells adhered to the plate (2-24h). $2\mu\text{M}$ calcein (stock $400\mu\text{M}$, 1 in 200, $25\mu\text{l}$ in 5ml PBS) and 10% acetic acid (1ml glacial acetic acid plus 9ml PBS) was prepared. The medium from each well was removed and replaced with $200\mu\text{l}$ calcein ($2\mu\text{M}$) and incubated for 1 hour at 37°C , but calcein was removed. $200\mu\text{l}$ acetic acid was added to the cells and incubated for 5-7 minutes with gentle agitation. The fluorescence of each well was read in plate reader 492nm excitation, 520nm emission. A standard curve was plotted and the derived equation used to convert fluorescence to cell number.

2.5.9.1. An *in vitro* cell invasion assay in presence of BD Falcon fluoroblok.

This protocol analysed different types of cell lines. These were TCL-1, a human villous trophoblast cell line, NIH/3T3 as negative control, a non-invasive mouse fibroblast cell line, and HT-1080 cells, a highly invasive human fibrosarcoma cell line as a positive control. The cell invasion assay upper surface of the insert membrane is coated with a uniform layer of dried basement membrane matrix solution. The basement membrane layer serves as a barrier for invasive cells from non-invasive cells. Invasive cells are able to degrade the matrix protein in the layer and to pass through pores of the polycarbonate membrane. However, the migration assay principle is different because it has the uncoated membrane that serves as a barrier for migratory cells from non-migratory cells. Migratory cells are able to extend protrusions towards chemoattractants and pass through pores of the polycarbonate membrane. Finally, cells are removed from the top of the membrane and the invaded and migratory cells are stained and quantified. Detection of cell invasion is quantified using Calcein AM.

The plate to be used was removed from -20°C storage and allowed to come to room temperature. 75µl of warm (37°C) media was added to the apical chamber interior of the insert wells of the invasion plate. The plate was allowed to rehydrate for 2 hours at 37°C, 5% CO₂. After rehydration, 50µl of medium was removed carefully from each apical chamber without disturbing the layer of BD Matrigel Matrix on the membrane. The system was then ready to use. For the migration plate, 25µl of medium in the apical chamber was added prior to cell addition. The cell suspension was prepared by trypsinizing cell monolayers and resuspending the cells in serum-free media at 5×10^5 cells/ml, determined by the haemocytometer. As this protocol uses different cell types HT-1080 cells, NIH/3T3 and TCL-1 cell lines, an optimal seeding density was first determined. Then 25µl of cell suspension (1.25×10^4 cells) was added to the apical chambers, and 25µl of serum-free medium added to each apical chamber in the uncoated system. The cells were grown to a~80% confluence in DMEM/ in 10% foetal calf serum (FCS) and antibiotic. Then, 200 µl of chemo attractant (5% FBS) was added to each of the basal chambers. Then, the BD Bio Coat Invasion system and the uncoated migration BD Falcon FluoroBlok™ 96-Multiwell insert plate was incubated overnight (depending on the cell type) at 37°C, 5% CO₂ atmosphere. Heparin (0,100ng/ml) and heparin-binding growth factors (0,1,10, 100nmol/L) were

added to the apical chamber. Following incubation, the medium was removed carefully from the apical chambers. This can be accomplished by flicking the contents into a waste container. The insert systems were transferred into a second 96-well plate containing (200µl of 4µg/ml Calcein AM in PBS. Then was incubated for 1 hour at 37°C, 5% CO₂. The Calcein AM solution was not removed from the lower chamber before reading fluorescence, because it is a non-fluorescent vital dye that is converted into green fluorescent calcein by cytosolic esterases. Readings were taken. The fluorescence of invaded cells was read at wavelengths of 497/517nm or 549/595 nm (EX/EM) on the bottom plate reader.

Data reduction:

Data is expressed as in the following equation

$$\% \text{ Invasion} = \frac{\text{Mean RFU of cells invaded through BD Matrigel Matrix coated membrane towards chemoattractant}}{\text{Mean RFU of cells migrated through uncoated BD FluoroBlok membranes towards chemoattractant}} \times 100$$

Mean RFU of cells migrated through uncoated BD FluoroBlok membranes towards chemoattractant.

The background was subtracted prior to the calculation of percent cell invasion.

RFU = relative fluorescent units.

2.5.9.2. Statistical Analysis.

For the invasion assay results these values were expressed as mean \pm SEM using the Mann-Whitney U test on computer software Stats Direct. A value of $p < 0.05$ was considered statistically significant.

2.6. Principle procedure of Polymerase Chain Reaction (PCR).

Polymerase Chain Reaction (PCR) is a technique for amplifying. Firstly, simply to create multiple copies of a rare piece of DNA and secondly to compare two different samples of DNA to see which is the more abundant. Because mRNA₂ is microscopic it is not possible to see which sample contains the most mRNA. However, if both samples are amplified at the same rate, it can be calculated which sample was the biggest to begin with by establishing which is the biggest after amplification. It is a polymerase enzyme that drives PCR. A polymerase will synthesize a complementary sequence of bases to any single strand of DNA providing it has a double stranded starting point. This is very useful because one can choose the gene the polymerase should amplify in a mixed DNA sample by adding small pieces of DNA complimentary to the gene of interest. These small pieces of DNA are known as primers because they prime the DNA sample ready for the polymerase to bind and to begin copying the gene of interest.

During PCR, changes in temperature are used to control the activity of the polymerase and the binding of primers. To begin the reaction the temperature is raised to 95°C. At this temperature all double-stranded DNA is “melted” in to single strands.

Real-Time (PCR)

Real-Time (PCR) is identical to simple PCR except that the progress of the reaction is monitored in “real-time”. There are a number of techniques that are used to allow the progress of a PCR reaction to be monitored. Real-time quantitative PCR is a powerful technique for advancing functional genomics (Qiagen, (a), 2013).

Reverse-transcriptase is a multifunctional enzyme with three distinct enzymatic activities an RNA-dependent DNA polymerase, a hybrid-dependent exoribonuclease (RNase H), and a DNA-dependent DNA polymerase (Qiagen, (a), 2013).

Stabilization using RNA Later

Immediate stabilization of RNA is necessary because changes in the gene expression pattern occur due to specific and nonspecific RNA degradation and transcriptional induction. It is important for a reliable q-gene expression analysis (Qiagen, (c), 2013).

2.6.1. Protocol 1: Stabilization of RNA.

The weight of the sample was stabilized in RNA later, and RNA Stabilization Reagent was estimated before the tissue was excised. For preserving the tissue, it is important to determine an appropriate volume of RNA later RNA Stabilization Reagent. Approximately 10 μ l reagent per 1mg of tissue was used, that is at least 10 volumes of the reagent. The correct amount of reagent was pipetted into collection tube. The excised tissue sample was cut into slices less than 0.5 cm thick for effective RNA stabilization, a procedure performed as quickly as possible. This tissue sample was completely submerged in the collection tube containing the RNA later, and RNA stabilization reagent to protect the RNA. Purification was the next step using the Qiagen kit (Qiagen, (d), 2013).

2.6.2. RNA purification.

RNA purification depends on the selective binding properties of a silica-based membrane with the speed of microspin technology. A specialised high-salt buffer system allows up to 100 μ g of RNA longer than 200 bases to bind to the RNAeasy silica membrane. Biological samples were first lysed and homogenised in the presence of a highly denaturing guanidine-thiocyanate-containing buffer, which

immediately inactivates RNase to ensure purification of intact RNA. Ethanol was added to provide appropriate binding conditions, and the sample was then applied to an RNeasy Mini spin column, where the total RNA binds to the membrane and contaminants are efficiently washed away. High-quality RNA was then eluted in 30-100µl of water (Qiagen, (d), 2013).

2.6.3. Disruption using a mortar and pestle.

The sample was frozen immediately and ground to a fine powder under liquid nitrogen. The suspension consisting of tissue powder and liquid nitrogen was transferred into a liquid-nitrogen cooled 2ml collection tube and the liquid nitrogen was allowed to evaporate without allowing the sample to thaw. Then, lysis buffer was added and continued as quickly as possible grinding the sample using the mortar and pestle to disrupted the sample. Complete disruption of cell walls and the plasma membranes of cells and organelles are absolutely required to release all the RNA contained in the sample. Different samples require different methods to achieve complete disruption. Incomplete disruption results in significant reduced RNA yields (Qiagen, (d), 2013).

2.6.4. Homogenization.

Homogenization is necessary to reduce the viscosity of the lysates produced by disruption. It shears high molecular weight genomic DNA and other high-molecular-weight cellular components to create a homogeneous lysate. Incomplete homogenization results in inefficient binding of RNA to the RNeasy spin column membrane and therefore significantly reduced RNA yields. Using QIA shredder homogenizers provides a fast and efficient way to homogenize cell and tissue lysates without contamination of samples (Qiagen,(d), 2013).

An aliquot of 700µl of lysate was loaded onto a QIAshredder spin column placed in a 2ml collection tube, and spun for 2 minutes at maximum speed in a microcentrifuge. The lysate was homogenized as it passed through the spin column (Qiagen, (d), 2013).

2.6.5. Protocol 2: extraction and purification of the total RNA from tissues using the RNeasy mini kit and spin technology as shown in Figure 25.

The tissue sample was removed from storage -80°C . The amount of tissue was not more than 30mg.

The RNeasy protect mini procedure using placental cells.



Figure 25. RNAeasy mini procedure was used for placental cells. Modified from (Qiagen, (d), 2013).

2.6.5.2. Extraction.

Thirty milligram of tissue were disrupted and the lysate in buffer RLT Plus was homogenized. Disruption using a mortar and pestle followed by homogenization using a QIAshredder homogenizer: the frozen, or RNAlater stabilized tissue is placed in liquid nitrogen, and ground thoroughly with a mortar and pestle. Then, decant tissue powder and liquid nitrogen into an RNAase-free, liquid-nitrogen-cooled, 2ml microcentrifuge tube. It is important that the tissue does not thaw, allowing the liquid nitrogen to evaporate. A 600 μ l volume of Buffer RTL is added. Then, pipetted the lysate directly in to a QIAshredder spin column placed in a 2ml collection tube, and centrifuge for 2 minutes at full speed (Qiagen, (c, d), 2013).

2.6.5.3. Purification.

The lysate was centrifuged for 3 minutes at 13,000rpm. The supernatant was carefully removed by pipette, and then transferred to a new 1.5ml microcentrifuge tube. It is important to use only this supernatant (lysate) in subsequent steps. One volume of 70% ethanol was added to the cleared lysate and mixed immediately by pipetting. An aliquot of 700 μ l of the sample, including the precipitate that had formed was transferred to an RNeasy spin column placed in a 2ml collection tube. The tube was centrifuged for 15 seconds at (13,000 rpm). The flow through was discarded. Then 700 μ l of the Buffer RW1 was added to the RNAeasy spin column and centrifuged for 15 seconds at 13,000 rpm to wash the spin column membrane. The flow through was again discarded. The collection tube was reused for the following steps: 7, 8. Then, 500 μ l of the Buffer RPE was added to the RNAeasy spin column and the flow through was discarded. This step was repeated. The RNAeasy spin column was placed in a new 2ml collection tube and centrifuged at full speed for 1 minute. The RNAeasy spin column was placed in a new 1.5ml collection tube and 50 μ l RNase-free water was added directly to the spin column membrane and centrifuged for 1 minute at 13,000 rpm to elute the RNA (Qiagen, (d), 2013).

2.6.5.4. Quantification of RNA.

The concentration of RNA was determined by measuring the ratio between absorbance at 260 nm (A_{260}) and 280 nm (A_{260}/A_{280}) in a spectrophotometer. This RNA purity was an estimate with respect to contaminants that absorbed the UV, such as protein. An absorbance of 1 unit at 260 nm corresponds to 44 μg of RNA per ml ($A_{260}=1 \rightarrow 44 \mu\text{g/ml}$) is based on an extinction coefficient calculated for RNA at neutral Ph, because the A_{260}/A_{280} ratio is influenced considerably by pH. When measuring RNA samples, the cuvettes must be RNAase-free (Qiagen, (d), 2013).

2.6.5.5. Purity of RNA.

For accurate values, measured the absorbance in 10 mM Tris·Cl, pH 7.5. Pure RNA has an A_{260}/A_{280} ratio of 1.9–2.1 in 10mM Tris·Cl, pH 7.5 and calibrating the spectrophotometer with this solution (Qiagen, (d), 2013).

2.6.5.6. Integrity of RNA.

Denaturing agarose checked the integrity and size distribution of total RNA gel electrophoresis and by using Agilent 2100 bioanalyzer. The Agilent 2100 bioanalyzer provides an RNA Integrity Number (RIN) (Qiagen, (d), 2013).

2.6.5.7. Protocol 3: Reverse-transcription with elimination of genomic DNA for quantitative, Real-Time PCR.

The template RNA was thawed on ice. The following were thawed at room temperature (15°-25°C): gDNA wipeout buffer, quantscript reverse transcriptase, quantscript RT Buffer, RT primer mix, and RNase-free water. The genomic DNA eliminator reaction on ice was prepared as in the table 4 (Qiagen, (b), 2013). This protocol was performed for 7 different placental tissue mRNA samples at dilutions (1 in 10, 1 in 50, and 1 in 100) were optimised. Using 10pg to 1µg RNA.

Component	Volume/reaction
gDNA wipeout buffer, 7x	2µl
Template RNA	(10-11µg RNA)
RNase-free water	Variable
Total volume	14 µl

Table 4. The genomic DNA eliminator reaction components (Qiagen, (b), 2013).

This was incubated for 2 minutes at 42° C. Then, the contents were immediately placed on ice. The reverse-transcriptase master mix was prepared on ice as in table 5.

Component	Volume/reaction
Reverse-transcriptase master mix	
Quantscript reverse transcriptase	1µl
Quantscript RT Buffer	4µl
RT primer mix	1µl
Entire genomic DNA eliminator reaction	14µl
Total volume	20µl

Table 5. Reverse-transcriptase reaction components. (Qiagen, (b), 2013).

The template RNA was added (14 μ l) to each tube containing the reverse-transcriptase master mix. This was mixed and stored on ice. Then, it was incubated for 15 minutes at 42° C. Then, the contents were immediately placed on ice. Then, the template RNA was incubated for 3 minutes at 95°C to inactivate quantscript reverse transcriptase. Then, an aliquot of each finished reverse transcriptase reaction was immediately added to real-time PCR mix. This was stored at -80°C.

2.6.5.8. Protocol 4. Real Time PCR and two-step RT-PCR using cyclser.

The 2x QuantiTect SYBR Green PCR Master Mix was thawed including template RNA, primers, and RNAase-free water. The individual solutions were mixed. As shown in Table 6, a reaction mix was prepared.

Component	Volume / Reaction
2x QuantiTect SYBR Green PCR Master Mix	25 μ l
Primer	2.5 μ l
Template RNA	12 μ l
RNase-free water	10.5 μ l
Total reaction time	50 μ l

Table 6. A reaction mix. (Qiagen, (e), 2013).

The reaction mix was prepared thoroughly, and appropriate volumes were dispensed into PCR tubes. The template RNA was added to the individual tubes containing the reaction mix. The real-time cycler was programmed according to the Table 7.

Step	Time	Temperature
PCR initial activation step	15 minutes	50°C
3-step cycling		
Denaturation	15s	95°C
Annealing	30s	55°C
Extension	30s	72°C
Number of cycles	40s	

Table 7. The real-time cycler programme. (Qiagen,(e), 2013).

The PCR tubes were placed in the real-time cycler, and the cycling programme was started. A melting curve was analysed for the PCR product. The specificity of PCR products was observed by agarose gel electrophoresis.

2.8. Principles of DNA Gel Electrophoresis.

Gel electrophoresis separates DNA fragments by size in a solid support medium (an agarose gel). DNA samples were pipetted into the sample wells, seen as dark slots at the top of the picture. Application of an electric current at the top (anodal, negative) end causes the negatively charged DNA to migrate (electrophorese) towards the bottom (cathodal, positive) end.

The gel contains small pores through which the DNA molecule pass and the rate of migration occurs at a speed inversely related to their length. Smaller fragments move

more quickly, and finish at the gel. Although each of the fragments of a single class of molecule is present in equimolar proportions, the smaller fragments include less mass of DNA.

2.8.1. Protocol 4. Agarose gel electrophoresis for mini casting tray.

A 1.5% of agarose gel was prepared to 35ml TBE Buffer and 315ml of deionized water. The solution was heated in a microwave for 1 to 3 minutes. The agarose mixture was carefully swirled once or twice during the microwaving. It was important that all agarose particles were dissolved. A 3 μ l of SYBER Safe DNA gel was added to the mixture in mini casting tray and allowed it to cool down. The RT-PCR product and DNA ladder of 100base pairs were thawed on ice. The gel was poured into casting tray with the well combs for 20-30 minutes at room temperature. The mini-casting tray was covered with 200ml of TBE Buffer completely. Then, a 10 μ l loading sample was transferred into epindorf tube and 2 μ l of Blue Juice gel loading Buffer on ice. A DNA ladder of 100base pairs was loaded into the sample well. A sample of 12 μ l was placed into the sample wells. The gel was run at 80V to 120V for 30-40 minutes.

2.8.1.2. The protocol specificity of PCR products by RNA agarose gel electrophoresis for mini casting tray was performed on (n=7) different placental tissue mRNA samples at a concentration of 100ng/ μ l for FGF2, and MK primer assay. The analysis required the DNA ladder and 2 internal standard markers (LM and UM). However, a gel electrophoresis representation analysis was performed on (n=7) different placental tissue mRNA samples at a concentration of 100ng/ μ l for FGF2, PLGF, MK, PTN and Succinate dehydrogenase complex subunit A (SDHA) primer assay using DNA ladder and 2 internal standard markers (LM and UM).

2.8.2. Statistical Analysis.

For the real-time PCR results of (n=7) different placental tissue mRNA samples at a concentration of 100ng/ μ l for FGF2, PLGF, MK, PTN and Succinate dehydrogenase complex subunit A (SDHA) primer assay values were expressed as medians. The 4 groups studied PE, PTL, TNL and TL values were expressed as mean \pm interquartile range (IQR). The comparisons using non-parametric statistical tests, the Mann-Whitney *U* test on computer software Stats Direct. The control (Term (TL+TNL) not in labour) and Pre-eclampsia (PE) of placental tissue mRNA expression with their sensitivity were compared. A value of $p < 0.05$ were considered statistically significant.

2.8.3. Photomicrographs of PCR.

Photomicrographs were taken for the agarose gel electrophoresis in the Lee Smith Foundation Laboratory using the camera Nikon (Kingston, UK).

Chapter 3

Results

3.1. Immunohistochemistry (IHC):

Expression of heparin-binding growth factor (HBGF) and their receptors.

This first technique I performed was immunohistochemical staining procedures for expression of fibroblast growth factor 2 (FGF2) that was localised showing negative staining for the trophoblast and the endothelial cells. These slides (n=56) in dilution 1 in 200 were incubated overnight at 2-8°C in the absence of citrate buffer in placental tissue of the four groups PE- Pre-eclampsia, TL-term labour, TNL-Term-non- Labour, and PTL-Pre-term Labour PE, PTL, TNL, and TL in the placenta as in Figure 26.

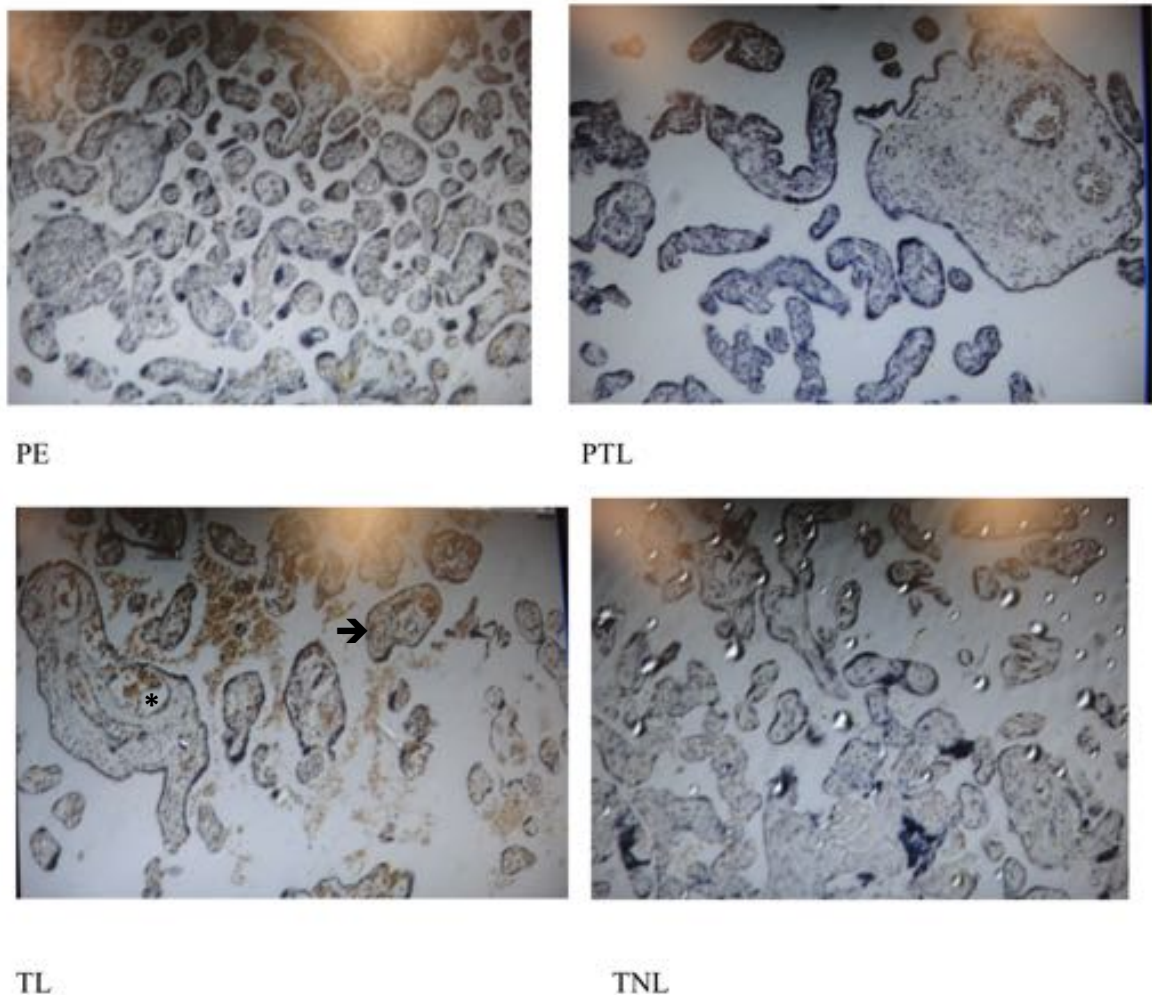


Figure 26. The expression of fibroblast growth factor 2 (FGF2) was distributed representative negative staining in the absence of citrate buffer in placental tissue with magnification of original x101. PE-Pre-eclampsia, PTL-Pre-term Labour, TNL-Term-non-Labour, and TL-term labour, (n = 56). The arrow indicate the trophoblast and (*) the endothelial cells.

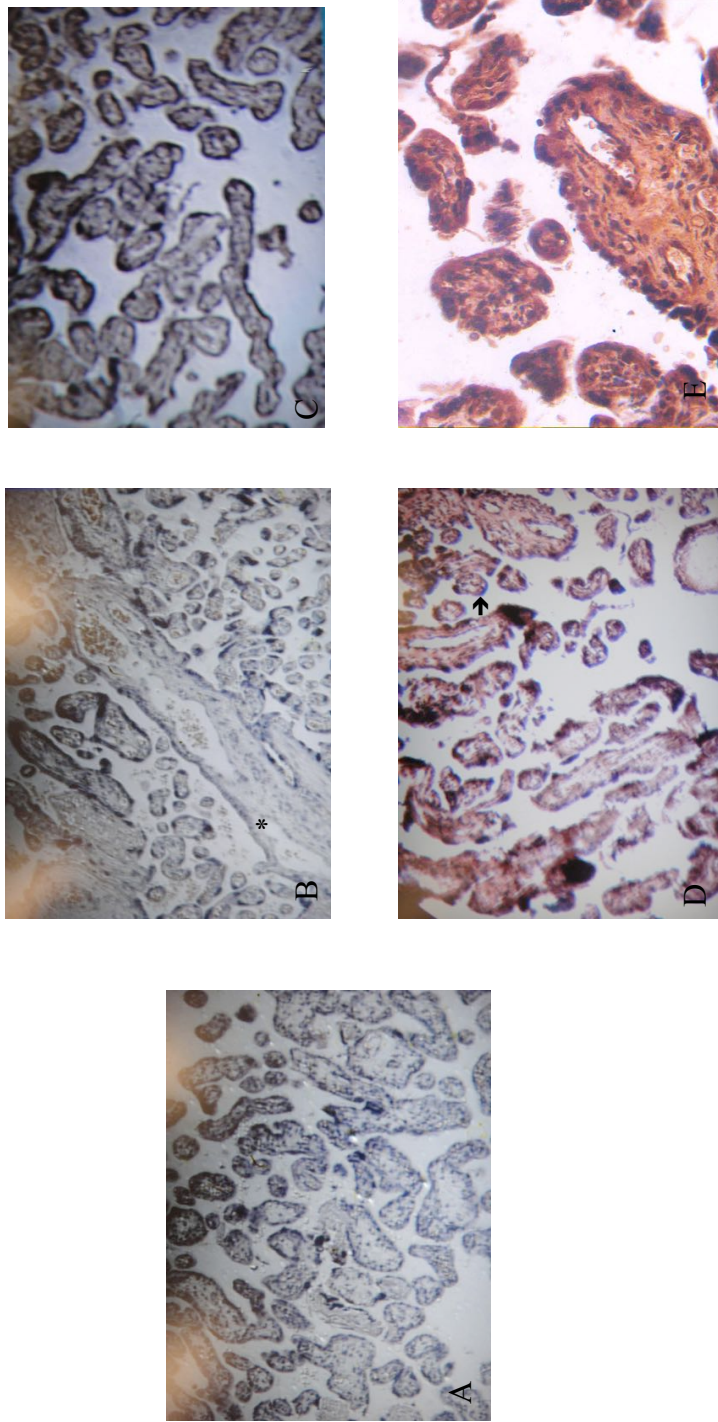


Figure 27. Immunohistochemical staining for expression of fibroblast growth factor 2 (FGF2) representative control A(0 = none) without antibody distributed in placental tissues with a magnification of original (x100) pre-eclampsia, (n=19). Positive staining in villous trophoblast (both syncytiotrophoblast and cytotrophoblast), and endothelial cells and a weaker staining in the mesenchyme, and in the core foetal vessels for B(1=weak) and D(3 = moderate to strong) pre-eclampsia (x100), C(2 = moderate) pre-term (x100), (n=17), and E(4 = strong) pre-eclampsia (x400), (n=19). The arrow indicates the trophoblast and (*) the endothelial cells.

In Figure 27. I confirmed staining, scored according to intensity, as grading was performed as 0 = none, 1 = weak, 2 = moderate, 3 = moderate to strong, and 4 = strong staining on human placenta samples of (n = 87) patients in dilution 1 in 200. These slides were incubated overnight at 2-8°C in the presence of citrate buffer for the expression of (FGF2), in PE-Pre-eclampsia, and PTL-Pre-term Labour distribution. There were numerous and prominent villous trophoblasts, but the villi were poorly vascularised with large number of syncytial knots. However, weaker staining in the mesenchyme, endothelial cells and in the core foetal vessels was observed in the placenta.

I performed immunohistochemical staining procedures to a series of heparin binding growth factors (HBGFs): vascular endothelial growth factor VEGF, placental growth factor PLGF, fibroblast growth factor 2 FGF2, hepatocyte growth factor HGF, platelet-derived growth factor PDGF-BB, and cluster differentiation CD105 (n = 87) except for heparin binding epidermal growth factor HB-EGF (n = 42), midkine MK (n = 35), and pleiotrophin PTN (n = 30), control using placental specimens in different dilutions, control (n = 51), and a negative control IgG (n = 10) specifically on human placenta samples of patients with normal pregnancy and pre-eclampsia all yielded positive staining results. The staining level was scored according to intensity and coverage (0 = negative, 1-20%= 1, 21- 40= 2, 41-60=3, 61-80=4, 81-100=5). The overall score was given as the product of intensity and coverage. I confirmed in the four groups PE- Pre-eclampsia, TL-term labour, TNL-Term-non- Labour, and PTL-Pre-term Labour distribution with numerous and prominent villous trophoblast, but the villi were poorly vascularised with large number of syncytial knots. There was irregular thickening of the trophoblastic basement membrane. However, the syncytiotrophoblast showed no degenerative changes. Figure 28a,b,c,d,e,f,g,h,i, and j. shows the characteristic feature of the villous trophoblast (both syncytiotrophoblast and cytotrophoblast), weaker staining in the mesenchyme, and in the core foetal vessels in the placenta for VEGF, PLGF, FGF2, HGF, PDGF- BB, and CD105 from women with pre-eclampsia. The villous trophoblast and the endothelial cells were analyzed.

Syncytial knots

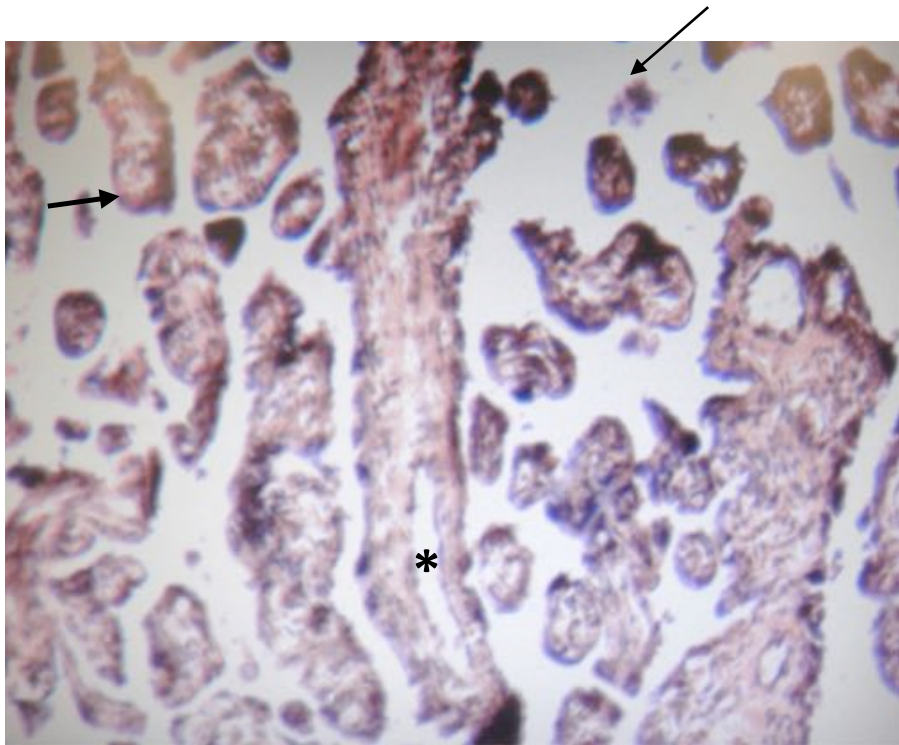


Figure 28a.

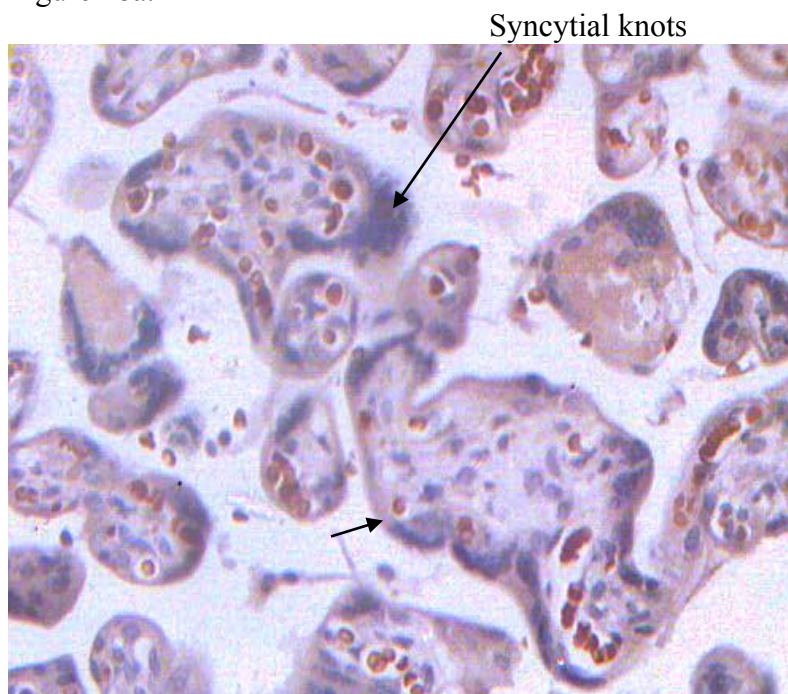


Figure 28b.

Immunohistochemical positive staining in villous trophoblast (both syncytiotrophoblast and cytotrophoblast), in Figure 28a. for fibroblast growth factor 2 (FGF2) expression, 3 = moderate to strong, Magnification of original (x101) and in Figure 28b. for placental growth factor (PLGF) expression, 2 = moderate distributed in placental tissues for Pre-eclampsia (PE), (n = 19). Magnification of original (x401). The arrows indicate the trophoblast and (*) the endothelial cells.



Figure 28c.

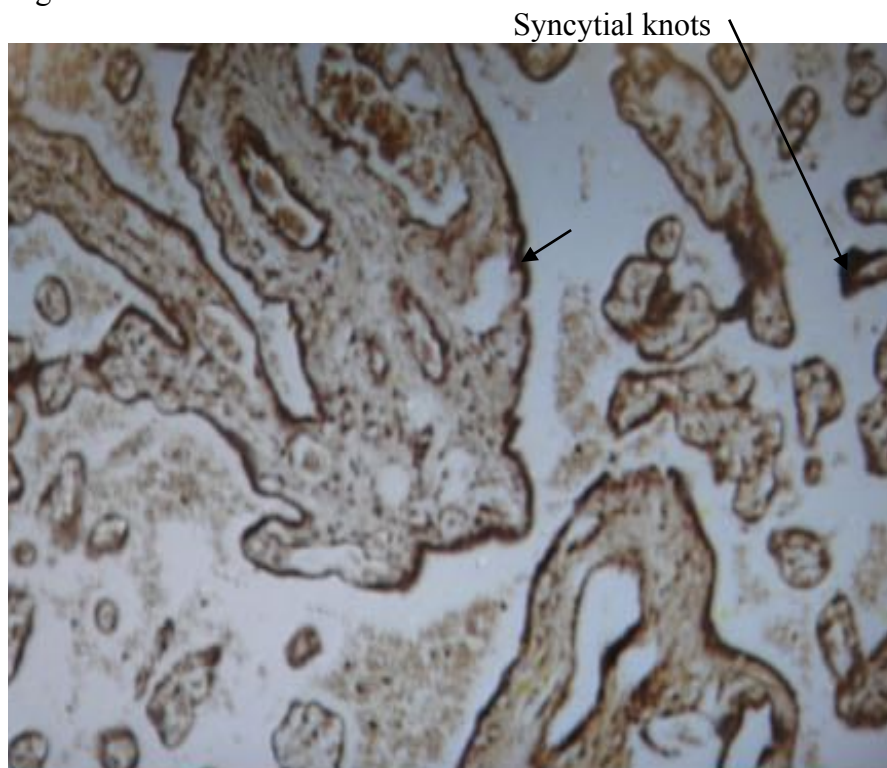


Figure 28d.

Immunohistochemical positive staining in villous trophoblast (both syncytiotrophoblast and cytotrophoblast), in Figure 28c for vascular endothelial growth factor, placental growth factor (VEGF) expression, 3 = moderate to strong, Magnification of original (x401), and in Figure 28d platelet-derived growth factor (PDGF-BB) expression, 4 = strong distributed in placental tissues for Pre-eclampsia (PE), (n = 19). Magnification of original (x101). The arrows indicate the trophoblast and (*) the endothelial cells.

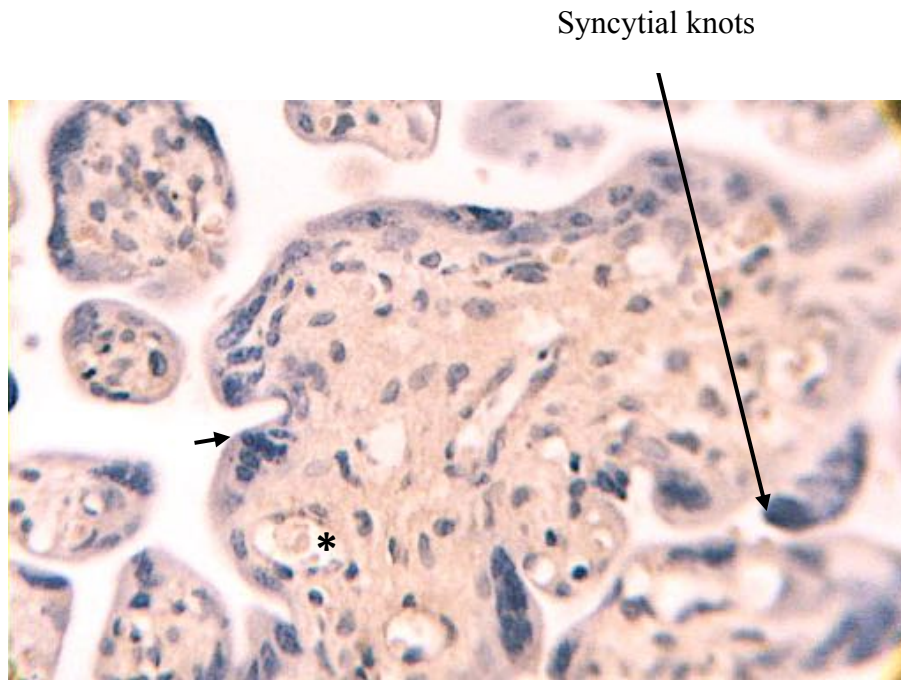


Figure 28e.

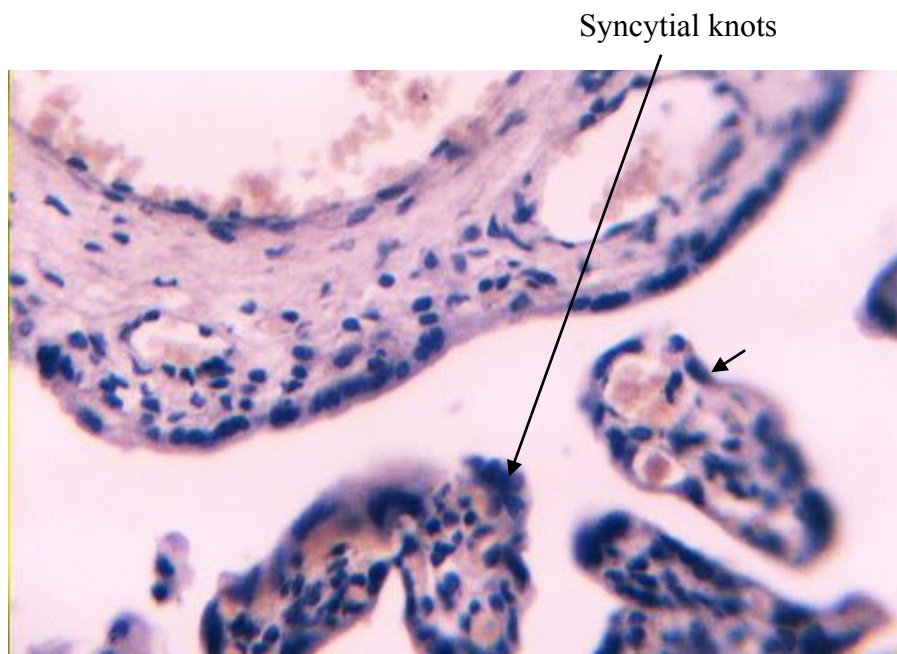


Figure 28f.

Immunohistochemical positive staining in villous trophoblast (both syncytiotrophoblast and cytotrophoblast), in Figure 28e for hepatocyte growth factor (HGF) expression, and in Figure 28f for cluster differentiation (CD105) expression, 2 = moderate, distributed in placental tissues for Pre-eclampsia (PE), (n = 19). Magnification of original (x401 and x402). The arrows indicate the trophoblast and (*) the endothelial cells.

Syncytial knots

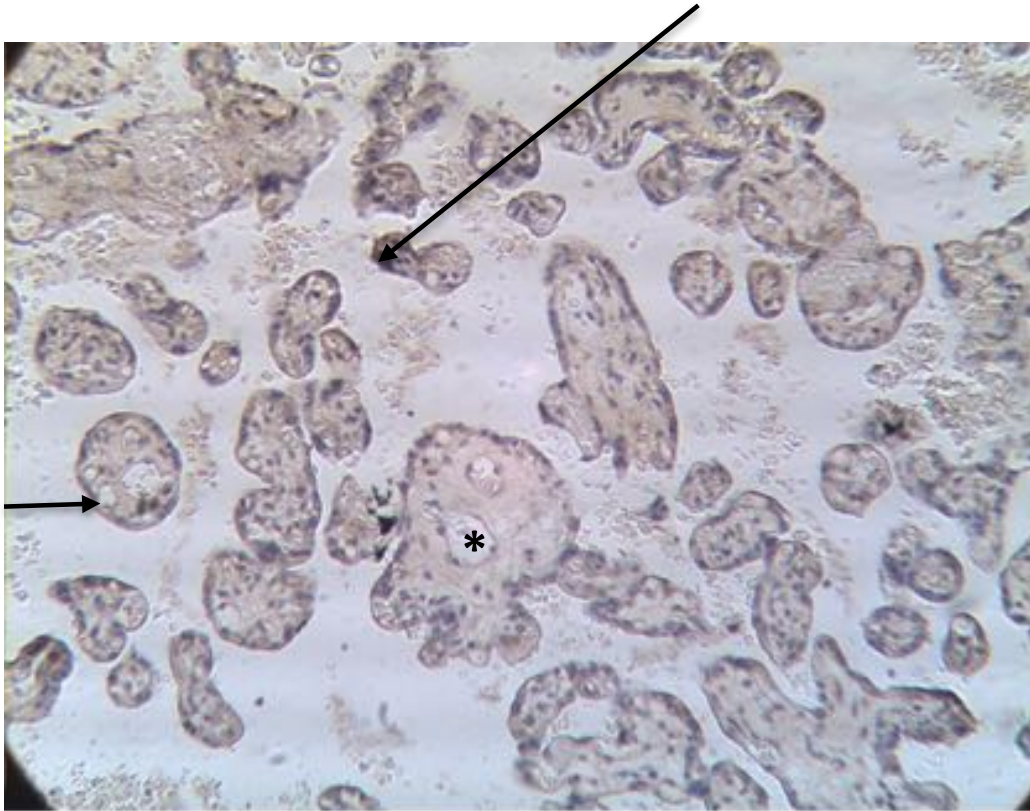


Figure 28g.

Immunohistochemical moderate to strong positive staining in villous trophoblast (both syncytiotrophoblast and cytotrophoblast), for heparin binding epidermal growth factor (HB-EGF) expression, 4 = strong, distributed in placental tissues for Pre-eclampsia (PE), (n = 21). Magnification of original (x401). The arrow indicate the trophoblast and (*) the endothelial cells.

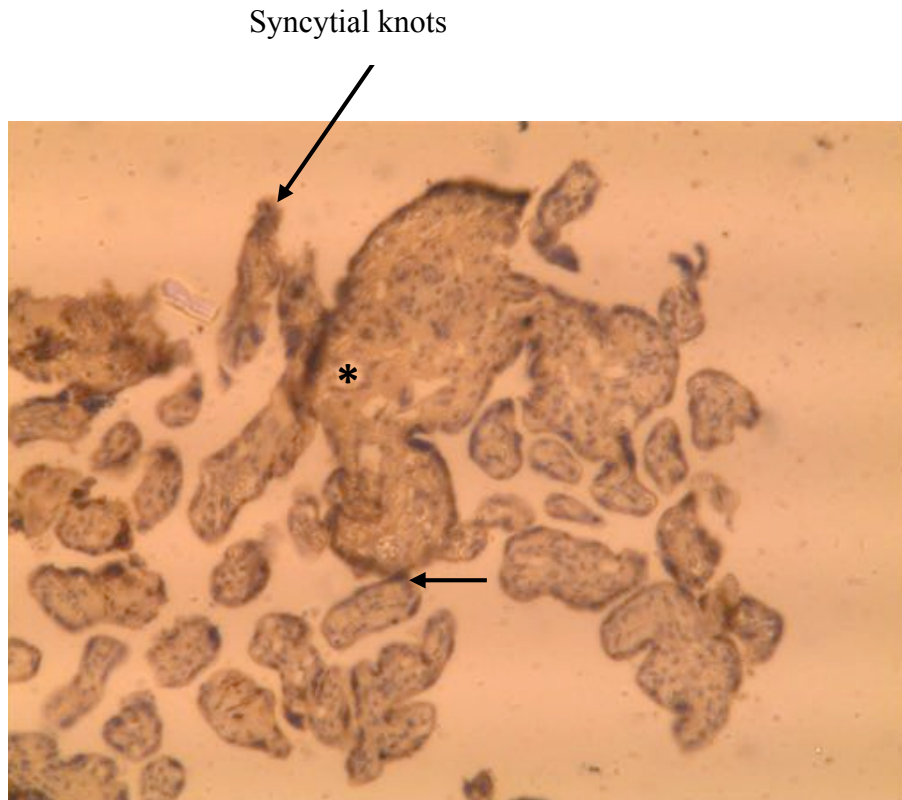


Figure 28h.



Figure 28i.

Immunohistochemical positive staining in villous trophoblast (both syncytiotrophoblast and cytotrophoblast), as in Figure 28h for midkine (MK) expression, (n = 19) and in Figure 28i for pleiotrophin (PTN) expression, (n = 19). 4 = strong, distributed in placental tissues for Pre-eclampsia (PE). Magnification of original (x401). The arrows indicate the trophoblast and (*) the endothelial cells.

Syncytial knots

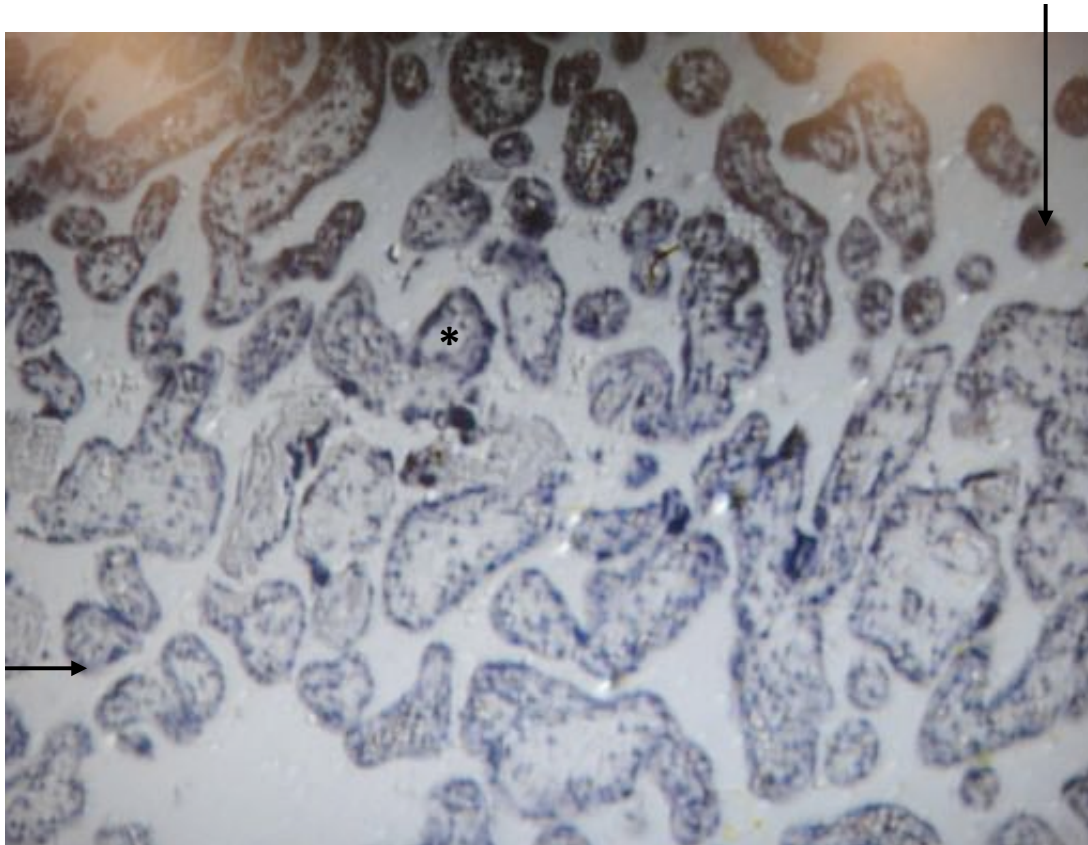


Figure 28J. Immunohistochemical representative negative control, 0 = none, without the antibody distributed in placental tissues for Pre-eclampsia (PE), (n = 19) in the absence of citrate buffer. Magnification of original (x401). The arrows indicate the trophoblast and the endothelial cells. The arrow indicate the trophoblast and (*) the endothelial cells.

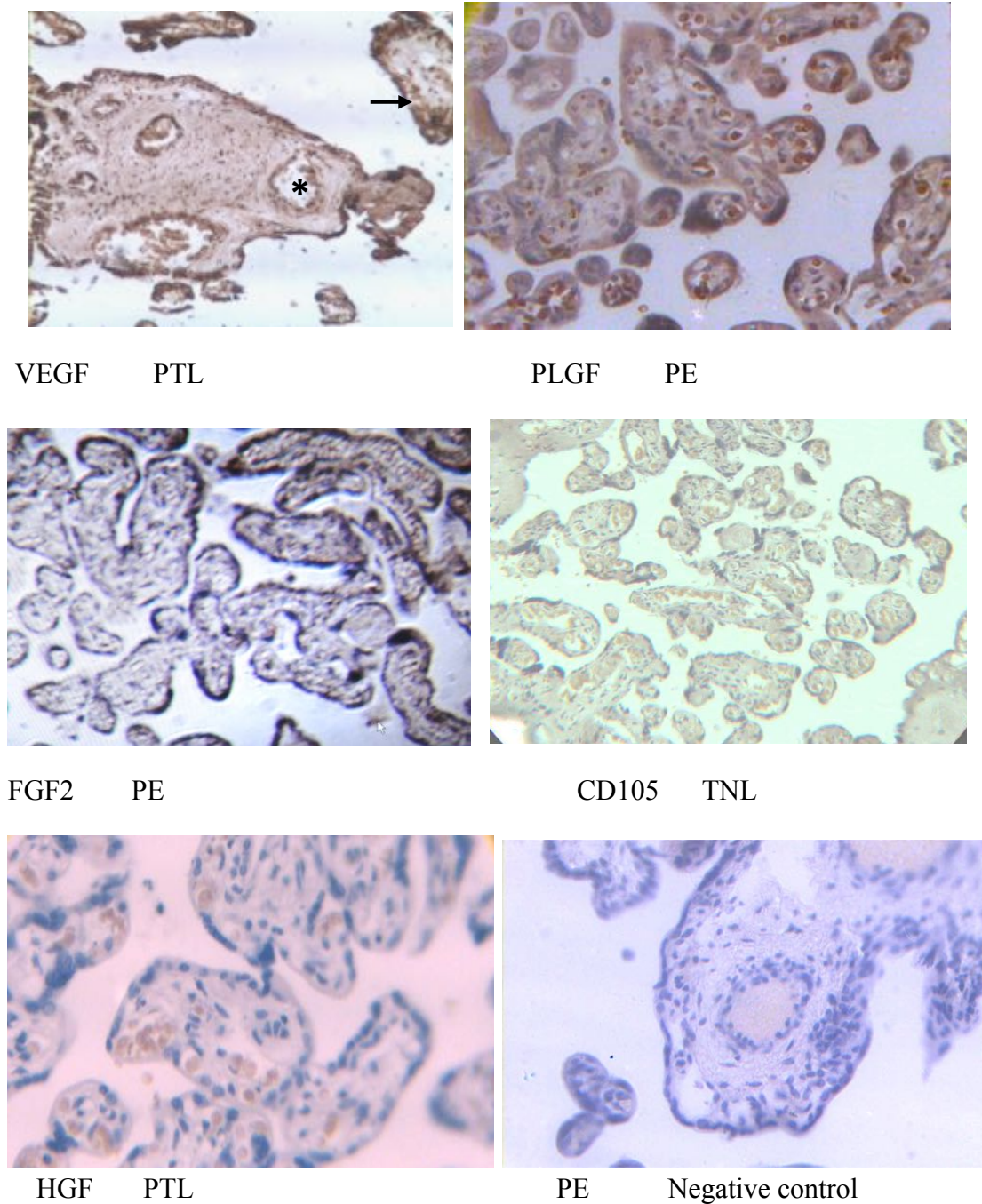
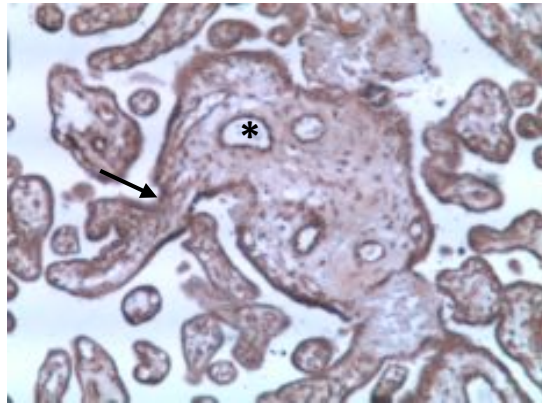
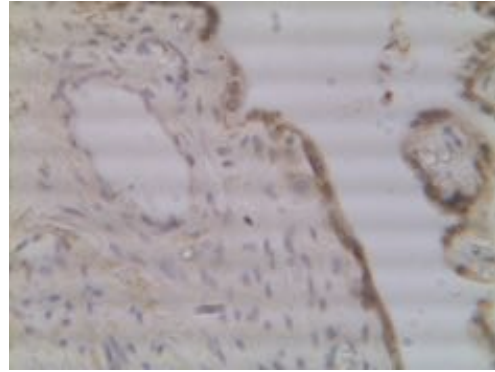


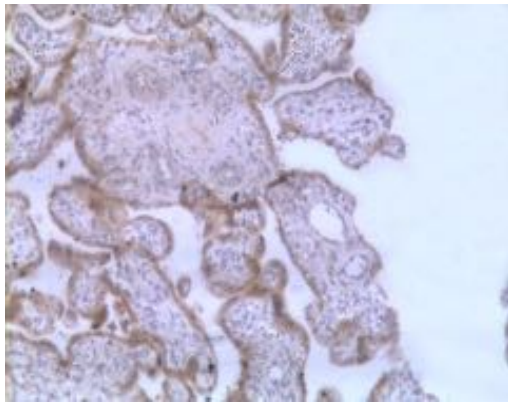
Figure 29. The expression of the vascular endothelial growth factor (VEGF), placental growth factor (PLGF), 2 = moderate, fibroblast growth factor 2 (FGF2), 4 = strong, and cluster differentiation (CD105), 3 = moderate to strong, hepatocyte growth factor (HGF) was distributed with 2 = moderate positive staining in villous trophoblast (both syncytiotrophoblast and cytotrophoblast), for PTL-Pre-term Labour, TL-term labour, and TNL-Term-non-Labour, (n = 68) at a magnification of original (x401). In the absence of (HBGFs) the placental tissue expressed negative staining with a magnification of original (x401). This control was confirmed without the antibody. The arrows indicate the trophoblast and (*) the endothelial cells.



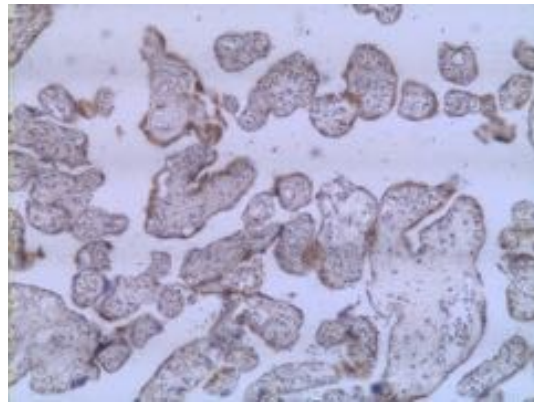
PDGF-BB PTL



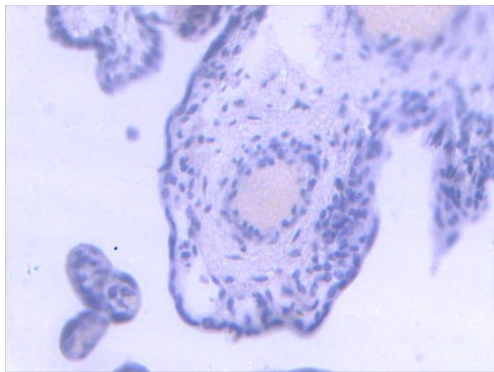
HBEGF PTL



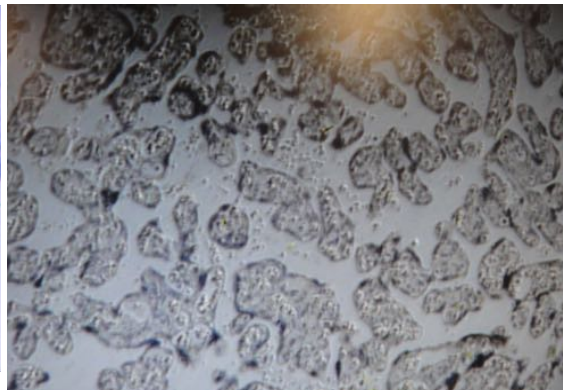
PTN PTL



MK TNL



PE Negative control



Human IgG TNL

Figure 30. The expression of the platelet-derived growth factor (PDGF-BB), 4=strong, heparin binding-epidermal growth factor (HB-EGF), 1=weak, pleiotrophin (PTN), and Midkine (MK), 2=moderate were distributed with moderate to strong positive staining in villous trophoblast (both syncytiotrophoblast and cytotrophoblast), and the endothelial cells, weaker staining in the mesenchyme, and in the core foetal vessels PTL-Pre-term Labour, TL-term labour, and TNL-Term-non-Labour, (n = 68) with a magnification of original (x401). The control showed negative staining, i.e.in the absence of the antibody of magnification of original (x401). The preincubation with non-specific binding of Human IgG confirmed negative staining and does not affect the staining intensity or pattern with a magnification of original (x101). The arrows indicate the trophoblast and (*) the endothelial cells.

The four groups of placenta tested in this study were as follows: preterm pre-eclampsia (PE after 20 weeks of gestation, $n = 19$), preterm labour (PTL before 36 weeks gestation, $n = 17$), term following labour onset (TL, $n = 21$) and term placenta (after 36 weeks gestation) not in labour (TNL, $n = 26$). The expression of HBGFs was confined to villous trophoblast and endothelial cells for VEGF, FGF2, PDGF-BB, CD105, HGF and PLGF. For HB-EGF, MK and PTN, expression was confined specifically to villous trophoblast. The colour intensity and clarity of staining was observed in the photomicrographs as shown in Figure 30A,B,C,D,E,F,G,H, and I. Values for each group as pre-eclampsia PE, versus pre-term PTL, term labour TL, and term non-labour TNL, are indicated using Mann-Whitney U test. As, each symbol corresponds to a different individual in the four placenta study groups. The horizontal red line indicates the median values. The expression of percentage scoring was used to establish differences between the four groups for VEGF, PLGF, HB-EGF, FGF2, CD105, PDGF-BB, HGF, MK, and PTN that provided additional information about the trophoblast and endothelial cells. The expression of VEGF in the large blood vessels was greater than in the trophoblast. For expression of PLGF and FGF2 the trophoblast exhibited greater staining intensity in the large blood vessels.

Comparisons between four groups:

The groups were compared by analysing differences between normal pregnancy and pre-eclampsia in various ways. The differences in expression associated with pre-eclampsia PE versus pre-term PTL, term labour TL, and term non-labour TNL. Then, differences between term (TL and TNL combined, $n = 47$) and preterm (PTL and PE combined, $n = 36$) and finally differences in expression according to the presence or absence of labour (TL and PTL combined, $n = 38$ versus PE and TNL combined, $n = 45$) were examined. In this way it is possible to determine whether differences in expression in pre-eclampsia are actually due to the fact these women tend to deliver preterm in the absence of labour.

Differences between groups with pre-eclampsia

The staining score for MK and PTN expression was significantly lower in pre-eclamptic versus other placenta groups in this study $p = 0.03$ and $p = 0.04$ Mann-Whitney U and Kruskal Wallis analysis of variance test respectively as shown in Figure 27G and H. The median for pre-eclampsia was lower than the other placenta groups. In contrast, the expression of PLGF as shown in Figure 28B was significantly higher in pre-eclampsia compared to other placenta groups studied $p = 0.02$ using the Mann-Whitney U test and Kruskal Wallis analysis of variance test. The VEGF, FGF2, HGF, PDGF-BB, PLGF, HB-EGF and CD105 investigated did not show a significant difference in staining expression in pre-eclampsia.

Comparison Term versus pre-term

As shown in Figure 28B the levels of PLGF were significantly higher in preterm compared to term placentas using the Mann-Whitney U test, $p = 0.05$ respectively. In Figure 28C the levels of CD105 were significantly higher in preterm compared to term placentas using the Mann-Whitney U test, $p = 0.01$. In Figure 28I, and K, the placental expression of HGF was significantly higher in pre-term compared to term placentas, as judged by obtaining a $p = 0.005$ by the Mann-Whitney U test. The expression of HGF staining intensity was significantly higher in pre-term compared to term placentas, $p = 0.003$ by the Mann-Whitney U test. The comparison of staining intensity revealed a significant difference in HGF expression which was higher for pre-term compared to term with $p = 0.02$ using the Mann-Whitney U test. The HBEGF expression was significantly higher in pre-term compared to term with $p = 0.02$ as in Figure 28F. The expression staining score was not significantly different in term compared to preterm according to gestational age in any of the following tested VEGF, FGF2, PDGF-BB, MK and PTN.

Comparison Labour versus non-labour

As shown in Figure 28D the levels of FGF2 were significantly lower in term labour compared to other groups using the Mann-Whitney *U* test, $p = 0.04$ respectively. The PLGF score was significantly lower after labour onset using the Mann-Whitney *U* test $p = 0.03$, as in Figure 28B and J. The expression of PLGF intensity score was significantly lower after labour onset using the Mann-Whitney *U* test $p = 0.02$ respectively. The HGF expression was significantly higher in pre-term labour compared to term with $p = 0.02$ using the Mann-Whitney *U* test as in Figure 28I and K. The HGF median was higher for pre-term labour than the term. The CD105 expression was higher in pre-term labour compared to term in Figure 28C. HBEGF expression was significantly higher in pre-term labour compared to term with $p = 0.03$. The PTN expression was significantly higher in pre-term labour compared to term with $p = 0.04$ using the Mann-Whitney *U* test as in Figure 28H. The VEGF, FGF2, PDGF-BB, and MK tested showed no significant difference in expression according to the presence or absence of labour.

Comparisons between each group versus all other.

The FGF2 expression was significantly different for staining and intensity score as ($p = 0.004$, and 0.02) for term Labour using the Mann-Whitney *U* test respectively. The VEGF expression was significantly different for staining score as $p=0.03$ for term Labour. The PTN expression was significantly different for staining and intensity score as ($p = 0.01$, and $p = 0.04$) for term non-Labour. The HBEGF expression was significantly different for staining and intensity score for term non-Labour and Pre-term Labour as ($p = 0.01$, and $p = 0.04$). There was a significant difference observed for HGF expression of staining and intensity score in pre-term Labour ($p = 0.01$ and $p = 0.001$) compared to term Labour ($p = 0.002$ and $p = 0.01$).

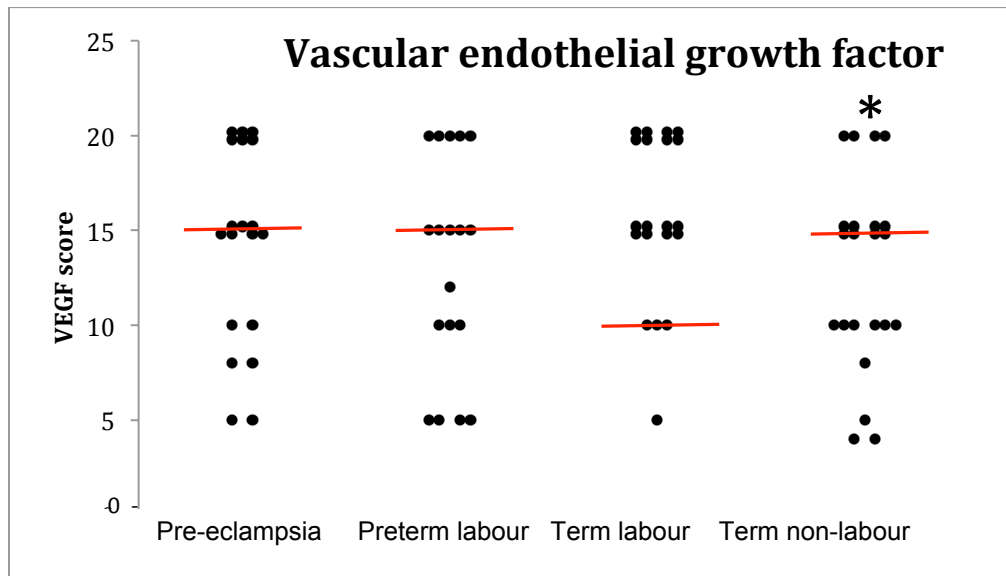


Figure 31A. The vascular endothelial growth factor (VEGF) expression staining score (intensity x coverage) in the four placenta groups studied. Values for each group as pre-eclampsia PE versus pre-term PTL, term labour TL, and term non-labour TNL (* $p < 0.05$ is considered statistically significant difference in staining in Term non-labour) using Mann-Whitney U test. The horizontal red line indicates the median values.

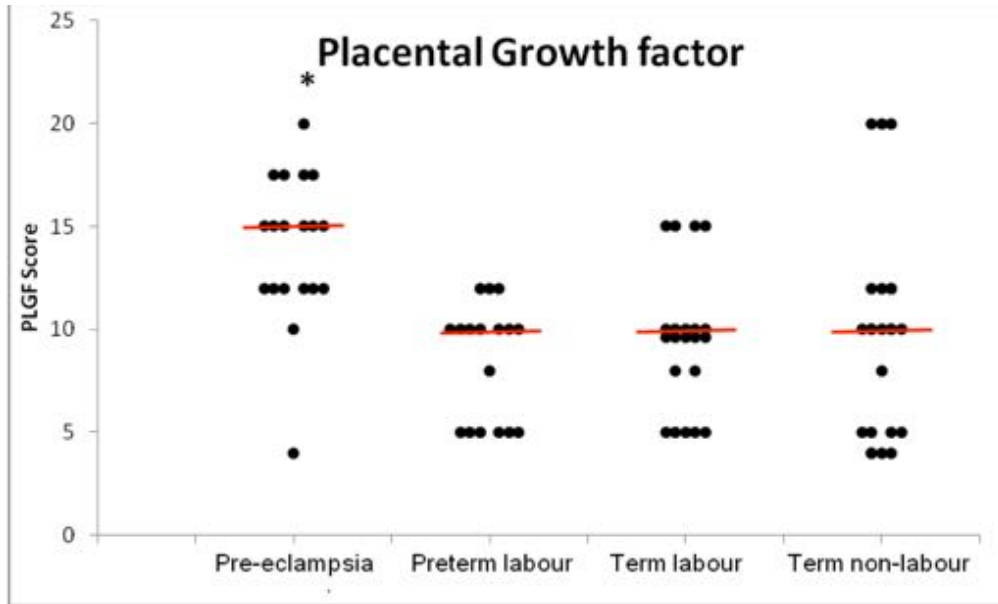


Figure 31B.

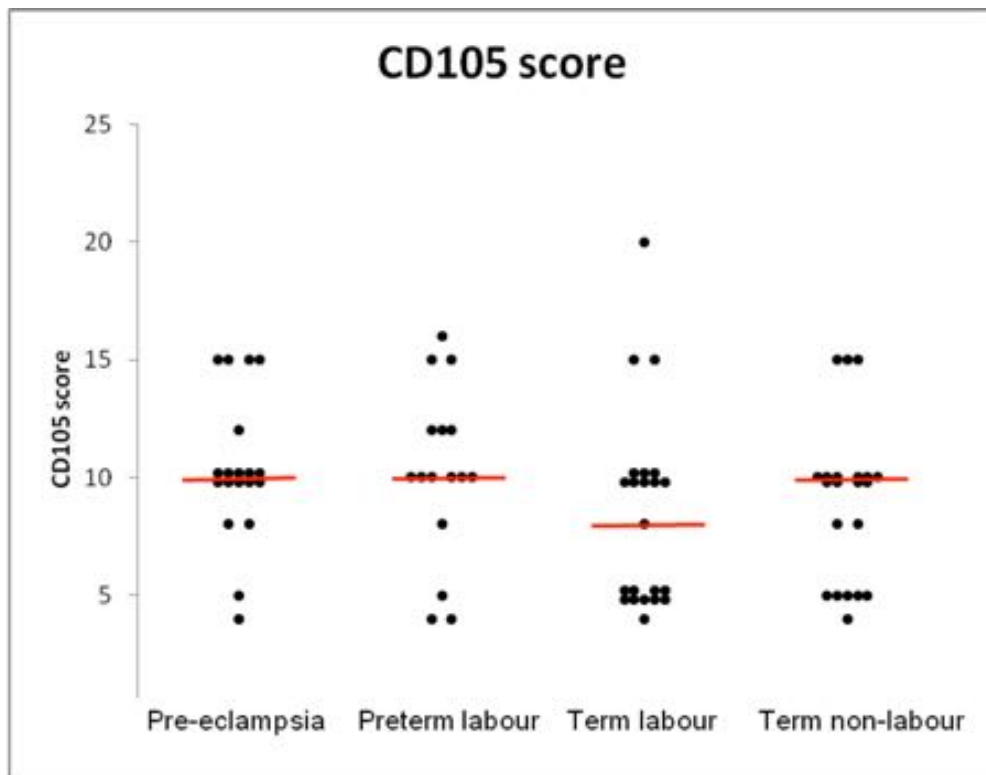


Figure 31C.

Figure 31B. represents the placental growth factor (PLGF) expression staining score (intensity x coverage) in the four placenta groups studied. Values for each group as pre-eclampsia PE versus pre-term PTL, term labour TL, and term non-labour TNL (*p <0.05 is considered statistically significant difference in staining in pre-eclampsia) using Mann-Whitney *U* test. Figure 31C. represents the cluster differentiation (CD105) expression staining score. The horizontal red line indicates the median values.

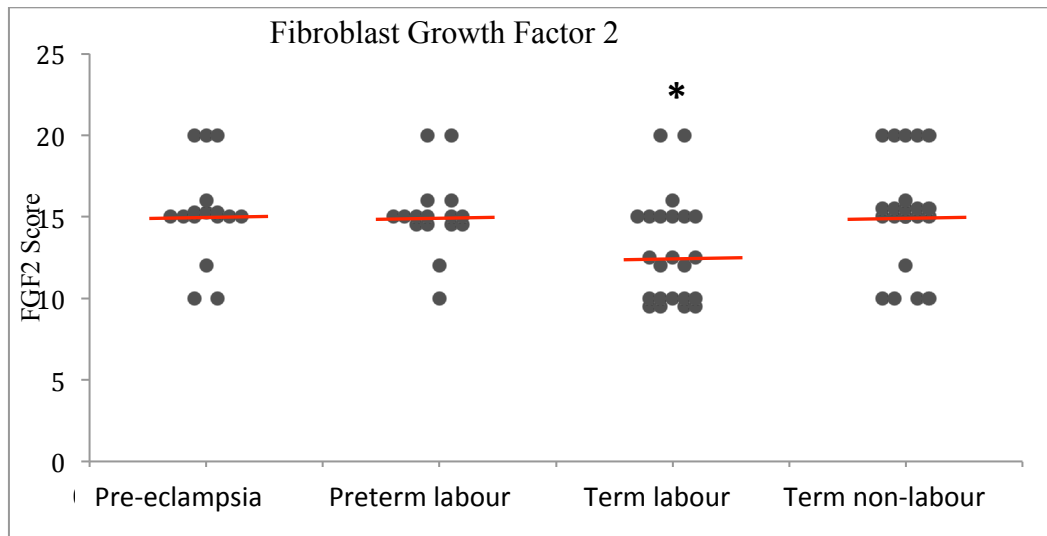


Figure 31D.

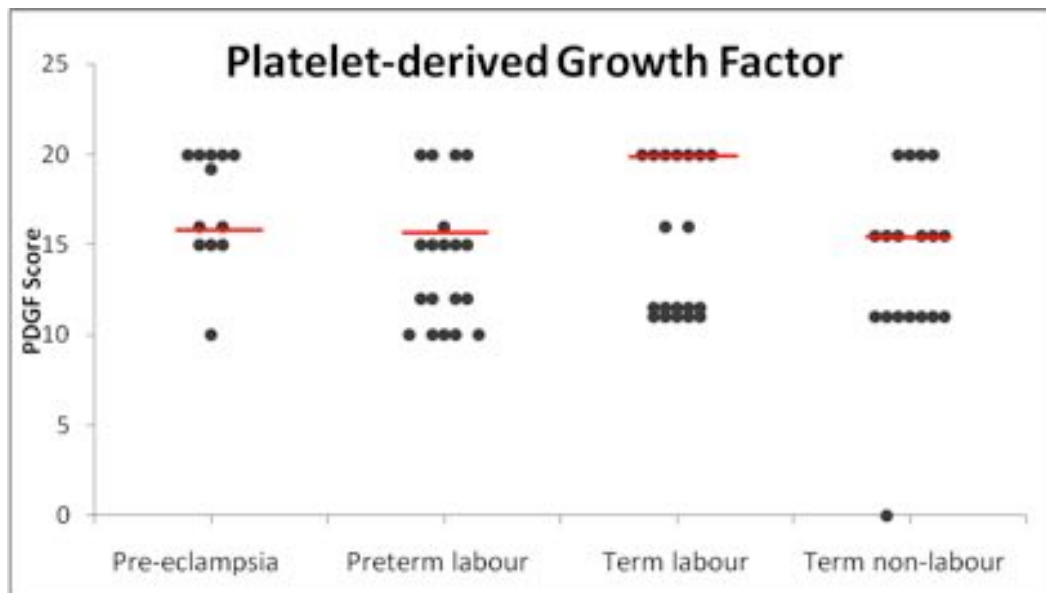


Figure 31E.

Figure 31D. represents the fibroblast growth factor 2 (FGF2) expression of staining score (intensity x coverage) in the four placenta groups studied. Values for each group as pre-eclampsia PE versus pre-term PTL, term labour TL, and term non-labour TNL (* $p < 0.05$ is considered statistically significant difference in staining in Term Labour) using Mann-Whitney U test. Figure 31E. represents the platelet-derived growth factor (PDGF) expression staining score in the four placenta groups studied. The horizontal red line indicates the median values.

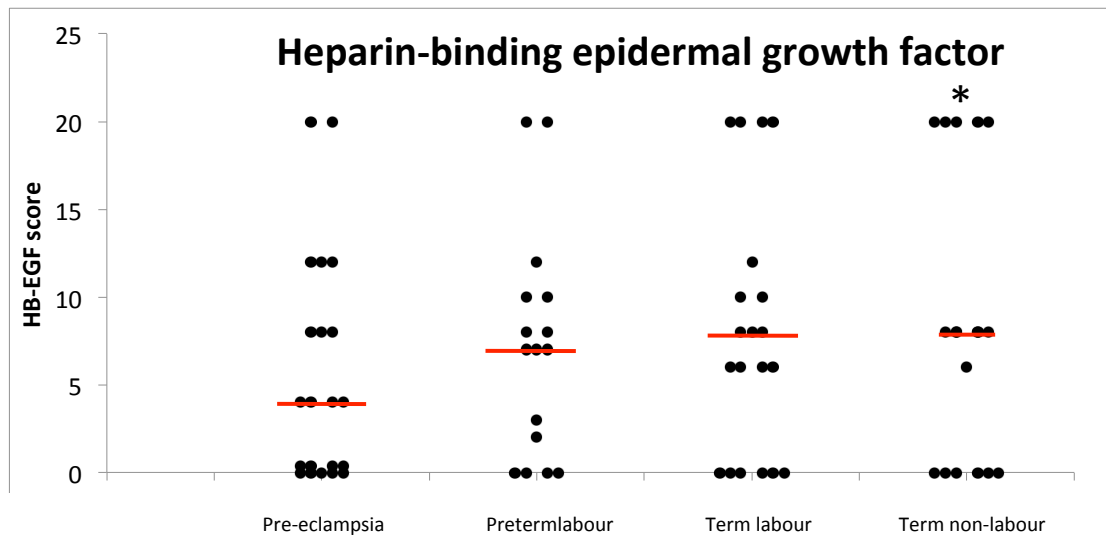


Figure 31F. The heparin-binding epidermal growth factor (HB-EGF) expression staining score (intensity x coverage) in four placenta groups studied. Values for each group as pre-eclampsia PE versus pre-term PTL, term labour TL, and term non-labour TNL (* $p < 0.05$ is considered statistically significant difference in staining in Term non-labour) using the Mann-Whitney U test. The horizontal red line indicates the median values.

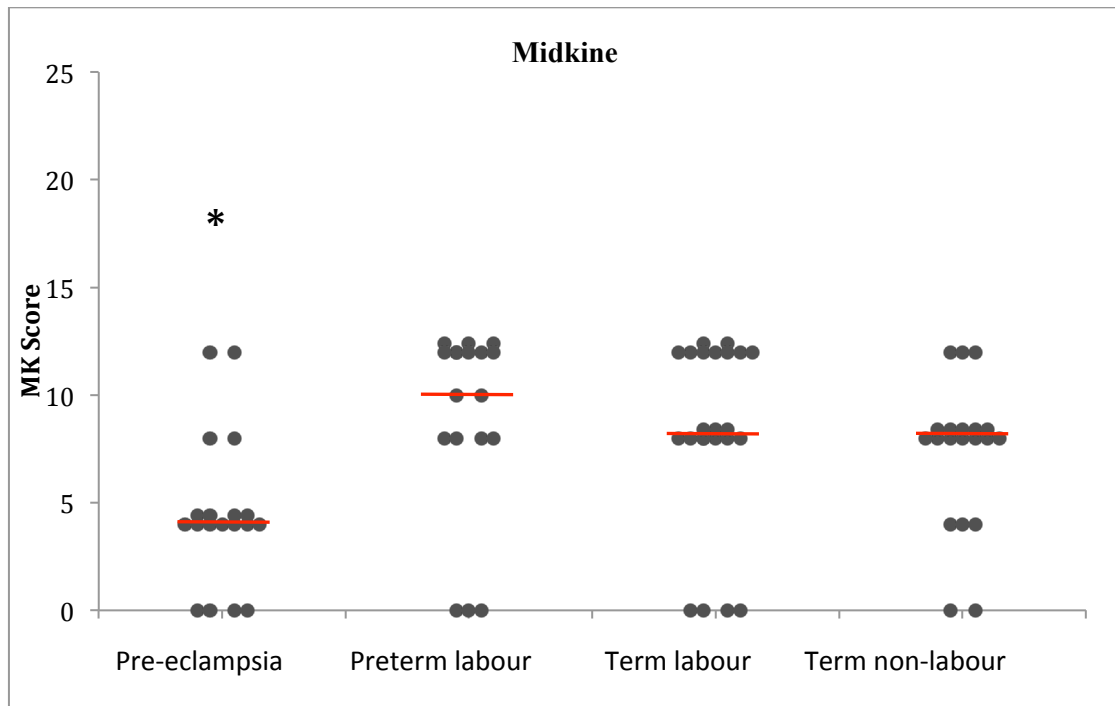


Figure 31G.

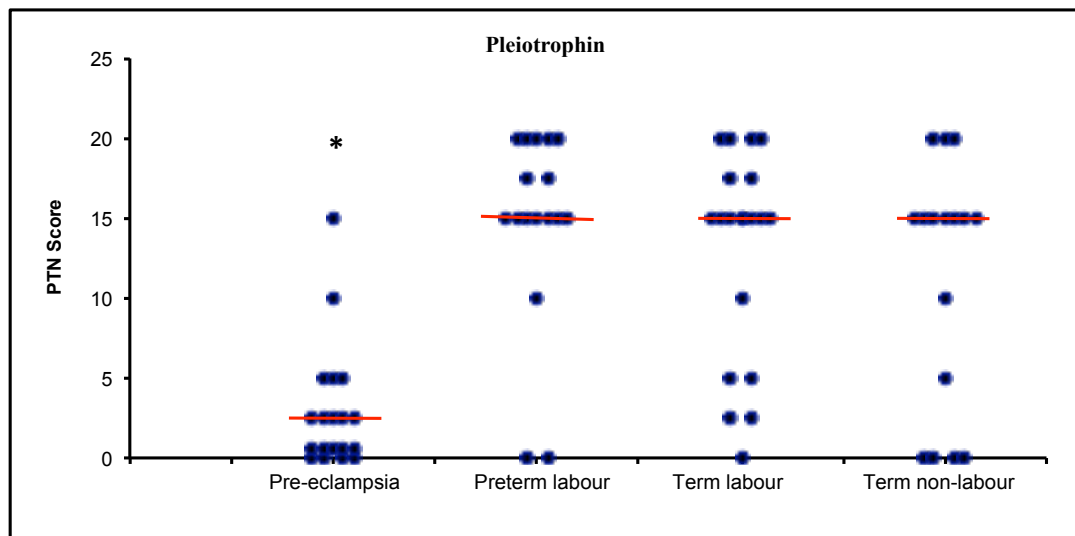


Figure 31H.

Figure 31G. represents the midkine (MK) expression staining score (intensity x coverage) in the four placenta groups studied. Values for each group as pre-eclampsia PE versus pre-term PTL, term labour TL, and term non-labour TNL (* $p < 0.05$ is considered statistically significant difference in staining in pre-eclampsia) using the Mann-Whitney U test. Figure 31H. represents the pleiotrophin (PTN) expression staining score values in the four placenta groups studied (* $p < 0.05$ is considered statistically significant difference in staining in pre-eclampsia using the Mann-Whitney U test). The horizontal red line indicates the median values.

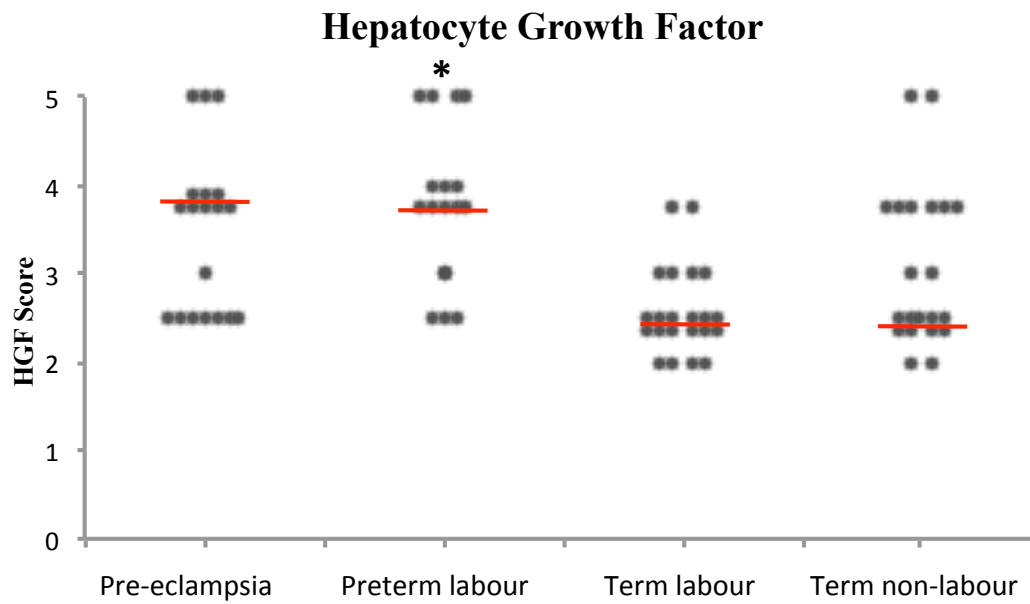


Figure 31I. represents the hepatocyte growth factor (HGF) expression staining score (intensity x coverage) in the four placenta groups studied. Values for each group as pre-eclampsia PE versus pre-term PTL, term labour TL, and term non-labour TNL (* $p < 0.05$ is considered statistically significant difference in staining in Pre-term Labour and Term Labour using the Mann-Whitney U test). The horizontal red line indicates the median values.

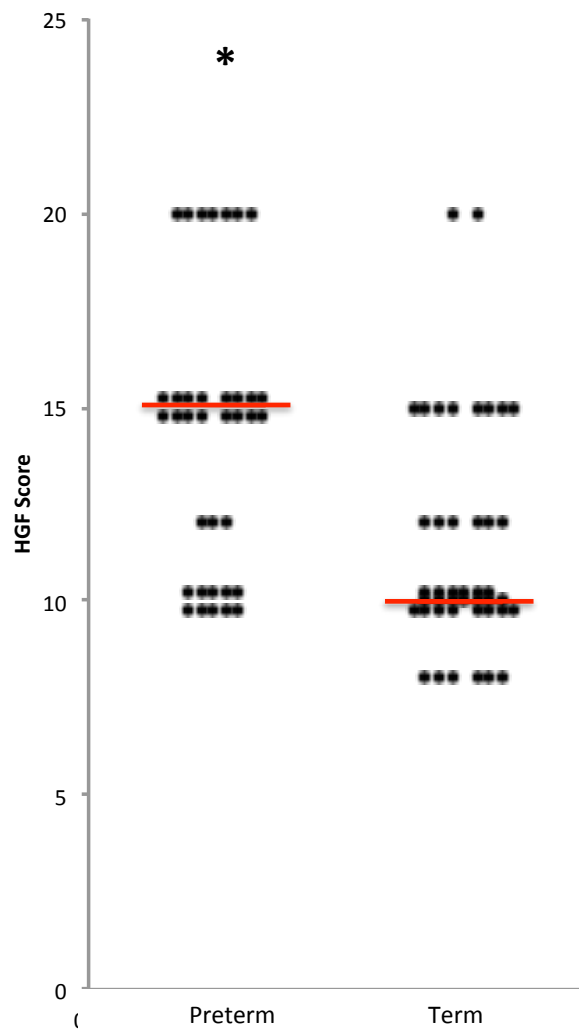


Figure 31K. represents the hepatocyte growth factor (HGF) expression staining score (intensity x coverage) in pre-term and term placenta groups studied (* $p < 0.05$ is considered statistically significant difference in staining in Pre-term) using the Mann-Whitney U test. The horizontal red line indicates the median values.

Correlation between growth factors

The Spearman rank correlation test was performed to investigate correlation relationships between the expression of variables that were non-grouped for the different growth factors VEGF, PLGF, FGF2, HGF, PDGF-BB, and CD105 (n = 87) and HB-EGF (n = 42), MK (n = 35) and PTN (n = 30) because data was not normally distributed as in the staining score table 8 and staining intensity table 9. The Rho represents Spearman's rank correlation coefficient. The p-value of < 0.05 and p-value of < 0.01 was considered statistically significant.

There was a significant positive correlation was shown between FGF2 and HGF expression (Rho = 0.35, p = 0.01**), HGF and PDGF expression (Rho = 0.43, p = 0.001) and for PLGF and CD105 expression (Rho = 0.31, p = 0.01**) shown in the staining score in table 8.

Whereas, all the other HBGFs expression showed a weak positive correlation between FGF2 and PDGF expression (Rho = 0.24, p = 0.08), HGF and VEGF expression (Rho = 0.06, p = 0.67), HGF and CD105 expression (Rho = 0.35, p = 0.79), HGF and PTN expression (Rho = 0.27, p = 0.86), VEGF and PLGF expression (Rho = 0.08, p = 0.48), VEGF and CD105 expression (Rho = 0.19, p = 0.10), VEGF and PTN expression (Rho = 0.39, p = 0.95), PLGF and VEGF expression (Rho = 0.08, p = 0.48), PLGF and MK expression (Rho = 0.06, p = 0.62), PLGF and PTN expression (Rho = 0.05, p = 0.58), PDGF and PTN expression (Rho = 0.22, p = 0.83), CD105 and MK expression (Rho = 0.41, p = 0.98), CD105 and PTN expression (Rho = 0.21, p = 0.19), MK and HBEGF expression (Rho = 0.18, p = 0.76) and PTN and HBEGF expression (Rho = 0.40, p = 0.92) respectively.

However, a negative correlation was observed for the reminder of HBGFs between staining score FGF2 and VEGF expression (Rho = -0.14, p = 0.27), FGF2 and PLGF expression (Rho = -0.04, p = 0.73), FGF2 and CD105 expression (Rho = -0.11, p = 0.41), FGF2 and MK expression (Rho = -0.25, p = 0.11), FGF2 and PTN expression (Rho = -0.01, p = 0.49), FGF2 and HBEGF expression (Rho = -0.32, p = 0.11), HGF and PLGF expression (Rho = -0.13, p = 0.35), HGF and MK expression (Rho = -0.08, p = 0.33), HGF and HBEGF expression (Rho = -0.36, p = 0.06), VEGF and PDGF expression (Rho = -0.01, p = 0.93), VEGF and MK expression (Rho = -0.19, p = 0.14), VEGF and HBEGF expression (Rho = -0.19, p = 0.19), PLGF and PDGF expression (Rho = -0.13, p = 0.36), PLGF and HBEGF expression (Rho = -0.07, p =

0.62), PDGF and CD105 expression (Rho = -0.22, p = 0.12), PDGF and MK expression (Rho = -0.05, p = 0.22), PDGF and HBEGF expression (Rho = -0.005, p = 0.50), CD105 and HBEGF expression (Rho = -0.12, p = 0.29) and MK and PTN expression (Rho = -0.06, p = 0.59) respectively as in table 8.

Growth Factors	Spearman's rank correlation coefficient Rho and p-values.								
	FGF2	HGF	PDGF	VEGF	PLGF	CD105	MK	PTN	HBEGF
FGF2		Rho=0.35	Rho=0.24	Rho=-0.14	Rho=-0.04	Rho=-0.11	Rho=-0.25	Rho=-0.01	Rho=-0.32
HGF	P=0.01**		Rho=0.43	Rho=0.06	Rho=-0.13	Rho=0.35	Rho=-0.08	Rho=0.27	Rho=-0.36
PDGF	P= 0.08	P=0.001**		Rho=-0.01	Rho=-0.13	Rho=-0.22	Rho=-0.05	Rho=0.22	Rho=-0.005
VEGF	P= 0.27	P= 0.67	P=0.93		Rho=0.08	Rho=0.19	Rho=-0.19	Rho=0.39	Rho=-0.19
PLGF	P= 0.73	P=0.35	P=0.36	P= 0.48		Rho=0.31	Rho=0.06	Rho=0.05	Rho=-0.07
CD105	P=0.41	P= 0.79	P=0.12	P=0.10	P=0.01**		Rho=0.41	Rho=0.21	Rho=-0.12
MK	P=0.11	P=0.33	P=0.22	P=0.14	P=0.62	P=0.98		Rho=-0.06	Rho=0.18
PTN	P= 0.49	P= 0.86	P=0.83	P=0.95	P=0.58	P=0.19	P=0.59		Rho=0.40
HBEGF	P= 0.11	P=0.06	P=0.50	P=0.19	P=0.62	P=0.29	P=0.76	P=0.92	

Table 8. Represents the correlation between expressions of staining score for Heparin-binding growth factors present in placental tissues. Abbreviations: Fibroblast growth factor 2 (FGF2), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), placental growth factor (PLGF), cluster differentiation (CD105) were of (n = 87), except for midkine (MK), (n = 35), pleiotrophin (PTN), (n = 30), and heparin binding epidermal growth factor (HBEGF), (n = 42), using the Spearman's rank correlation test. The Rho represents Spearman's rank correlation coefficient. The p-value of < 0.01** and p-value of < 0.001** is considered statistically significant.

Growth Factors	Spearman's rank correlation coefficient Rho and p-values.								
	FGF2	HGF	PDGF	VEGF	PLGF	CD105	MK	PTN	HBEGF
FGF2		Rho=0.34	Rho=0.28	Rho=0.19	Rho=-0.01	Rho=-0.16	Rho=-0.10	Rho=0.27	Rho=-0.31
HGF	P=0.01**		Rho=0.50	Rho=0.03*	Rho=-0.06	Rho=0.02*	Rho=-0.29	Rho=0.33	Rho=-0.28
PDGF	P=0.04*	P=0.0001**		Rho=0.02*	Rho=-0.07	Rho=-0.07	Rho=-0.04	Rho=0.20	Rho=-0.03
VEGF	P= 0.14	P= 0.57	P=0.57		Rho=0.08	Rho=0.15	Rho=0.35	Rho=0.21	Rho=-0.45
PLGF	P= 0.46	P=0.64	P=0.30	P= 0.75		Rho=0.26	Rho=-0.07	Rho=0.45	Rho=0.22
CD105	P=0.11	P= 0.57	P=0.32	P= 0.90	P= 0.98		Rho=0.36	Rho=0.17	Rho=-0.14
MK	P=0.28	P=0.06	P=0.40	P=0.97	P=0.34	P=0.98		Rho=0.22	Rho=0.29
PTN	P=0.84	P=0.90	P=0.76	P= 0.81	P=0.97	P= 0.75	P=0.16		Rho=-0.08
HBEGF	P= 0.11	P=0.12	P=0.46	P=0.02	P=0.83	P=0.27	P=0.82	P=0.40	

Table 9. Correlation between expression of staining intensity for Heparin-binding growth factors present in placental tissues. Abbreviations: Fibroblast growth factor 2 (FGF2), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), placental growth factor (PLGF), cluster differentiation (CD105) were of (n = 87), except for midkine (MK), (n = 35), pleiotrophin (PTN), (n = 30), and heparin binding epidermal growth factor (HBEGF), (n = 42), using the Spearman's rank correlation test. The Rho represents Spearman's rank correlation coefficient. The p-value of < 0.05* and p-value of < 0.01** and < 0.0001** is considered statistically significant.

In addition, there was a significant positive correlation between staining intensity as shown in table 9 for HGF and FGF2 expression (Rho = 0.35, p = 0.01**), PDGF versus FGF2 expression (Rho = 0.24, p = 0.04*), and PDGF versus HGF expression (Rho = 0.50, p = 0.0001**), HGF and CD105 expression (Rho = 0.02*, p = 0.57) and VEGF and PDGF expression (Rho = 0.02*, p = 0.57). Whereas, remainder of HBGFs expression showed a both weak positive correlation between staining intensity for

HGF and PTN expression (Rho = 0.33, p = 0.90), VEGF and PLGF expression (Rho = 0.08, p = 0.75), VEGF and CD105 expression (Rho = 0.15, p = 0.90), VEGF and MK expression (Rho = 0.35, p = 0.97), VEGF and PTN expression (Rho = 0.21, p = 0.81), PLGF and VEGF expression (Rho = 0.08, p = 0.75), PLGF and CD105 expression (Rho = 0.26, p = 0.98), PLGF and MK expression (Rho = 0.07, p = 0.34), PLGF and PTN expression (Rho = 0.45, p = 0.97), PLGF and HBEGF expression (Rho = 0.22, p = 0.83), PDGF and PTN expression (Rho = 0.20, p = 0.76), CD105 and MK expression (Rho = 0.36, p = 0.98), CD105 and PTN expression (Rho = 0.17, p = 0.75), MK and PTN expression (Rho = 0.22, p = 0.16) and MK and HBEGF expression (Rho = 0.29, p = 0.82) respectively.

However, a weak negative correlation between staining intensity for FGF2 and VEGF expression (Rho = 0.19, p = 0.14), FGF2 and PLGF expression (Rho = -0.01, p = 0.46), FGF2 and CD105 expression (Rho = -0.16, p = 0.11), FGF2 and MK expression (Rho = -0.10, p = 0.28), FGF2 and PTN expression (Rho = -0.27, p = 0.84), FGF2 and HBEGF expression (Rho = -0.31, p = 0.11), HGF and PLGF expression (Rho = -0.06, p = 0.64), HGF and MK expression (Rho = -0.29, p = 0.06), HGF and HBEGF expression (Rho = -0.28, p = 0.12), VEGF and HBEGF expression (Rho = -0.45, p = 0.02), PLGF and PDGF expression (Rho = -0.07, p = 0.30), PDGF and CD105 expression (Rho = -0.07, p = 0.32), PDGF and MK expression (Rho = -0.04, p = 0.40), PDGF and HBEGF expression (Rho = -0.03, p = 0.46), CD105 and HBEGF expression (Rho = -0.14, p = 0.27) and PTN and HBEGF expression (Rho = -0.08, p = 0.40) respectively.

Growth Factors (a)	Mann-Whitney <i>U</i> test			
	PE	PTL	TNL	TL
Median	15	15	15	12
FGF2	P=0.23	P=0.09	P=0.22	P=0.004*
HGF	15 P=0.27	15 P=0.01**	10 P=0.93	10 P=0.001**
VEGF	15 P=0.36	15 P=0.18	10 P=0.09	15 P=0.03*
PLGF	15 P=0.02*	10 P=0.41	10 P=0.28	10 P=0.21
MK	4 P=0.03*	10 P=0.43	8 P=0.25	8 P=0.26
PTN	3 P=0.04*	15 P=0.39	15 P=0.01**	15 P=0.09
HB-EGF	4 P=0.33	7 P=0.03	8 P=0.01**	8 P=0.16

Growth Factors (b)	Mann-Whitney <i>U</i> test			
	PE	PTL	TNL	TL
Median	16	16	20	15
PDGF	P=0.68	P=0.92	P=0.26	P=0.24
CD105	10 P=0.07	10 P=0.32	10 P=0.42	8 P=0.06

Table 10 a and b. Represents the comparison of expression in staining score for Heparin binding growth factors present in placental tissues using the Mann-Whitney *U* test. Abbreviations: Fibroblast growth factor 2 (FGF2), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), placental growth factor (PLGF) and cluster differentiation (CD105), midkine (MK), pleiotrophin (PTN), heparin binding epidermal growth factor (HB-EGF). PE- Pre-eclampsia, PTL-Pre-term Labour, TNL-Term-non-Labour, and TL-term labour. Comparisons between each group versus all other groups combined. The median is given above and p-values are shown. The p-value of < 0.05* and p-value of < 0.01** is considered statistically significant.

Table 10. a and b. represents the comparison of expression in staining score for (FGF2), pre-eclampsia, (m=15, p=0.23), pre-term labour, (m=15, p=0.09), term-non-labour, (m=15, p=0.22), and term labour (m=15, p=0.0004*), HGF for pre-eclampsia,(m=15, p=0.27), pre-term labour,(m=15, p=0.01*),term-non-labour,(m=10, p=0.93),and term labour (m=10, p=0.001**), PDGF for pre-eclampsia, (m=16, p=0.68), pre-term labour,(m=16, p= 0.92), term-non-labour, (m=20, p= 0.26), and term labour, (m=15, p=0.24), VEGF for pre-eclampsia,(m=15, p=0.02**), pre-term labour,(m=10, p= 0.41), term-non labour,(m=10, p=0.09), and term labour (m=15, p= 0.03*), PLGF for pre-eclampsia, (m=15, p=0.02*), pre-term labour,(m=10, p= 0.41), term-non-labour, (m=10, p=0.28), and term labour (m=10, p=0.21), and CD105 for pre-eclampsia,(m=10, p=0.07), pre-term labour,(m=10, p=0.32), term-non-labour, ,(m=10, p= 0.42), and term labour, (m=8, p=0.06), MK for pre-eclampsia,(m=4, p= 0.03*), pre-term labour,(m=10, p=0.43), term-non-labour, (m=8, p=0.25), and term labour (m=8, p=0.26), PTN for pre-eclampsia,(m=3, p=0.04), pre-term labour,(m=15, p=0.39), term-non--labour, (m=15, p=0.01**), and term labour (m=15, p= 0.09), HB-EGF for pre-eclampsia,(m=4, p= 0.33), pre-term labour,(m=7, p= 0.03), term-non-labour, (m=8, p=0.01*), and term labour (m=8, p=0.16) using the Mann-Whitney *U* test. The p-value of < 0.05* and p-value of < 0.01** is considered statistically significant.

Growth factors	Mann-Whitney <i>U</i> test			
	PE	PTL	TNL	TL
Median FGF2	3 P= 0.26	3 P= 0.24	3 P= 0.38	3 P= 0.02*
HGF	3 P= 0.22	3 P= 0.01**	2 P= 0.46	2 P= 0.002**
PDGF	4 P= 0.55	3.5 P= 0.56	4 P= 0.21	3 P= 0.20
VEGF	3 P= 0.34	3 P= 0.19	3 P= 0.09	3 P= 0.04*
PLGF	2 P= 0.02*	2 P= 0.40	2 P= 0.21	2 P= 0.14
CD105	2 P= 0.07	2 P= 0.11	2 P= 0.29	2 P= 0.02*
MK	2 P= 0.52	3 P= 0.44	2 P= 0.38	2.5 P= 0.42
PTN	2.5 P= 0.67	3 P= 0.29	3 P= 0.32	1.5 P= 0.07
HB-EGF	3 P= 0.30	2 P= 0.04*	4 P= 0.03	3 P= 0.42

Table 11. Represents the comparison of expression in staining intensity for Heparin binding growth factors present in placental tissues using the Mann-Whitney *U* test. Abbreviations: Fibroblast growth factor 2 (FGF2), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), placental growth factor (PLGF) and cluster differentiation (CD105), midkine (MK), pleiotrophin (PTN), heparin binding epidermal growth factor (HB-EGF). PE-Pre-eclampsia, PTL-Pre-term Labour, TNL-Term-non-Labour, and TL-term labour. Comparisons between each group versus all other groups combined. The median is given above and p-values are shown. The p-value of < 0.05* and p-value of < 0.01** is considered statistically significant.

Table 11. represents the comparison of expression in staining intensity for FGF2, pre-eclampsia, (m=3, p=0.26), pre-term labour, (m=3, p=0.24), term-non-labour, (m=3, p=0.38), and term labour (m=3, p=0.02*), HGF for pre-eclampsia, (m=3, p=0.22), pre-term labour, (m=3, p=0.01**), term-non-labour, (m=2, p=0.46), and term labour (m=2, p=0.002**), PDGF for pre-eclampsia, (m=4, p=0.55), pre-term labour, (m=3.5, p=0.56), term-non-labour, (m=4, p=0.21), and term labour (m=3, p=0.20), VEGF for pre-eclampsia, (m=3, p=0.34), pre-term labour, (m=3, p=0.19), term-non-labour, (m=3, p=0.09), and term labour (m=3, p=0.04*), PLGF for pre-eclampsia, (m=2, p=0.02*), pre-term labour, (m=2, p=0.40), term-non-labour, (m=2, p=0.21), and term labour (m=2, p=0.14), and CD105 for pre-eclampsia, (m=2, p=0.07), pre-term labour, (m=2, p=0.11), term-non-labour, (m=2, p=0.29), and term labour, (m=2, p=0.02*), MK for pre-eclampsia, (m=2, p=0.52), pre-term labour, (m=3, p=0.44), term-non-labour, (m=2, p=0.38), and term labour (m=2.5, p=0.42), PTN for pre-eclampsia, (m=2.5, p=0.67), pre-term labour, (m=3, p=0.29), term-non-labour, (m=3, p=0.32), and term labour, (m=1.5, p=0.07), HB-EGF for pre-eclampsia, (m=3, p=0.30), pre-term labour, (m=2, p=0.04*), term-non-labour, (m=4, p=0.03), and term labour (m=3, p=0.42) using the Mann-Whitney *U* test. The p-value of < 0.05* and p-value of < 0.01** is considered statistically significant.

Growth factors	Mann-Whitney <i>U</i> test	
	Labour versus Non-Labour	Term versus Pre-term
Median=M FGF2	M=15 M=15 P= 0.04*	M=15 M=15 P= 0.91
HGF	M=15 M=15 P= 0.02*	M=15 M=15 P= 0.005*
PDGF	M= 20 M=15 P= 0.25	16 P= 0.35
VEGF	M=15 M=15 P= 0.19	M=15 M=15 P= 0.33
PLGF	M=10 M=10 P= 0.03*	M=10 M=10 P= 0.05*
CD105	M=10 M=10 P= 0.98	M=10 M=10 P= 0.01**
MK	M=12 M=7 P= 0.20	M=10 M=8 P= 0.50
PTN	M=4 M=13.5 P= 0.04 *	M=10 M=5 P= 0.27
HB-EGF	M= 6 M=20 P= 0.03 *	M=7 M=20 P= 0.02*

Table 12. Represents the comparison of expression in staining score for Heparin binding growth factors present in placental tissues using the Mann-Whitney *U* test. Abbreviations: Fibroblast growth factor 2 (FGF2), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), placental growth factor (PLGF) and cluster differentiation (CD105), midkine (MK), pleiotrophin (PTN), heparin binding epidermal growth factor (HB-EGF). PE-Pre-eclampsia, PTL-Pre-term Labour, TNL-Term-non-Labour, and TL-term labour. Comparisons are labour (TL + PTL) versus non-labour (PE + TNL) and Term (TL + TNL) versus Pre-term (PE + PTL). The medians and p-values are shown. The p-value of < 0.05* and p-value of < 0.01** is considered statistically significant.

Table 12. represents comparison of labour (TL + PTL) versus non-labour (PE + TNL) and term versus pre-term (PE + PTL) in placental tissues and comparison of expression in staining score for FGF2, labour versus non-labour of (m=15, m=15, p=0,04*), and term versus pre-term of (m=15, m=15, p=0.91), HGF, labour versus non-labour of (m=15, m=15, p=0.02*), and term versus pre-term of (m=15, m=15, p=0.005*), PDGF, labour versus non-labour of (m=20, m=15, p=0.25), and term versus pre-term of (m=16, p=0.35), VEGF, labour versus non-labour of (m=20, m=15, p=0.19), and term versus pre-term of (m=15, m=15, p=0.33), PLGF, labour versus non-labour of (m=10, m=10, p=0.03*), and term versus pre-term of (m=10, m=10, p=0.05*), CD105, labour versus non-labour of (m=10, m=10, p=0.98), and term versus pre-term of (m=10, m=10, p=0.01**), MK, labour versus non-labour of (m=12, m=7, p=0.20), and term versus pre-term of (m=10, m=8, p=0.05), PTN, labour versus non-labour of (m=4, m=13.5, p=0.04*), and term versus pre-term of (m=10, m=5, p=0.27), HB-EGF, labour versus non-labour of (m=6, m=20, p=0.03*), and term versus pre-term of (m=7, m=20, p=0.02*) using the Mann-Whitney *U* test. The p-value of < 0.05* and p-value of < 0.01** is considered statistically significant.

Growth factors	Mann-Whitney <i>U</i> test	
	Labour versus Non-Labour	Term versus Pre-term
Median=M	M=3 M=3	M=3 M=3
FGF2	P= 0.11	P= 0.10
HGF	M=2 M=3 P= 0.70	M=2 M=3 P= 0.003*
PDGF	M=3 M=4 P= 0.18	M=4 M=4 P= 0.51
VEGF	M=3 M=3 P= 0.21	M=3 M=3 P= 0.36
PLGF	M=2 M=2 P= 0.02*	M=2 M=2 P= 0.56
CD105	M=2 M=2 P= 0.80	M=2 M=2 P= 0.99
MK	M=3 M=2 P= 0.33	M=2 M=2 P= 0.52
PTN	M=2 M=3 P= 0.16	M=3 M=3 P= 0.26
HB-EGF	M=2 M=4 P= 0.06	M=4 M=2 P= 0.022

Table 13. Represents the comparison of expression in staining intensity for Heparin binding growth factors present in placental tissues using the Mann-Whitney *U* test. Abbreviations: Fibroblast growth factor 2 (FGF2), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), placental growth factor (PLGF) and cluster differentiation (CD105), midkine (MK), pleiotrophin (PTN), heparin binding epidermal growth factor (HB-EGF). PE-Pre-eclampsia, PTL-Pre-term Labour, TNL-Term-non-Labour, and TL-term labour. Comparisons are labour (TL + PTL) versus non-labour (PE + TNL) and Term (TL + TNL) versus Pre-term (PE + PTL). The median is given above and p-values are shown. The p-value of < 0.05* and p-value of < 0.01** is considered statistically significant.

Table 13. represents comparison of labour (TL + PTL) versus non-labour (PE + TNL) and term versus pre-term (PE + PTL) in placental tissues and comparison of expression in staining score for FGF2, labour versus non-labour of (m=3, m=3, p=0.11), and term versus pre-term of (m=3, m=3, p=0.10), HGF, labour versus non-labour of (m=2, m=3, p=0.70), and term versus pre-term of (m=2, m=3, p=0.003*), PDGF, labour versus non-labour of (m=3, m=4, p=0.18), and term versus pre-term of (m=4, m=4, p=0.51), VEGF, labour versus non-labour of (m=3, m=3, p=0.21), and term versus pre-term of (m=3, m=3, p=0.36), PLGF, labour versus non-labour of (m=2, m=2, p=0.02*), and term versus pre-term of (m=2, m=2, p=0.56), CD105, labour versus non-labour of (m=2, m=2, p=0.80), and term versus pre-term of (m=2, m=2, p=0.99), MK, labour versus non-labour of (m=3, m=2, p=0.33), and term versus pre-term of (m=2, m=2, p=0.52), PTN, labour versus non-labour of (m=2, m=3, p=0.16), and term versus pre-term of (m=3, m=3, p=0.26), HB-EGF, labour versus non-labour of (m=2, m=4, p=0.06), and term versus pre-term of (m=4, m=2, p=0.22) using the Mann-Whitney *U* test. The p-value of < 0.05* and p-value of < 0.01** is considered statistically significant.

Growth factors	Kruskal Wallis test
FGF2	T = 7.28 P = 0.06
HGF	T = 14.26 P = 0.0001**
PDGF	T = 1.61 P = 0.66
VEGF	T = 5.55 P = 0.14
PLGF	T = 14.76 P = 0.0001*
CD105	T = 4.70 P = 0.19
MK	T = 10.97 P = 0.003*
PTN	T = 10.10 P = 0.004*
HB-EGF	T = 9.34 P = 0.05*

Table 14. Represents the comparison of expression in staining scoring results for each Heparin binding growth factors present in placental tissues. Abbreviations: Fibroblast growth factor 2 (FGF2), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), placental growth factor (PLGF) and cluster differentiation (CD105), midkine (MK), pleiotrophin (PTN), heparin binding epidermal growth factor (HB-EGF). Comparison here is between the four groups overall using the Kruskal Wallis analysis of variance. PE-Pre-eclampsia, PTL- Pre-term Labour, TNL-Term-non-Labour, and TL-term labour. The t values and p values are shown. The p-value of $< 0.05^*$ and p-value of $< 0.01^{**}$ is considered statistically significant.

Table 14. represents the comparison of expression in staining scoring and is between the four groups overall for FGF2 ($t=7.28$, $p=0.06$), HGF ($t=14.26$, $p=0.0001^{**}$), PDGF ($t=1.61$, $p=0.66$), VEGF ($t=5.55$, $p=0.14$), PLGF ($t=14.76$, $p=0.0001^{*}$), and CD105 ($t=4.70$, $p=0.19$), MK ($t=10.97$, $p=0.003^{*}$), PTN ($t=10.10$, $p=0.004^{*}$), HB-EGF ($t=9.34$, $p=0.05^{*}$) using the Kruskal Wallis analysis of variance test. The p-value of $< 0.05^{*}$ and p-value of $< 0.01^{**}$ is considered statistically significant.

Table 15, represents the comparison of expression in staining scoring and is between the four groups overall for FGF2 ($t=5.50$, $p=0.14$), HGF ($t=11.9$, $p=0.01^{*}$), PDGF ($t=1.83$, $p=0.61$), VEGF ($t=4.36$, $p=0.22$), PLGF ($t=1.41$, $p=0.001^{**}$), and CD105 ($t=6.42$, $p=0.09$), MK ($t=2.18$, $p=0.70$), PTN ($t=2.91$, $p=0.57$), HB-EGF ($t=2.91$, $p=0.57$) using the Kruskal Wallis analysis of variance test. The p-value of $< 0.05^{*}$ and p-value of $< 0.01^{**}$ is considered statistically significant.

Growth factors	Kruskal Wallis test
FGF2	T = 5.50 P = 0.14
HGF	T = 11.9 P = 0.01**
PDGF	T = 1.83 P = 0.61
VEGF	T = 4.36 P = 0.22
PLGF	T = 1.41 P = 0.001**
CD105	T = 6.42 P = 0.09
MK	T = 2.18 P = 0.70
PTN	T = 2.91 P = 0.57
HB-EGF	T = 2.91 P = 0.57

Table 15. Comparison expression of staining intensity results for each Heparin binding growth factors present in placental tissues. Abbreviations: Fibroblast growth factor 2 (FGF2), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), placental growth factor (PLGF) and cluster differentiation (CD105), midkine (MK), pleiotrophin (PTN), heparin binding epidermal growth factor (HB-EGF). Comparison here is between the four groups overall using the Kruskal Wallis analysis of variance. PE-Pre-eclampsia, PTL-Pre-term Labour, TNL-Term-non-Labour, and TL-term labour. The t values and p values are shown. The p-value of $< 0.05^*$ and p-value of $< 0.01^{**}$ is considered statistically significant.

3.2. Growth factor receptor localisation: for expression of VEGFR1, VEGFR2 and FGF2R2 receptor in the human placenta.

I identified VEGFR1, VEGFR2, and FGF2R2 receptor expression in the human placenta. The VEGFR1, (soluble Fms-like tyrosine kinase1 is called sFlt), VEGFR2 and FGF2 receptor study group comprised term placenta (after 36 weeks gestation) not in labour (n=22), preterm labour (before 36 weeks gestation, n=6), term following labour onset (n=21), and pre-eclampsia (after 20 weeks of gestation, n=12). Immunohistochemistry staining was performed and positive results obtained. The colour intensity and clarity of staining was observed in the photomicrographs as shown in Figure 32, 33, and 34. Characterization of FGF2R1, VEGFR1, and VEGFR2 receptors and its placental tissue distribution would facilitated the understanding of the role of FGF2, VEGF receptors present in the human placenta. The interactions of growth factors with specific, high affinity cognate receptors located in plasma membrane of target cells that triggers events inside the cells which eventually lead to DNA synthesis. The FGF2R-1, VEGF receptor-1 (VEGFR-1, FLt-1), and VEGF receptor-2 (VEGFR-2, Flk-1/KDR) consists of protein-tyrosine kinases. These participate in vasculogenesis and angiogenesis during placental development. As shown in Figure 32, 33 and 34, VEGFR-1 (sflt-1)/VEGFR-2 receptors expression for the four groups PE-Pre-eclampsia, TL-term labour, TNL-Term-non-Labour, and PTL-Pre-term Labour was distributed to the villous and extra villous trophoblast, villous mesenchyme, Hofbauer cells and maternal decidua cells. The receptor PLGF expression was abundant in villous trophoblast. FGFR-1 receptor expression was localised to the villous and extra villous trophoblast, villous mesenchyme.

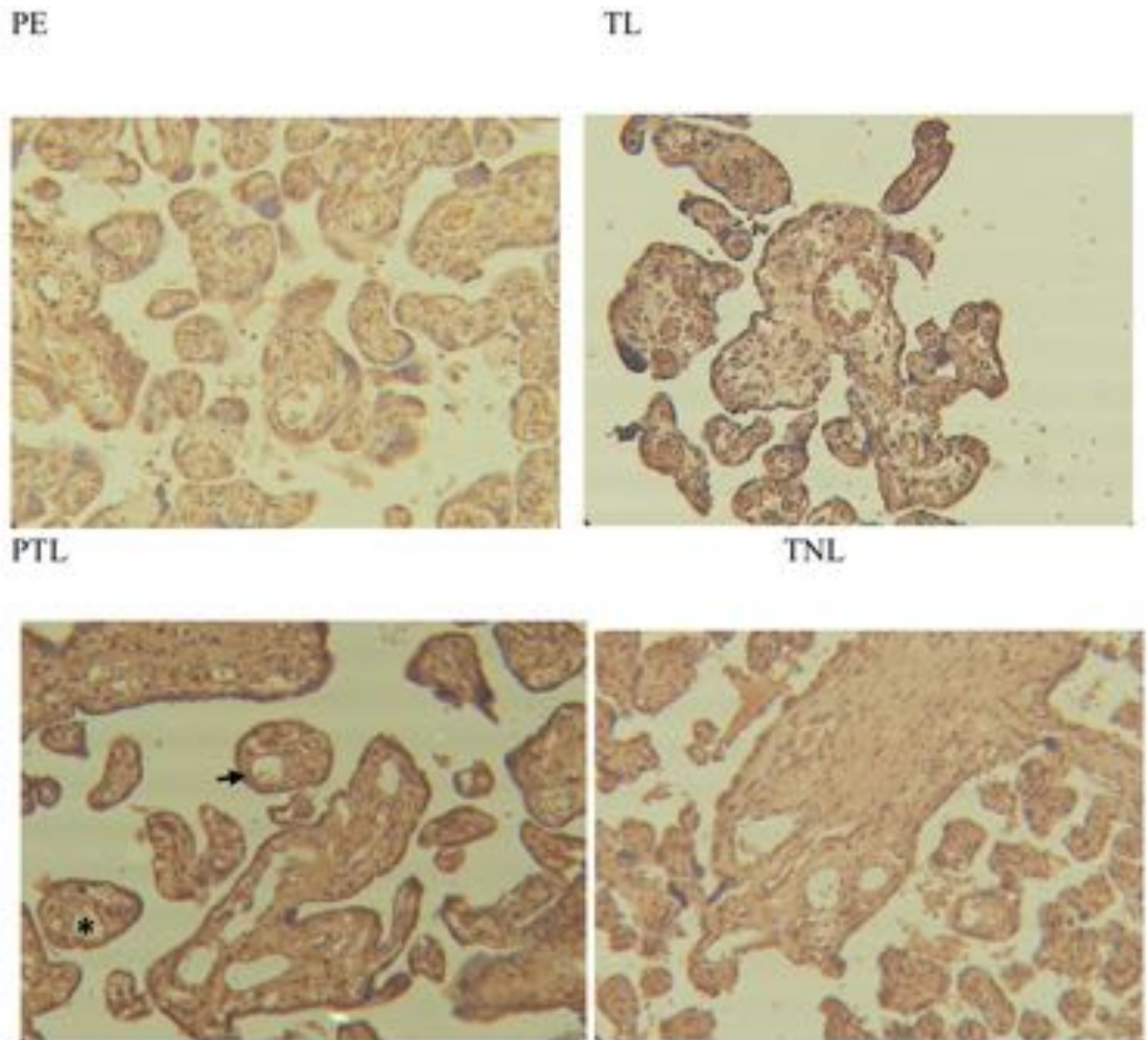
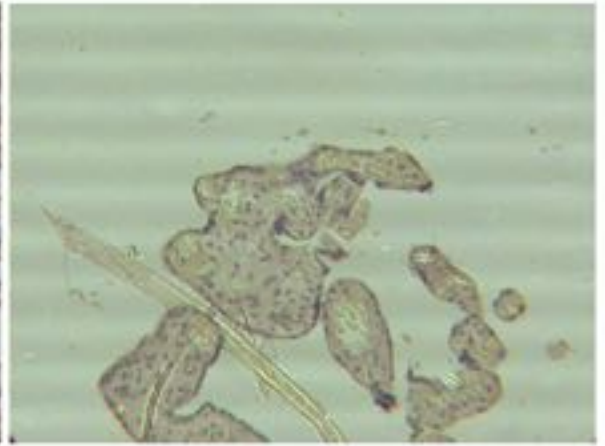
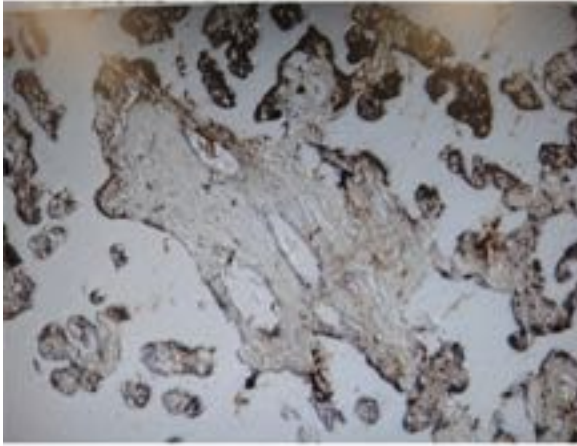


Figure 32. Immunohistochemical moderate to strong positive staining in villous trophoblast (both syncytiotrophoblast and cytotrophoblast), weaker staining in the mesenchyme, and in the core foetal vessels for fibroblast growth factor 2 (FGF2) receptor expression distributed in placental tissues for the four groups PE-Pre-eclampsia, (n = 12), and PTL-Pre-term Labour, (n = 6), TL-term labour, (n = 22), and TNL-Term-non- Labour, (n = 21), 3= moderate to strong with a magnification of x401. The arrows indicate the trophoblast and (*) the endothelial cells.

PE

TL



PTL

TNL

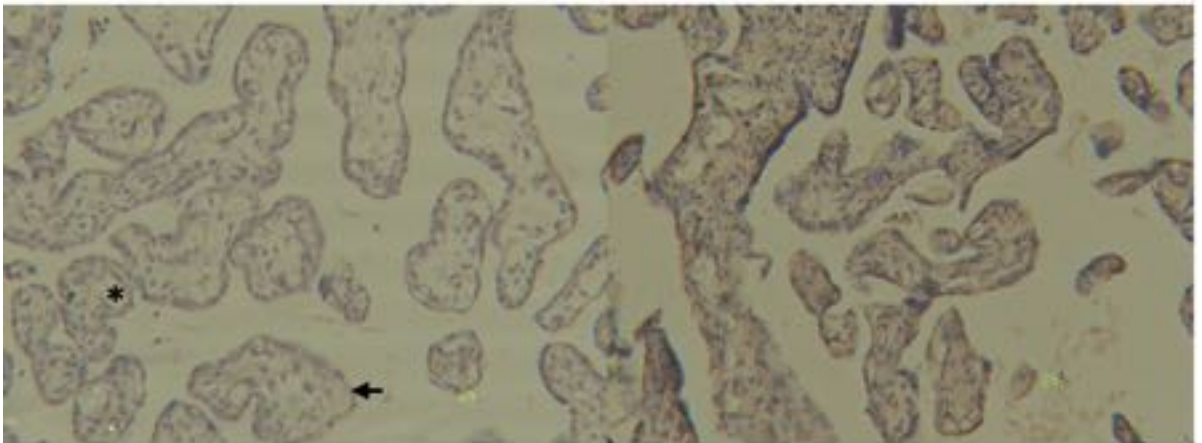
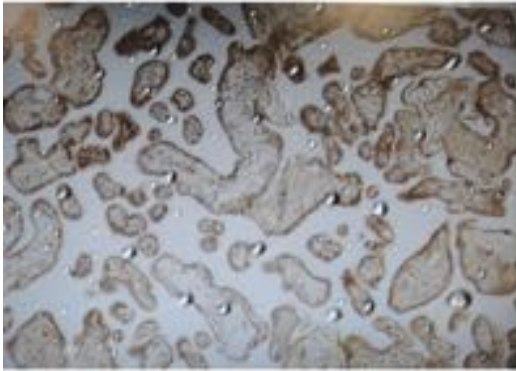
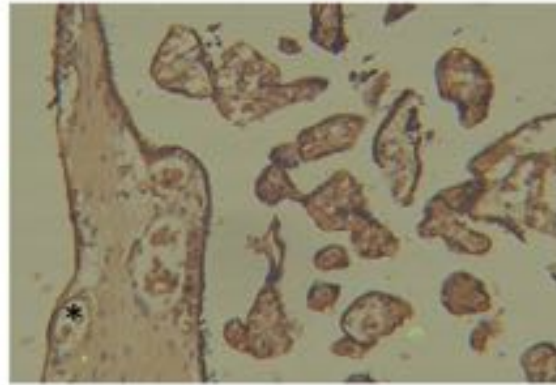


Figure 33. Immunohistochemical moderate to strong positive staining in villous trophoblast (both syncytiotrophoblast and cytotrophoblast), weaker staining in the mesenchyme, and in the core foetal vessels for vascular endothelial growth factor (VEGF) receptor I expression distributed in placental tissues for the four groups PE-Pre-eclampsia, (n = 12), and PTL-Pre-term Labour, (n = 6), TL-term labour, (n = 22), and TNL-Term-non- Labour, (n = 21), 3=moderate to strong with a magnification of original x401. The arrows indicate the trophoblast and (*) the endothelial cells.

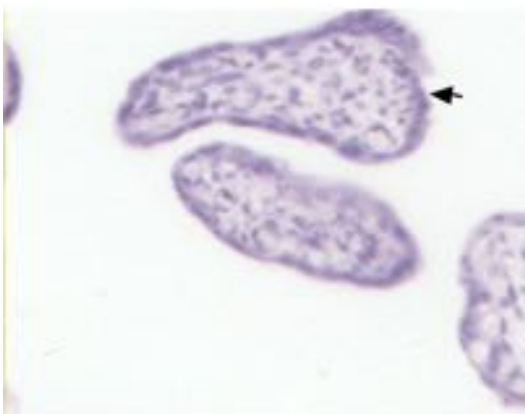
PE



TL



PTL



TNL

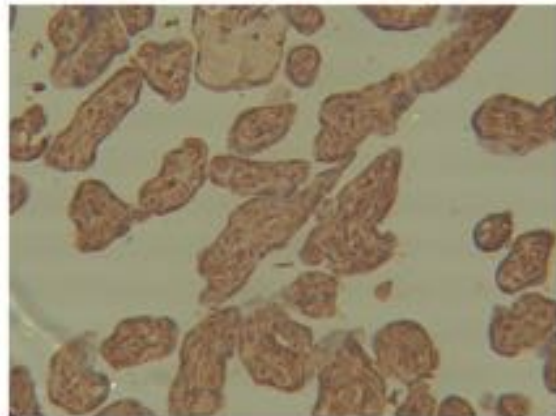


Figure 34. Immunohistochemical moderate to strong positive staining prominent in villous trophoblast (both syncytiotrophoblast and cytotrophoblast), weaker staining in the mesenchyme, and in the core foetal vessels for vascular endothelial growth factor (VEGF) receptor II expression in placental tissues for the four groups PE-Pre-eclampsia, (n = 12), and PTL-Pre-term Labour, (n = 6), TL-term labour, (n = 22), and TNL-Term-non- Labour, (n = 21), 3 = moderate to strong with a Magnification of x 401 The arrows indicate the trophoblast and (*) the endothelial cells.

Comparisons between four groups.

The groups were compared by analysing differences between normal pregnancy and pre-eclampsia. The results of FGF2R1 (n =24), VEGFR1 (n=20) and VEGFR2 (n=16) were examined.

Differences between groups with pre-eclampsia.

The staining score for the expression TL, TNL and PTL for VEGFR1 and VEGFR2, FGF2R1 was significantly lower in pre-eclamptic compared to TL, TNL and PTL other placentas in this study ($p < 0.05$, Mann-Whitney *U* test) as shown in table 18. In contrast, the expression of VEGFR2 as shown in table 16. and Figure 34. was significantly higher in pre-eclampsia compared to other placenta groups studied ($p < 0.05$) using the Mann-Whitney *U* test and Kruskal Wallis analysis of variance test. The VEGFR1 and VEGFR2, FGF2R1 investigated did not show a significant difference in staining in pre-eclampsia.

Comparison Term versus pre-term.

As shown in table 20. and Figure 34. the expression of staining score of VEGFR2 were significantly lower in (preterm, m=12) compared to (term, m=15) placentas using the Mann-Whitney *U* test, $p = 0.06$. In Figure 33. the expression of staining score of VEGFR1 showed no difference between (term, m=10) and (preterm, m=10) placentas giving the Mann-Whitney *U* test, $p = 0.37$. In table 20 the placental expression of FGF2R1 was similar in (pre-term, m=12) versus (term, m=12) placentas, as judged by obtaining a $p = 0.38$ compared to (labour, m=12) and (non-labour, m=13.5) obtaining a $p = 0.53$ by the Mann-Whitney *U* test respectively. The expression of staining score was not significantly different in term compared to preterm according to gestational age in any of the following tested FGF2R1, VEGFR1 and VEGFR2.

Comparison Labour versus non-labour

The expression of staining score of FGF2R2 showed no significance difference in term labour (m=12) compared to other groups using the (Mann-Whitney *U* test, $p = 0.53$) in table 20. The VEGFR1 expression of staining score showed no significance difference after labour onset using the Mann-Whitney *U* test $p = 0.16$. The comparison of staining score expression was not associated with significant difference in VEGFR1 and VEGFR2 expression as the Mann-Whitney *U* test, (m=10, $p = 0.16$) and (m=10, $p = 0.37$). The comparison of staining intensity for labour and non-labour did not reveal a significant difference in VEGFR1 and VEGFR2 expression of (m=2, $p = 0.58$) and (m=2, $p = 0.27$) using the Mann-Whitney *U* test. VEGFR1 and VEGFR2 expression did not reveal a significant difference in pre-term labour compared to term (m=2, $p = 0.41$ and m=4, $p = 0.18$). The VEGFR1, VEGFR2 and FGF2R2 showed no significant difference in expression according to the presence or absence of labour.

Growth Factor Receptors	Mann-Whitney <i>U</i> test P= value; Median= value			
	PE	PTL	TNL	TL
FGF2 R-1	M= 12 P= 0.57	M=12.5 P=0.57	M= 15 P=0.40	M=21 P= 0.48
VEGF R-1	M= 10 P=0.57	M=10 P=0.57	M=10 P=0.52	M= 10 P= 0.29
VEGFR-2	M=13.5 P=0.33	M=13.5 P=0.33	M=15 P=0.21	M=15 P=0.36

Table 16. Comparison for expression of staining score for fibroblast growth factor 2 receptor (FGF2R-1), vascular endothelial growth factor (VEGFR-1, and VEGFR 2) receptors in placental tissues using the Mann-Whitney *U* test. PE-Pre-eclampsia, (n = 12), PTL-Pre-term Labour, (n = 6), TNL-Term-non-Labour, (n = 21), and TL-term labour, (n = 22). Comparisons between each group versus all other groups combined. The medians and p values are shown.

Table 16. represents the comparison for expression of staining score for FGF2 R-1, pre-eclampsia (m = 12, p = 0.57); pre-term Labour (m = 12.5, p = 0.57), term-non-Labour (m = 15, p=0.40), and term labour (m= 21, p = 0.48). VEGFR-1, for pre-eclampsia (m = 10, p = 0.57), pre-term Labour (m = 10, p = 0.57), term-non-Labour (m = 10, p = 0.52), and term labour (m = 10, p = 0.29). VEGFR-2, for pre-eclampsia (m = 13.5, p = 0.33), pre-term Labour (m = 13.5, p = 0.33), term-non-Labour (m = 15, p = 0.21), and term labour (m = 15, p = 0.36).

Growth Factor Receptors	Mann-Whitney <i>U</i> test P= value; Median= value			
	PE	PTL	TNL	TL
FGF2 R-1	M= 3 P= 0.48	M= 3 P=0.48	M= 3 P= 0.52	M= 3 P= 0.04*
VEGF R-1	M= 2 P= 2.00	M= 2 P= 0.57	M= 2 P= 2.00	M= 2 P=2.00
VEGFR-2	M= 4 P= 0.33	M=4 P= 0.33	M= 4 P=1.00	M=4 P= 0.78

Table 17. Comparison for expression of staining intensity for fibroblast growth factor receptor 2 (FGF2R-2), vascular endothelial growth factor (VEGFR-1, and VEGFR 2) receptors in placental tissues using the Mann-Whitney *U* test. PE-Pre-eclampsia, (n = 12), PTL-Pre-term Labour, (n = 6), TNL-Term-non-Labour, (n = 21), and TL-term labour, (n = 22). Comparisons between each group versus all other groups combined. The medians and p values are shown.

Table 17. represents the comparison for expression of staining intensity for FGF2 R-1, for pre-eclampsia (m = 3, p = 0.48); pre-term Labour (m = 3, p = 0.48), term-non-Labour (m = 3, p = 0.52), and term labour (m = 3, p = 0.04*). VEGFR-1, for pre-eclampsia (m = 2, p = 2.00), pre-term Labour (m = 2, p = 0.57), term-non-Labour (m = 2, p = 2.00), and term labour (m = 2, p = 2.00). VEGFR-2, for pre-eclampsia (m = 4, p = 0.33), pre-term Labour (m = 4, p = 0.33), term-non-Labour (m = 4, p = 1.00), and term labour (m = 4, p = 1.00) using the Mann-Whitney *U* test.

Growth factors	Mann-Whitney <i>U</i> test	
	Labour and non-labour P= value; median= value	Term and Pre-term P= value; median= value
FGF2 R-1	M=12 M=13.5 P= 0.53	M=12 M=12 P= 0.38
VEGF R-1	M=10 M=10 P=0.16	M=10 M=10 P= 0.37
VEGF R-2	M=15 M=15 P= 0.27	M=15 M=12 P=0.06

Table 18. Comparison for expression of staining score for fibroblast growth factor 2 receptor (FGF2R-1), vascular endothelial growth factor (VEGFR-1, and VEGFR 2) receptors present in placental tissues using the Mann-Whitney *U* test. PE-Pre-eclampsia, (n = 12), PTL-Pre-term Labour, (n = 6), TNL-Term-non-Labour, (n = 21), and TL-term labour, (n = 22). Comparisons are labour (TL + PTL) versus non-labour (PE + TNL) and Term (TL + TNL) versus Pre-term (PE + PTL). The medians and p-values are shown.

Table 18. represents the comparison for expression of staining score for FGF2 R-1, for labour (TL + PTL) versus non-labour (PE + TNL), (m = 12, m = 13.5, p = 0.53), term (TL + TNL) versus pre-term (PE + PTL), (m = 12, m = 12, p = 0.38). VEGFR-1, for labour (TL + PTL) versus non-labour (PE + TNL), (m = 10, m = 10, p = 0.16), and term (TL + TNL) versus pre-term (PE + PTL), (m = 10, m = 10, p = 0.37). VEGFR-2, of labour (TL + PTL) versus non-labour (PE + TNL), (m = 15, m = 15, p = 0.27), and term (TL + TNL) versus pre-term (PE + PTL), (m = 15, m = 12, p = 0.06) using the Mann-Whitney *U* test.

Growth Factor Receptor	Mann-Whitney <i>U</i> test	
	Labour and non-labour P= value; median= value	Term and Pre-term P= value; median= value
FGF2 R-1	M=3 M=3 P= 0.91	M=3 M=3 P= 0.09
VEGF R-1	M=2 M=2 P= 0.58	M=2 M=2 P= 0.41
VEGF R-2	M=4 M=4 P= 0.27	M=4 M=4 P=0.18

Table 19. Comparison for expression of staining intensity for fibroblast growth factor 2 receptor (FGF2R-1), vascular endothelial growth factor (VEGFR-1, and VEGFR 2) receptors present in placental tissues using the Mann-Whitney *U* test. PE-Pre-eclampsia, (n = 12), PTL-Pre-term Labour, (n = 6), TNL-Term-non-Labour, (n = 21), and TL-term labour, (n = 22). Comparisons are labour (TL + PTL) versus non-labour (PE + TNL) and Term (TL + TNL) versus Pre-term (PE + PTL). The medians and p-values are shown.

Table 19. represents the comparison for expression of staining intensity for FGF2R-1, for labour (TL + PTL) versus non-labour (PE + TNL), (m = 3, m = 3, p = 0.91), term (TL + TNL) versus pre-term (PE + PTL), (m = 3, m = 3, p = 0.09). VEGFR-1, for labour (TL + PTL) versus non-labour (PE + TNL), (m = 2, m = 2, p = 0.58), term (TL + TNL) versus pre-term (PE + PTL), (m = 2, m = 2, p = 0.41). VEGFR-2, for labour (TL + PTL) versus non-labour (PE + TNL), (m = 4, m = 4, p = 0.27), term (TL + TNL) versus pre-term (PE + PTL), (m = 4, m = 4, p = 0.18) using the Mann-Whitney *U* test.

Growth factors receptor	Kruskal-Wallis test
FGF2 R-1	T = 0.14 P = 0.99
VEGF R-1	T = 1.07 P = 0.78
VEGF R-2	T = 3.75 P = 0.29

Table 20. Comparison for expression of staining scoring results for fibroblast growth factor 2 receptor (FGF2R-1), vascular endothelial growth factor (VEGFR-1, and VEGFR 2) receptors present in placental tissues. Comparison here is between the four groups overall using the Kruskal Wallis analysis of variance. PE-Pre-eclampsia, (n = 12), PTL-Pre-term Labour, (n = 6), TNL-Term-non-Labour, (n = 21), and TL-term labour, (n = 22). The t values and p values are shown.

Table 20. represents the comparison for expression of staining scoring results for FGF2R-1, (t = 0.14, p= 0.99), VEGFR-1, for (t = 1.07, p= 0.78) and VEGFR-2, for (t= 3.75, p= 0.29) using Kruskal Wallis analysis of variance test.

Growth factor receptors	Kruskal-Wallis test
FGF2 R-1	T = 0.94 P = 0.82
VEGF R-1	T = 0.46 P = 0.93
VEGF R-2	T = 3.42 P = 0.33

Table 21. Comparison for expression of staining intensity results for fibroblast growth factor 2 receptor (FGF2R-1), vascular endothelial growth factor (VEGFR-1, and VEGFR 2) receptors present in placental tissues. Comparison here is between the four groups overall using the Kruskal Wallis analysis of variance. PE-Pre-eclampsia, (n = 12), PTL-Pre-term Labour, (n = 6), TNL-Term-non-Labour, (n = 21), and TL-term labour, (n = 22). The t values and p values are shown.

Table 21. represents the comparison for expression of staining scoring results for FGF2R-1, of (t = 0.94, p = 82), VEGFR-1, of (t = 46, p = 93), and VEGFR-2, of (t = 3.42, p = 0.33) using Kruskal Wallis analysis of variance test.

Correlation between growth factors receptors

The Spearman rank correlation test was used to investigate relationship between the expression of the different VEGFR1, VEGFR2 and FGF2R2 because data was not normally distributed. FGF2R2 and VEGFR1 expression (Rho = 0.26, p = 0.13) and FGF2R2 and VEGFR2 (Rho = 0.14, p = 0.30) respectively showed a positive correlation between staining score as in table 23. There was a negative correlation between the staining score for VEGFR1 and VEGFR2 expression (Rho = 0.21, p = 0.22).

In addition there was a positive correlation between FGFR2 staining intensity and VEGFR2 (Rho = 0.08, p = 0.38) expression and for VEGFR1 and VEGFR2 expression (Rho = 0.43, p = 0.07) respectively as shown in table 23. FGFR2 and VEGFR1 (Rho = -0.38, p = 0.05) expression showed a negative correlation between staining intensity. There was no significant correlation of the coefficient between staining intensity for VEGFR1, VEGFR2 and FGF2R2 expression.

Growth Factor Receptors	Spearman's rank correlation coefficient Rho and p-values		
	FGF2R-1	VEGFR=1	VEGFR=2
FGF2R-1		Rho =0.26	Rho = 0.14
VEGFR=1	P= 0.13		Rho = 0.21
VEGFR=2	P= 0.30	P= 0.22	

Table 22. Correlation between expression of staining score for fibroblast growth factor 2 receptor (FGF2R-1), vascular endothelial growth factor (VEGFR-1 and VEGFR-2) receptors in placental tissues. PE-Pre-eclampsia, (n = 12), PTL-Pre-term Labour, (n = 6), TNL-Term-non-Labour, (n = 21), and TL-term labour, (n = 22). Using the Spearman's rank correlation test. The Rho represents Spearman's rank correlation coefficient.

Table 22. represents the correlation between for expression of staining score for FGF2 R-1 and VEGFR-1, of (Rho = 0.26, p = 0.13), FGF2R-1 and VEGFR-2 of (Rho = 0.14, p = 0.30), and VEGFR-1 and VEGFR-2, for (Rho = 0.21, p = 0.22) using the Spearman's rank correlation test.

Growth factors Receptors	Spearman's rank correlation coefficient Rho and p-values		
	FGF2	VEGFR=1	VEGFR=2
FGF2 R-1		Rho =-0.38	Rho = 0.08
VEGFR=1	P=0.05		Rho = 0.43
VEGFR=2	P= 0.38	P= 0.07	

Table 23. Correlation between expressions of staining intensity for fibroblast growth factor 2 (FGF2), vascular endothelial growth factor (VEGFR-1 and VEGFR-2) receptors present in placental tissues. PE-Pre-eclampsia, (n = 12), PTL-Pre-term Labour, (n = 6), TNL-Term-non-Labour, (n = 21), and TL-term labour, (n = 22). Using the Spearman's rank correlation test. The Rho represents Spearman's rank correlation coefficient.

Table 23. represents the correlation between for expression of staining intensity for FGF2R-1 and VEGFR-1, of (Rho = -0.38, p = 0.05), FGF2R-1 and VEGFR-2 of (Rho = 0.08, p = 0.38), and VEGFR-1 and VEGFR-2, of (Rho = 0.43, p = 0.07) using the Spearman's rank correlation test.

Chapter 4

Invasion

4.1. Cell Invasion Assay: standard curve calibration.

Cell invasion and migration are important events in placental development. An *in vitro* model, applying Matrigel is representative of *in vivo* invasion. This cell invasion assay study showed that invasive cells can penetrate a barrier consisting of basement membrane components in response to chemoattractant. Invasion and migration is fundamental for functions in cellular processes such as angiogenesis, embryonic development, immune response, metastasis and invasion of cancer cells. This second technique, I succeeded in performing the experiment for standard curve calibration in a 96-wells invasion assay. These results used different cell types such as the Human fibrosarcoma HT1080 invasive cells (positive control), and human villous trophoblast cell-line TCL-1 were reproduced in 4 further experiments. Cell invasion was detected and was quantified using Calcein-acetomethylester (AM). This Calcein-AM moiety generated free calcein. Internalized by the cells and intracellular esterases cleaved the acetomethylester. It's the free calcein that fluoresces brightly. Its from this fluoresces that quantitate these number of cells that have migrated and invaded using standard curve. The fluorescence of each well was read on Fluorometer in a fluorescence plate reader with a 492nm excitation, and 520nm emission settings. The fluorescence measurement was performed at relative fluorescence units (RFU) of 1000 gain and 2000 gain that were converted to number of cells as shown in Figure 35A and B. A standard curve was plotted and the derived equation used to convert fluorescence to cell number.

Invasion studies Protocol A

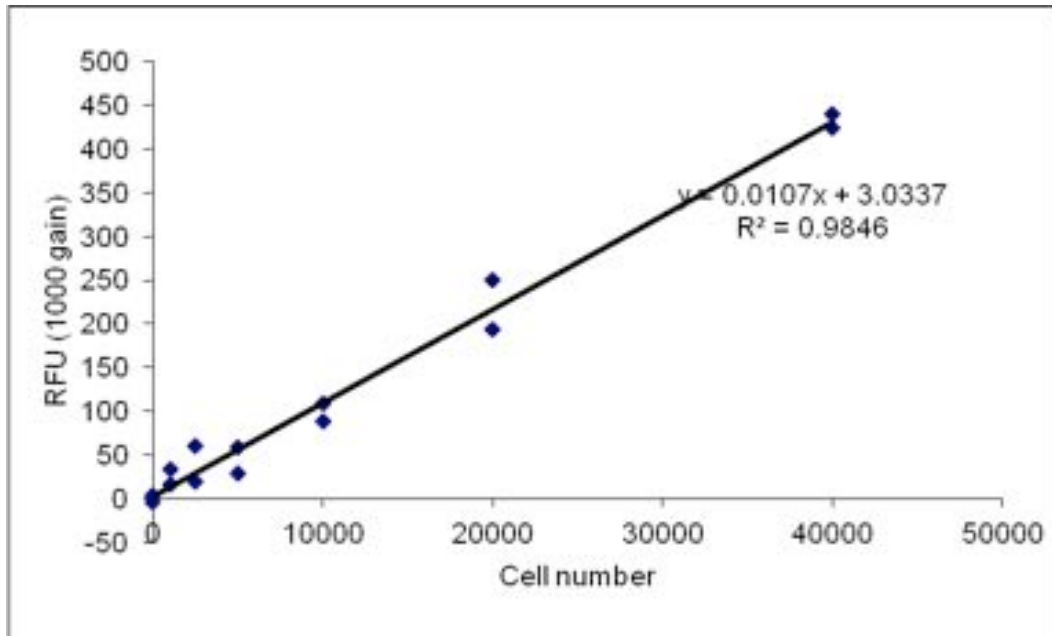


Figure 35A. Represents the % of assumed invasion for the following cell line human trophoblast TCL-1 with the relative fluorescent unit (RFU) at 1000 gain.

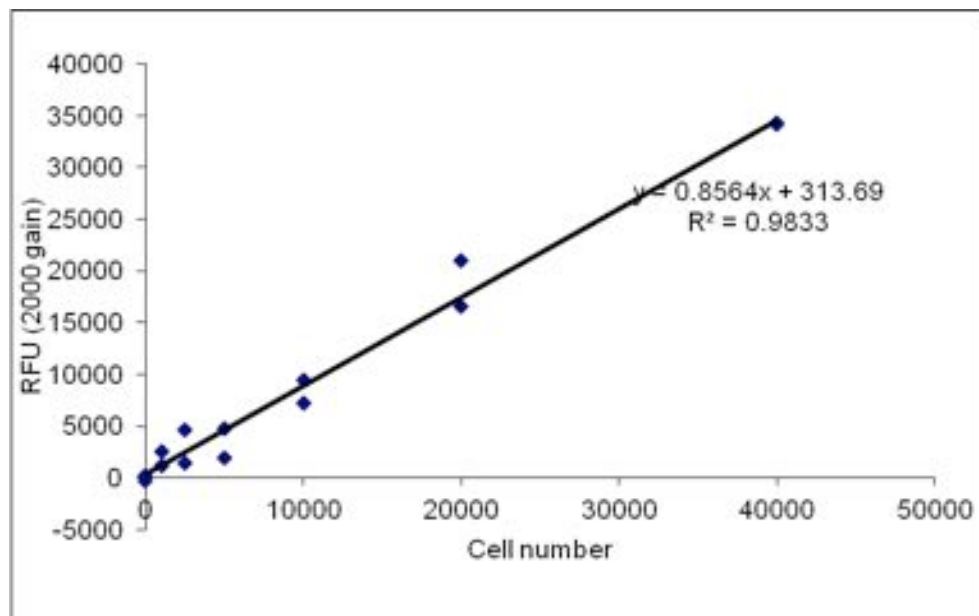


Figure 35B. Represents the % of assumed invasion for the following cell line human trophoblast TCL-1 with the relative fluorescent unit (RFU) at 2000 gain.

4.2. Invasion and Migration studies.

The role of these heparin-binding growth factors on trophoblast invasion was determined using *in vitro* studies on immortalized cultured human villous trophoblast cell-line TCL-1. I performed the villous trophoblast invasion and migration assay with all the cells raised and developed cell culture for Human fibrosarcoma HT1080 invasive cells (positive control) and NIH 3T3 mouse embryonic fibroblast non-invasive cells (negative control). The cell invasion assay using BD Falcon FluoroBlok were the upper surface of the insert membrane is coated with a uniform layer of dried basement membrane matrix solution. The basement membrane layer serves as a barrier for invasive cells from non-invasive cells. Invasive cells are able to degrade the matrix protein in the layer, and pass through pores of the polycarbonate membrane. However, the migration assay principle is different because it has the uncoated membrane that serves as a barrier for migratory cells from non-migratory cells. Migratory cells are able to extend protrusions towards chemoattractants and pass through pores of the polycarbonate membrane. Finally, cells are removed from the top of the membrane and the invaded and migratory cells are stained and quantified. Detection of cell invasion is quantified using Calcein AM. Using this invasion assay the validated controls are shown in Figure 36, which represents the percentage of invasion of cells for HT1080 ($83.2\% \pm 1.28$, $p < 0.05$), NIH 3T3 ($27.8\% \pm 2.64$, $p < 0.05$) and invasive cell-line TCL-1 ($61.6\% \pm 1.25$, $p < 0.05$). Both the 3T3/NIH and TCL-1 were significantly different from those for HT1080 ($p < 0.05$ versus HT1080 and 3T3).

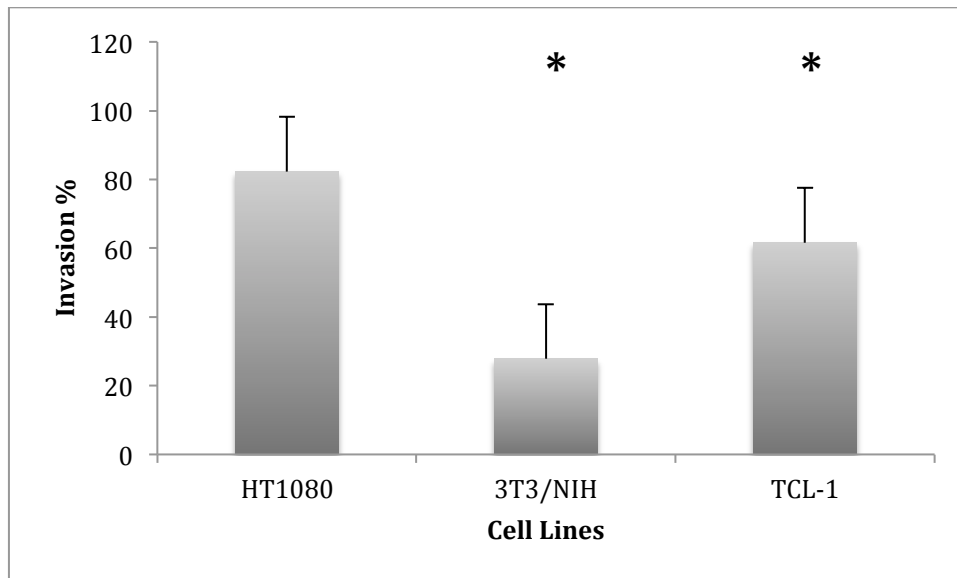


Figure 36. Represents the percentage of invasion presented as mean \pm SEM for the following cell lines HT 1080 (+ve control) of (n = 3, 83.2% \pm 1.28%, $p < 0.05$), 3T3/NIH (-ve control) (n = 3, 27.8% \pm 2.64%, $p < 0.05$), and human trophoblast TCL-1 cell line at (n = 3, 61.6% \pm 1.25%, $p < 0.05$) 0nmol/l (* $p < 0.05$ vs. HT1080 and 3T3/NIH, Mann-Whitney *U* test).

The invasion assay in Figure 36. for the following cell lines HT 1080 (+ve control) of (n=3, 83.2% \pm 1.28%, $p < 0.05$), 3T3/NIH (-ve control) (n=3, 27.8% \pm 2.64%, $p < 0.05$), and human trophoblast TCL-1 cell line at (n=3, 61.6% \pm 1.25%, $p < 0.05$) 0nmol/l (* $p < 0.05$ vs. HT1080 and 3T3/NIH, Mann-Whitney *U* test).

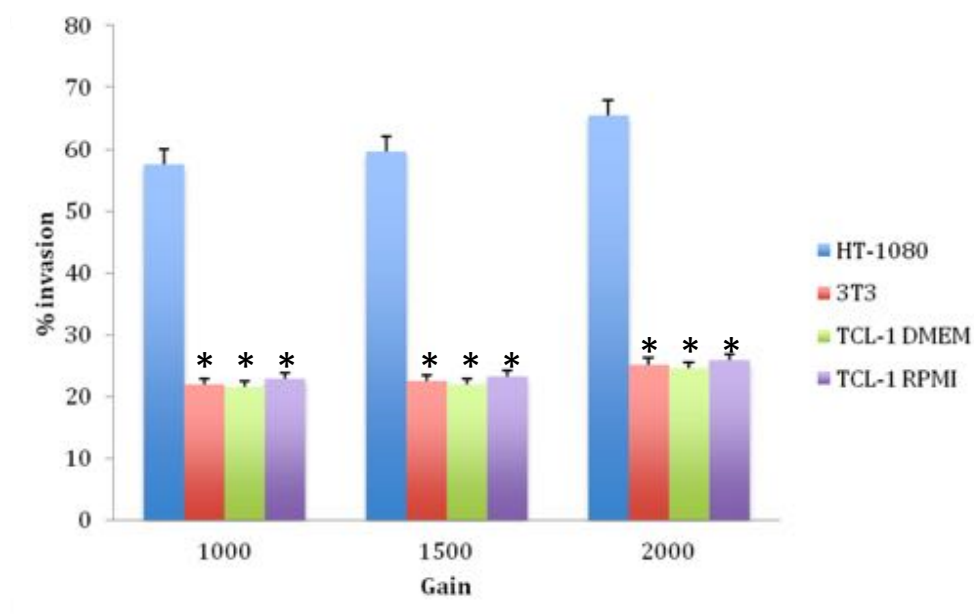


Figure 37. Represents the percentage of invasion presented as mean \pm SEM for the following cell lines HT 1080 (+ve control) of (n = 3, 57.6% \pm 10.8%, $p < 0.05$) 1000 gain, (n = 3, 59.7% \pm 11.1%, $p < 0.05$) 1500 gain, (n = 3, 65.5% \pm 12.3%, $p < 0.05$) 2000 gain 3T3/NIH (-ve control) (n = 3, 21.9% \pm 5.1%, $p < 0.05$) 1000 gain, (n = 3, 22.5% \pm 5.2%, $p < 0.05$) 1500 gain, (n = 3, 25.2% \pm 5.8%, $p < 0.05$) 2000 gain and human trophoblast TCL-1 cell line in DMEM at (n = 3, 21.5% \pm 5.0%, $p < 0.05$) 1000 gain, (n = 3, 22.0% \pm 5.1% , $p < 0.05$) 1500 gain, (n = 3, 24.6% \pm 5.7% , $p < 0.05$) 2000 gain. The TCL-1 cell line in RPMI at (n = 3, 22.9% \pm 5.2%, $p < 0.05$) 1000 gain, (n = 3, 23.2% \pm 5.3%, $p < 0.05$) 1500 gain, (n = 3, 25.9% \pm 5.9%, $p < 0.05$) 2000 gain (* $p < 0.05$ vs. HT1080 and 3T3/NIH, Mann-Whitney U test) at 1000,1500 and 2000 gains.

The invasion assay in Figure 37. represents the percentage of invasion presented as mean \pm SEM for the following HT 1080 (+ve control) of (n = 3, 57.6% \pm 10.8%, $p < 0.05$) 1000 gain, (n = 3, 59.7% \pm 11.1%, $p < 0.05$) 1500 gain, (n = 3, 65.5% \pm 12.3%, $p < 0.05$) 2000 gain 3T3/NIH (-ve control) (n=3, 21.9% \pm 5.1%, $p < 0.05$) 1000 gain, (n = 3, 22.5% \pm 5.2%, $p < 0.05$) 1500 gain, (n = 3, 25.2% \pm 5.8%, $p < 0.05$) 2000 gain and human trophoblast TCL-1 cell line in DMEM at (n = 3, 21.5% \pm 5.0%, $p < 0.05$)1000 gain, (n = 3, 22.0% \pm 5.1% , $p < 0.05$) 1500 gain, (n = 3, 24.6% \pm 5.7% , $p < 0.05$) 2000 gain. The TCL-1 cell line in RPMI at (n = 3, 22.9% \pm 5.2%, $p < 0.05$) 1000 gain, (n = 3, 23.2% \pm 5.3%, $p < 0.05$) 1500 gain, (n = 3, 25.9% \pm 5.9%, $p < 0.05$) 2000 gain (* $p < 0.05$ vs. HT1080 and 3T3/NIH, Mann-Whitney U test) at 1000,1500 and 2000 gains.

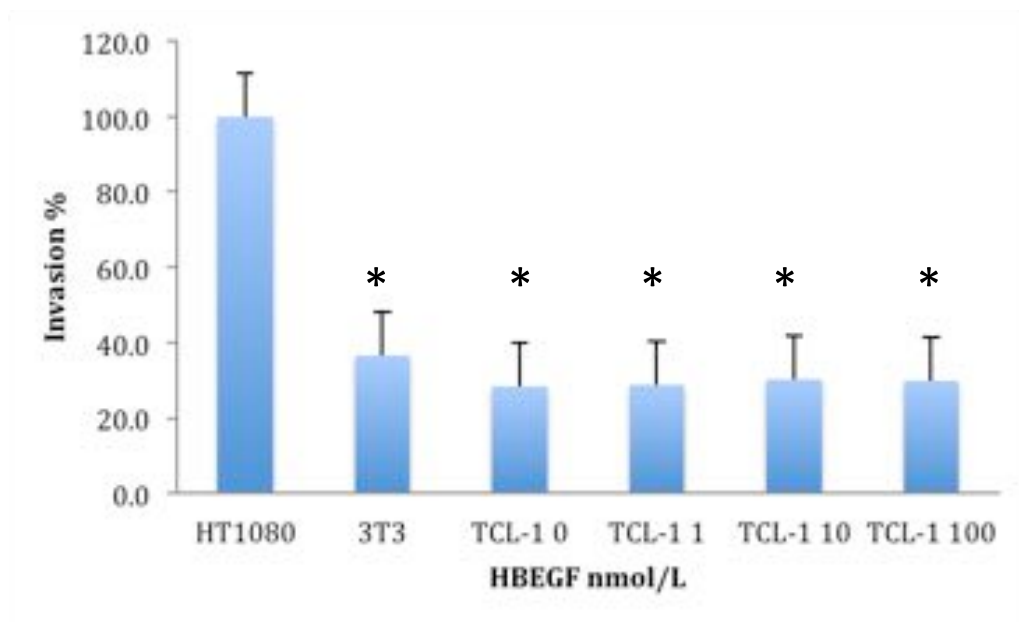


Figure 38. Represents the percentage of invasion presented as mean \pm SEM for the following cell lines HT 1080 (+ve control) of (n = 3, 100% \pm 20.1%, $p < 0.05$), 3T3/NIH (-ve control) (n = 3, 36.5% \pm 7.5%, $p < 0.05$), and human trophoblast TCL-1 cell line with the presence of heparin binding-epidermal growth factor (HB-EGF) expression at (n = 3, 28.5% \pm 2.3%, $p < 0.05$) 0nmol/l, (n = 3, 28.7% \pm 1.5%, $p < 0.05$) 1nmol/l, (n = 3, 30.2% \pm 3.0%, $p < 0.05$) 10nmol/l and (n = 3, 29.9% \pm 2.4%, $p < 0.05$) 100nmol/l. (* $p < 0.05$ vs. HT1080 and 3T3, Mann-Whitney *U* test).

The invasion assay in Figure 38. for the following cell lines HT 1080 (+ve control) of (n = 3, 100% \pm 20.1%, $p < 0.05$), 3T3/NIH (-ve control) (n = 3, 36.5% \pm 7.5%, $p < 0.05$), and human trophoblast TCL-1 cell line with the presence of heparin binding-epidermal growth factor (HB-EGF) expression at (n = 3, 28.5% \pm 2.3%, $p < 0.05$) 0nmol/l, (n = 3, 28.7% \pm 1.5%, $p < 0.05$) 1nmol/l, (n = 3, 30.2% \pm 3.0%, $p < 0.05$) 10nmol/l and (n = 3, 29.9% \pm 2.4%, $p < 0.05$) 100nmol/l. (* $p < 0.05$ vs. HT1080 and 3T3, Mann-Whitney *U* test). RPMI media was preferred for TCL-1 cell line at 1500 gain to represent the percentage of invasion.

An *in vitro* model has been developed to investigate the effect of heparin and HBGFs on placental trophoblast invasion. I performed the villous trophoblast invasion assay and selected HB-EGF, VEGF, PLGF, FGF2, PDGF, HGF, MK and PTN for this study using BD FluoroBlok that has a uniform coated layer with Matrigel Matrix. The percentage of trophoblast invasion for TCL-1 in the presence of HB-EGF, VEGF, PLGF, FGF2, PDGF, HGF, MK and PTN at the concentrations 0,1, 10,100nmol/L

was investigated. MK and PTN role has never been examined in trophoblast invasion during placental development. Profiles are shown in the Figures 37A, 38B, 39C, 40D, 41E, 42F, 43G and 44H giving the percentage of trophoblast invasion for TCL-1 in the presence of HB-EGF, VEGF, PLGF, FGF2, PDGF, HGF, MK and PTN at the concentrations 0,1,10, and 100nmol/L are shown. To compare 0 versus growth factor and 0 heparin the Mann-Whitney *U* test was used. This study suggests a role for some of the growth factors expressed in placenta on migration and invasion.

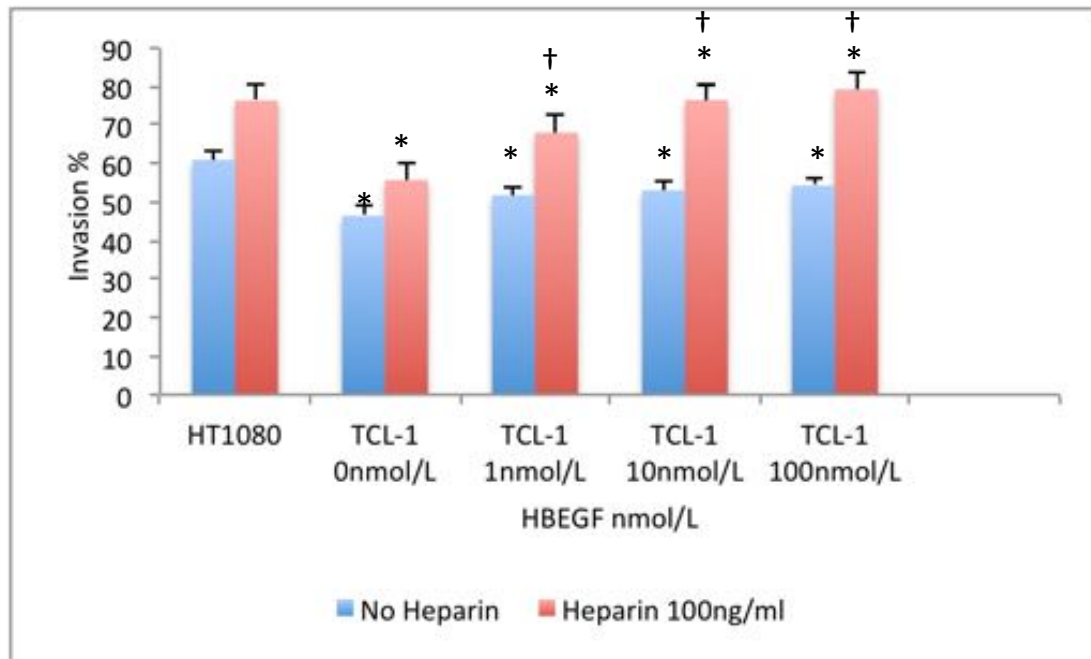


Figure 39A. Represents the percentage of invasion presented as mean \pm SEM for the following cell lines HT 1080 of ($n = 3$, $60.95\% \pm 15.70\%$; and $76.9\% \pm 6.26\%$, $p < 0.05$), and TCL-1 with the presence of heparin-binding epidermal growth factor (HB-EGF) expression at ($n = 3$, $46.43\% \pm 4.40\%$, $p < 0.05$) 0nmol/l, ($n = 3$, $51.794.39\%$, $p < 0.05$) 1nmol/l, ($n = 3$, $53.06\% \pm 2.58\%$ $p < 0.05$) 10nmol/l and ($n = 3$, $54.28\% \pm 2.26\%$, $p < 0.05$) 100nmol/l. In the presence of heparin at ($n=3$, $55.72\% \pm 6.38\%$, $p < 0.05$) 0ng/ml, ($n = 3$, $68.06\% \pm 4.45\%$, $p < 0.05$) 1ng/ml, ($n = 3$, $76.51\% \pm 6.80\%$, $p < 0.05$) 10ng/ml, and ($n = 3$, $79.30\% \pm 6.84\%$, $p < 0.05$) 100ng/ml. (* $p < 0.05$ vs. 0 growth factor, Mann-Whitney U test; † $p < 0.05$ vs. 0 heparin).

14.3.2.2. Figure 39A. presents the increase in trophoblast invasion in the presence of HT1080 ($60.95\% \pm 15.70$, $m = 57.25$, $p < 0.05$), and HB-EGF was significantly higher than in cells with no heparin ($46.43\% \pm 4.40$, $m = 42.8$, $p < 0.05$), ($51.79\% \pm 4.39$, $m = 55.0$, $p < 0.05$), ($53.06\% \pm 2.58$, $m = 42.8$, $p < 0.05$), ($54.28\% \pm 2.26$, $m = 52.5$, $p < 0.05$) at concentrations of 0,1, 10, 100nmol/L, respectively. The effect of trophoblast invasion in the presence of HB-EGF with no heparin produced a small increase but was significant with increasing concentrations. In the presence of heparin at concentrations of 0,1,10, and 100ng/ml the values were again higher. The differences in HB-EGF stimulated invasion with heparin were significant for the concentrations 0, 1, 10, and 100nmol/L ($55.723\% \pm 6.38$, $m = 55.5$, $p < 0.05$), ($68.06\% \pm 4.45$, $m = 65.7$, $p < 0.05$), ($76.51\% \pm 6.80$, $m = 72.2$, $p < 0.05$), ($79.30\% \pm 6.84$, $m = 66.1$, $p < 0.05$), and HT1080 ($76.9\% \pm 6.26$, $m = 82.6$, $p < 0.05$) respectively.

There was a small increase with increasing dose of HB-EGF, which was enhanced by heparin. The medians were increased in absence and presence of heparin.

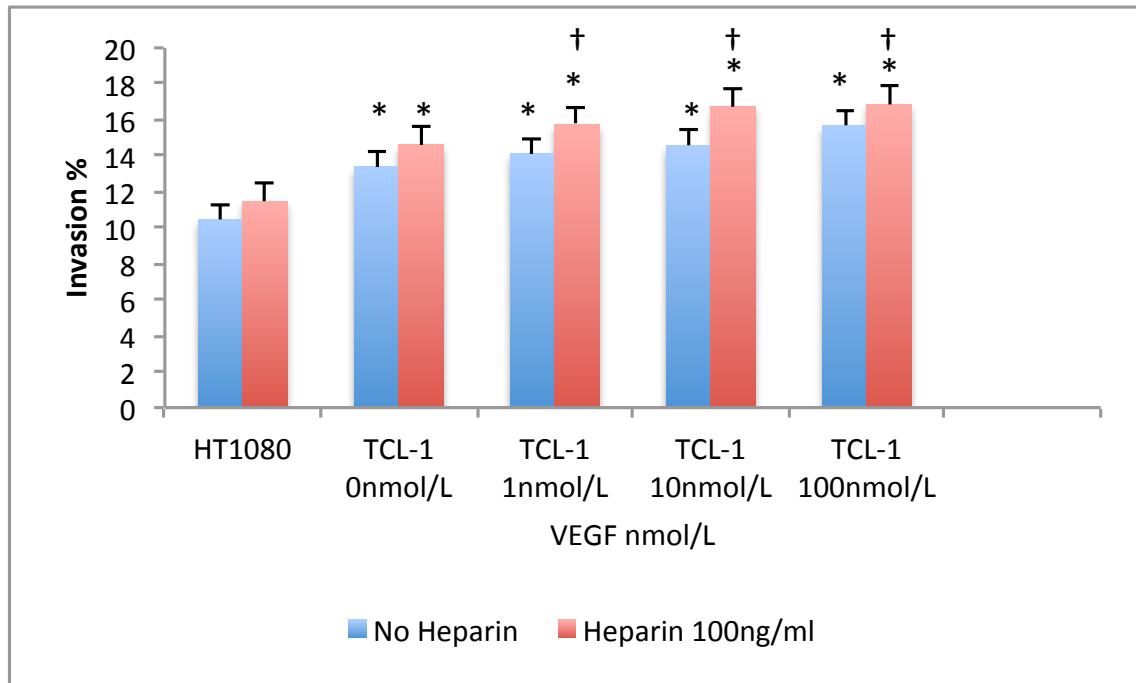


Figure 40B. Represents the percentage of invasion presented as mean \pm SEM for the following cell lines HT 1080 of ($n = 3$, $10.46\% \pm 18.68$; and $11.48\% \pm 19.56\%$, $p < 0.05$), and TCL-1 with the presence of vascular endothelial growth factor (VEGF) expression at ($n = 3$, $13.38\% \pm 0.39\%$, $p < 0.05$) 0nmol/l, ($n = 3$, $14.10\% \pm 0.99\%$, $p < 0.05$) 1nmol/l, ($n = 3$, $14.56\% \pm 1.69\%$, $p < 0.05$) 10nmol/l and ($n = 3$, $15.70\% \pm 2.20\%$, $p < 0.05$) 100nmol/l. In the presence of heparin at ($n = 3$, $14.60\% \pm 1.03\%$, $p < 0.05$) 0ng/ml, ($n = 3$, $15.73\% \pm 1.55\%$, $p < 0.05$) 1ng/ml, ($n = 3$, $16.73\% \pm 2.18\%$, $p < 0.05$) 10ng/ml, and ($n = 3$, $16.85\% \pm 1.67\%$, $p < 0.05$) 100ng/ml. (* $p < 0.05$ vs. 0 growth factor, Mann-Whitney U test; † $p < 0.05$ vs. 0 heparin).

14.3.2.3. In Figure 40B. presents the effect of trophoblast invasion in the presence of HT1080 ($10.46\% \pm 18.68$, $m = 10.53$, $p < 0.05$), and VEGF was significantly higher than in cells with no heparin ($13.38\% \pm 0.39$, $m = 11.32$, $p < 0.05$), ($14.10\% \pm 0.99$, $m = 12.74$, $p < 0.05$), ($14.56\% \pm 1.69$, $m = 12.95$, $p < 0.05$), ($15.70\% \pm 2.20$, $m = 14.24$, $p < 0.05$) for concentrations 0, 1, 10, 100nmol/L, respectively. The increase in trophoblast invasion in the presence of VEGF at increasing concentrations was small. VEGF stimulated a trophoblast invasion in the presence of heparin at concentrations of 0,1,10, and 100ng/ml their values were again higher. The differences in VEGF stimulated invasion with heparin were significant ($14.60\% \pm 1.03$, $m = 11.32$, $p < 0.05$), ($15.73\% \pm 1.55$, $m = 11.88$, $p < 0.05$), ($16.73\% \pm 2.18$, $m = 11.96$, $p < 0.05$), ($16.85\% \pm 1.67$, $m = 12.03$, $p < 0.05$) for the concentrations 0, 1, 10, and 100nmol/L, HT1080 ($11.48\% \pm 19.56$, $m = 10.28$, $p < 0.05$) respectively. VEGF response was

greater in presence of heparin. The medians were increased in absence and presence of heparin.

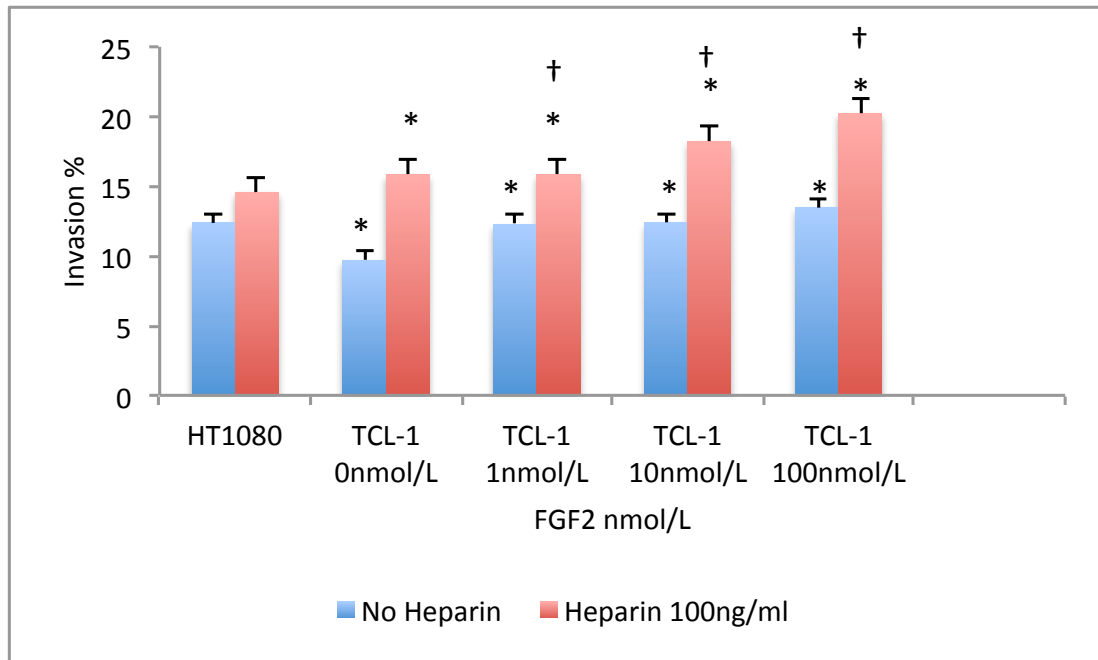


Figure 41C. Represents the percentage of invasion presented as mean \pm SEM for the following cell lines HT 1080 of ($n = 3$, $24.7\% \pm 2.08\%$, and $29.17\% \pm 1.56\%$, $p < 0.05$), and TCL-1 with the presence of fibroblast growth factor 2 (FGF2) expression at ($n = 3$, $9.69\% \pm 1.54\%$, $p < 0.05$) 0nmol/l, ($n = 3$, $12.28\% \pm 1.65\%$, $p < 0.05$) 1nmol/l, ($n = 3$, $12.41\% \pm 0.58\%$, $p < 0.05$) 10nmol/l and ($n = 3$, $13.47\% \pm 0.98\%$, $p < 0.05$) 100nmol/l. In the presence of heparin at ($n = 3$, $15.87\% \pm 1.05\%$, $p < 0.05$) 0ng/ml, ($n = 3$, $15.88\% \pm 0.91\%$, $p < 0.05$) 1ng/ml, ($n = 3$, $18.22\% \pm 2.15\%$, $p < 0.05$) 10ng/ml, and ($n = 3$, $20.24\% \pm 3.63\%$, $p < 0.05$) 100ng/ml. (* $p < 0.05$ vs. 0 growth factor, Mann-Whitney U test; † $p < 0.05$ vs. 0 heparin).

14.3.2.3. Figure 41C. presents the effect in trophoblast invasion in the presence of for HT1080 ($24.7\% \pm 2.08$, $m = 24.6$, $p < 0.05$), FGF2 was significantly higher than in cells with no heparin ($9.69\% \pm 1.54$, $m = 7.84$, $p = 0.05$), ($12.28\% \pm 1.65$, $m = 11.80$, $p < 0.05$), ($12.41\% \pm 0.58$, $m = 12.09$, $p < 0.05$), and ($13.47\% \pm 0.98$, $m = 12.28$, $p < 0.05$) at concentrations of 0, 1, 10, 100nmol/L, was small respectively. FGF2 stimulated trophoblast invasion in the presence of heparin at increasing concentrations of 0, 1, 10, and 100ng/ml produced values that were again higher. The differences in FGF2 stimulated invasion with heparin were significant ($15.87\% \pm 1.05$, $m = 13.40$, $p < 0.05$), ($15.88\% \pm 0.91$, $m = 17.98$, $p < 0.05$), ($18.22\% \pm 2.15$, $m = 25.79$, $p < 0.05$), and ($20.24\% \pm 3.63$, $m = 27.71$, $p < 0.05$) for the concentrations 0, 1, 10, and 100nmol/L, HT1080 of ($29.17\% \pm 1.56$, $m = 29.17$, $p < 0.05$) respectively. Thus, the FGF2 response was enhanced following co-incubation in the presence of heparin. The medians were increased in absence and presence of heparin.

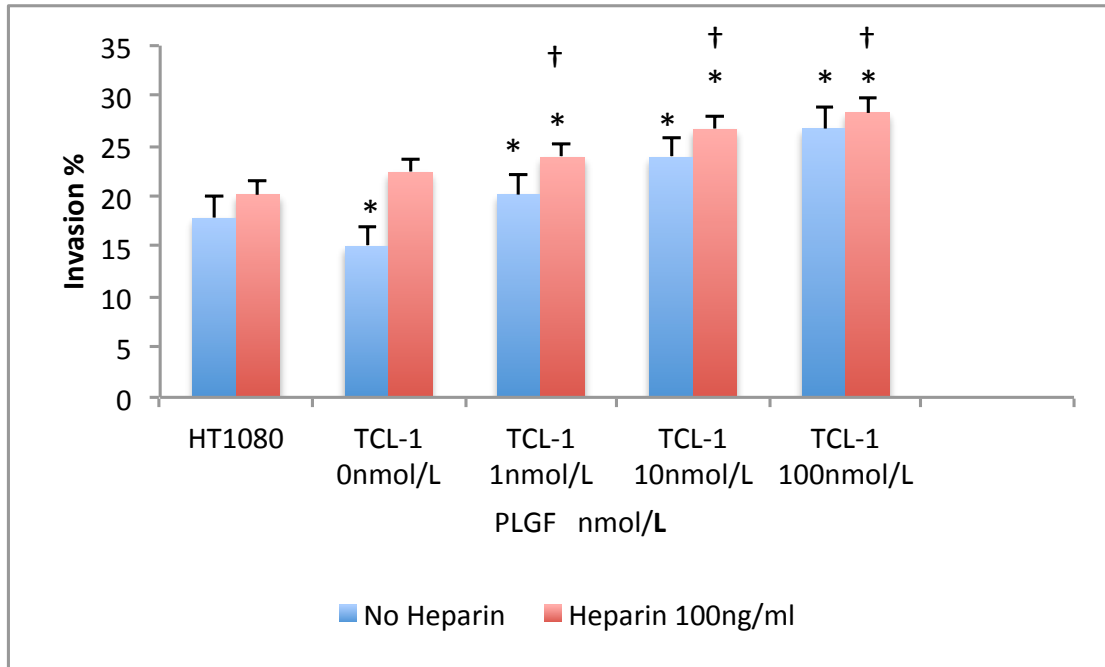


Figure 42D. Represents the percentage of invasion presented as mean \pm SEM for the following cell lines HT 1080 of ($n = 3$, 17.84% \pm 2.88%, and 20.16% \pm 9.87%, $p < 0.05$), and TCL-1 with the presence of placental growth factor (PLGF) expression at ($n = 3$, 15.02% \pm 2.70%, $p < 0.05$) 0nmol/l, ($n = 3$, 20.18% \pm 2.88%, $p < 0.05$) 1nmol/l, ($n = 3$, 23.88% \pm 7.19%, $p < 0.05$) 10nmol/l and ($n = 3$, 26.72% \pm 10.18%, $p < 0.05$) 100nmol/l. In the presence of heparin at ($n = 3$, 22.33% \pm 4.03%, $p < 0.05$) 0ng/ml, ($n = 3$, 23.89% \pm 6.51%, $p < 0.05$) 1ng/ml, ($n = 3$, 26.64% \pm 7.80%, $p < 0.05$) 10ng/ml, and ($n = 3$, 28.30% \pm 8.82%, $p < 0.05$) 100ng/ml (* $p < 0.05$ vs. 0 growth factor, Mann-Whitney U test; † $p < 0.05$ vs. 0 heparin).

14.3.2.4. Figure 42D. presents the effect of trophoblast invasion in the presence of HT1080 (17.84% \pm 2.88, $m = 8.00$, $p < 0.05$), for PLGF was significantly higher than in cells with no heparin (15.02% \pm 2.70, $m = 21.36$, $p < 0.05$), (20.18% \pm 2.88, $m = 35.52$, $p < 0.05$), (23.88% \pm 7.19, $m = 51.10$, $p < 0.05$), and (26.72% \pm 10.18, $m = 54.71$, $p < 0.05$) at concentrations of 0, 1, 10, 100nmol/L, respectively. The increase in trophoblast invasion was stimulated in the presence of PLGF were small for increasing concentrations. PLGF stimulated trophoblast invasion in the presence of heparin at concentrations of 0, 1, 10, and 100ng/ml the values were again higher but showed small increases. The differences in PLGF stimulated invasion with heparin were significant (22.33% \pm 4.03, $m = 13.08$, $p < 0.05$), (23.89% \pm 6.51, $m = 37.85$, $p < 0.05$), (26.64% \pm 7.80, $m = 44.16$, $p < 0.05$), and (28.30% \pm 8.82%, $m = 46.73$, $p < 0.05$), at increasing concentrations of 0, 1, 10, and 100nmol/L and HT1080 of (20.16% \pm 9.87, $m = 24.44$, $p < 0.05$) respectively. There was a small change with

increasing dose of PLGF, which showed very little effect by heparin. The medians were increased in absence and presence of heparin.

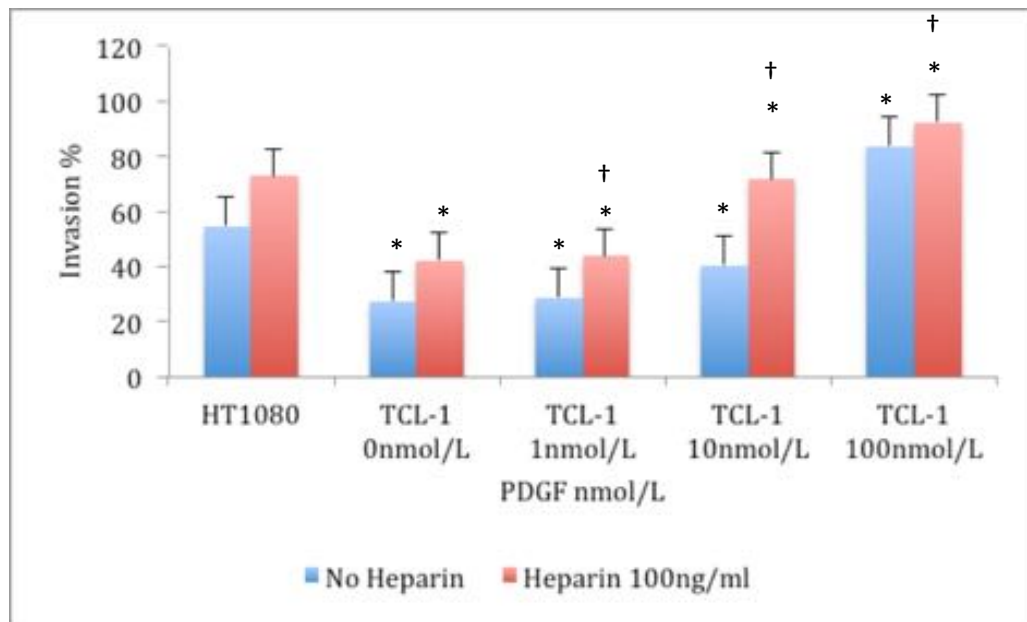


Figure 43E. Represents the percentage of invasion presented as mean \pm SEM for the following cell lines HT 1080 of ($n = 3$, $54.99\% \pm 3.65\%$, and $72.68\% \pm 5.22\%$, $p < 0.05$), and TCL-1 with the presence of platelet-derived growth factor (PDGF) expression at ($n = 3$, $27.31\% \pm 4.50\%$, $p < 0.05$) 0nmol/l, ($n = 3$, $28.18\% \pm 6.91\%$, $p < 0.05$) 1nmol/l, ($n = 3$, $40.22\% \pm 18.74\%$, $p < 0.05$) 10nmol/l and ($n = 3$, $83.51\% \pm 3.80\%$, $p < 0.05$) 100nmol/l. In the presence of heparin at ($n = 3$, $42.03\% \pm 6.79\%$, $p < 0.05$) 0ng/ml, ($n = 3$, $43.87\% \pm 6.91\%$, $p < 0.05$) 1ng/ml, ($n = 3$, $71.50\% \pm 18.74\%$, $p < 0.05$) 10ng/ml, and ($n = 3$, $92.1\% \pm 8.85\%$, $p < 0.05$) 100ng/ml (* $p < 0.05$ vs. 0 growth factor, Mann-Whitney U test; † $p < 0.05$ vs. 0 heparin).

14.3.2.5. Figure 43E. presents the effect of trophoblast invasion was stimulated in the presence of HT1080 ($54.28\% \pm 5.58$, $m = 7.66$, $p < 0.05$), and PDGF, was presented in Figure 36E, was significantly higher than in cells with no heparin ($27.31\% \pm 4.50$, $m = 27.98$, $p < 0.05$), ($28.18\% \pm 6.91$, $m = 28.93$, $p < 0.05$), ($40.22\% \pm 18.74$, $m = 41.07$, $p < 0.05$), and ($83.51\% \pm 3.80$, $m = 93.49$, $p < 0.05$) at concentrations of 0, 1, 10, 100nmol/L, respectively. PDGF stimulated trophoblast invasion with a slight change in the presence of heparin for lower concentrations, but its values were pronounced and were significantly higher for the concentrations. In the presence of heparin at 0, 1, 10, and 100ng/ml the values were higher. The differences in PDGF stimulated invasion with heparin were significant ($42.03\% \pm 6.79$, $m = 36.62$, $p < 0.05$), ($43.87\% \pm 6.91$, $m = 37.31$, $p < 0.05$), ($71.50\% \pm 18.74$, $m = 56.81$, $p < 0.05$), and ($92.1\% \pm 8.85$,

m= 88.80, p <0.05) at concentrations of 0, 1, 10, and 100nmol/L, HT1080 (72.68% ± 5.22, m = 5.93, p <0.05), respectively. Thus, PDGF response was enhanced with following co-incubation in the presence of heparin only for higher doses. The medians were increased in absence and presence of heparin.

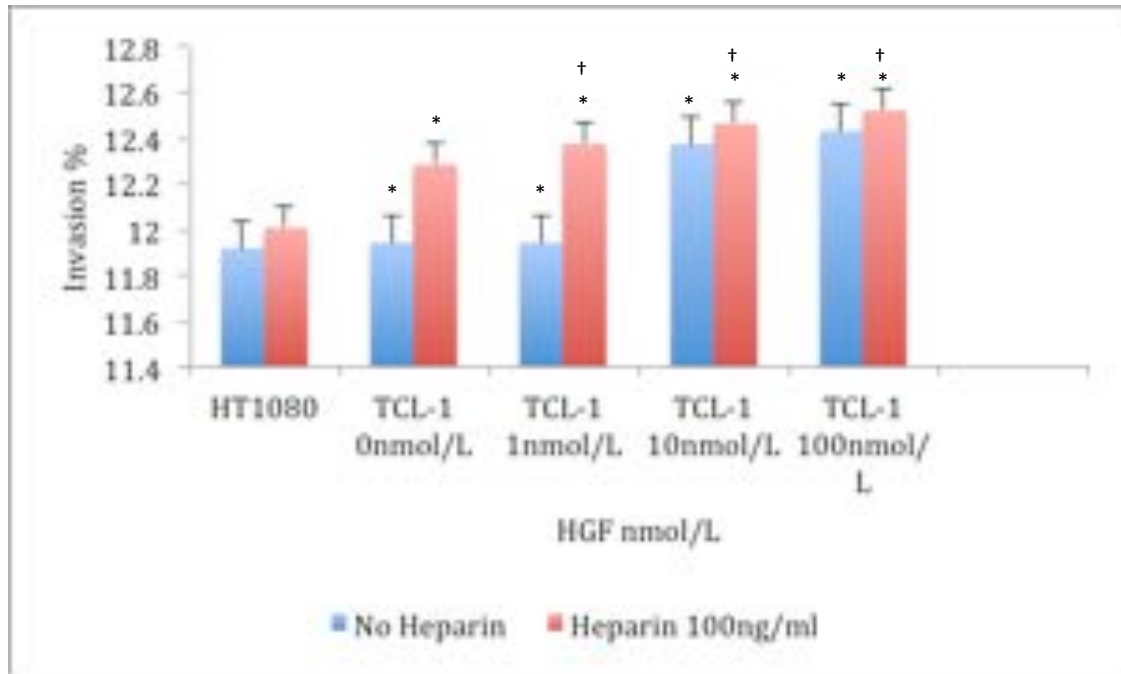


Figure 44F. Represents the percentage of invasion presented as mean \pm SEM for the following cell lines HT 1080 of ($n = 3$, $11.92\% \pm 0.58\%$, $12.01\% \pm 0.133\%$ and, $p < 0.05$), and TCL-1 with the presence of hepatocyte growth factor (HGF) expression at ($n = 3$, $11.94\% \pm 0.61\%$, $p < 0.05$) 0nmol/l, ($n = 3$, $11.94\% \pm 0.055\%$, $p < 0.05$) 1nmol/l, ($n = 3$, $12.37\% \pm 0.10\%$, $p < 0.05$) 10nmol/l and ($n = 3$, $12.43\% \pm 0.20\%$, $p < 0.05$) 100nmol/l. In the presence of heparin at ($n = 3$, $12.28\% \pm 0.15\%$, $p < 0.05$) 0ng/ml, ($n = 3$, $12.37\% \pm 0.18\%$, $p < 0.05$) 1ng/ml, ($n = 3$, $12.46\% \pm 0.02\%$, $p < 0.05$) 10ng/ml, and ($n = 3$, $12.52\% \pm 0.14\%$, $p < 0.05$) 100ng/ml. (* $p < 0.05$ vs. 0 growth factor, Mann-Whitney U test; † $p < 0.05$ vs. 0 heparin).

14.3.2.6. Figure 44F presents the effect of trophoblast invasion in the presence of HT1080 ($11.92\% \pm 0.58$, $m = 11.01$, $p < 0.05$), and HGF was significantly higher than in cells with no heparin ($11.94\% \pm 0.61$, $m = 11.91$, $p < 0.05$), ($11.94\% \pm 0.055$, $m = 11.93$, $p < 0.05$), ($12.37\% \pm 0.10$, $m = 12.46$, $p < 0.05$), and ($12.43\% \pm 0.20$, $m = 12.53$, $p < 0.05$) at concentrations of 0, 1, 10, 100nmol/L, respectively. The trophoblast invasion was stimulated in the presence of HGF showed a significant dose dependent rise with increasing concentrations. In the presence of heparin at 0, 1, 10, and 100ng/ml there values were again higher. The differences in HGF stimulated invasion with heparin were significant ($12.28\% \pm 0.15$, $m = 12.58$, $p < 0.05$), ($12.37\% \pm 0.18$, $m = 12.35$, $p < 0.05$), ($12.46\% \pm 0.02$, $m = 12.46$, $p < 0.05$), ($12.52\% \pm 0.14$, $m = 12.58$, $p < 0.05$) at concentrations of 0, 1, 10, and 100nmol/L, and HT1080 ($12.01\% \pm 0.133$, $m = 12.45$, $p < 0.05$) respectively. Trophoblast invasion in the presence of HGF with

heparin presented small changes for increasing concentrations. The effect of heparin was only observed at higher concentrations. Thus, heparin modifies the HGF response. The medians were increased in the absence and presence of heparin.

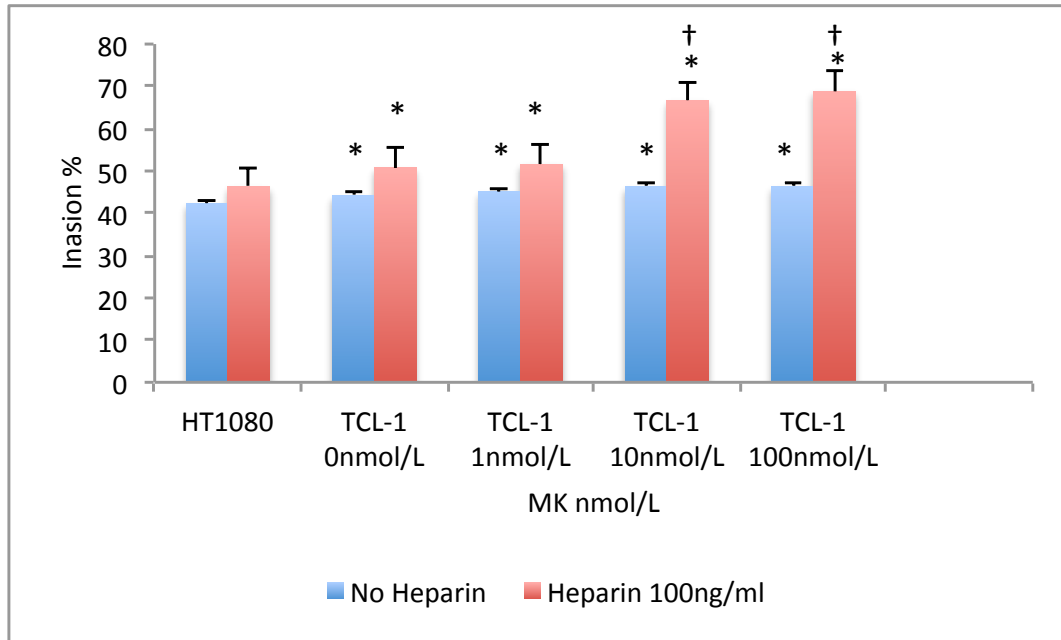


Figure 45G. Represents the percentage of invasion presented as mean \pm SEM for the following cell lines HT 1080 of ($n = 3$, $42.38\% \pm 1.54\%$, and $46.27\% \pm 4.97\%$, $p < 0.05$), and TCL-1 with the presence of Midkine (MK) expression at ($n = 3$, $44.16\% \pm 5.80\%$, $p < 0.05$) 0nmol/l, ($n = 3$, $45.11\% \pm 4.09\%$, $p < 0.05$) 1nmol/l, ($n = 3$, $46.23\% \pm 4.95\%$, $p < 0.05$) 10nmol/l and ($n = 3$, $46.30\% \pm 5.57\%$, $p < 0.05$) 100nmol/l. In the presence of heparin at ($n = 3$, $50.69\% \pm 1.84\%$, $p < 0.05$) 0ng/ml, ($n = 3$, $51.63\% \pm 2.58\%$, $p < 0.05$) 1ng/ml, ($n = 3$, $66.58\% \pm 5.72\%$, $p < 0.05$) 10ng/ml, and ($n = 3$, $68.82\% \pm 5.11\%$, $p < 0.05$) 100ng/ml. (* $p < 0.05$ vs. 0 growth factor, Mann-Whitney U test; † $p < 0.05$ vs. 0 heparin).

14.3.2.7. Figure 45G presents the effect of trophoblast invasion of HT1080 ($42.38\% \pm 1.54$, $m = 10.71$, $p < 0.05$), and were similar to the control for increasing concentrations of MK at 0, 1, 10, and 100nmol/L ($44.16\% \pm 5.80$, $m = 40.17$, $p < 0.05$), ($45.11\% \pm 4.09$, $m = 41.49$, $p < 0.05$), ($46.23\% \pm 4.95$, $m = 42.82$, $p < 0.05$), and ($46.30\% \pm 5.57$, $m = 46.05$, $p < 0.05$) with no heparin. MK stimulated trophoblast invasion in the presence of heparin and showed similar values for the lower concentrations, but were significant for the higher concentrations. The values were the same in the presence of heparin for 0 and 1mmol/L ($50.69\% \pm 1.84$, $m = 53.40$, $p < 0.05$), ($51.63\% \pm 2.58$, $m = 53.42$, $p < 0.05$), HT1080 ($46.27\% \pm 4.97$, $m = 11.83$, $p < 0.05$), but were significantly higher for the concentrations 10, and 100nmol/L ($66.58\% \pm 5.72$, $m = 40.17$, $p < 0.05$), and ($68.82\% \pm 5.11$, $m = 41.49$, $p < 0.05$). Therefore, MK effect was strikingly augmented as the largest dose response was

enhanced following co-incubation with higher doses of heparin. The medians were increased in absence and presence of heparin.

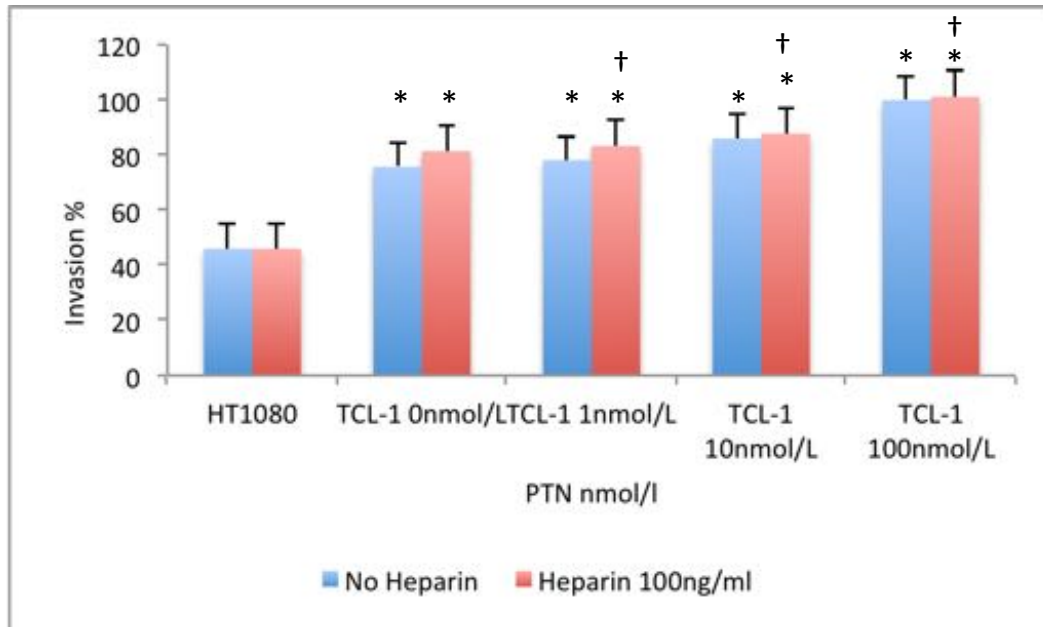


Figure 46H. Represents the percentage of invasion presented as mean \pm SEM for the following cell lines HT 1080 of ($n = 3$, $45.67\% \pm 2.88\%$, and $45.96\% \pm 9.87\%$, $p < 0.05$), and TCL-1 with the presence of Pleiotrophin (PTN) expression at ($n = 3$, $75.65\% \pm 12.83\%$, $p < 0.05$) 0nmol/l, ($n = 3$, $78.09\% \pm 3.03\%$, $p < 0.05$) 1nmol/l, ($n = 3$, $85.94\% \pm 8.02\%$, $p < 0.05$) 10nmol/l and ($n = 3$, $99.84\% \pm 6.35\%$, $p < 0.05$) 100nmol/l. In the presence of heparin at ($n = 3$, $81.38\% \pm 13.39\%$, $p < 0.05$) 0ng/ml, ($n = 3$, $83.25\% \pm 6.73\%$, $p < 0.05$) 1ng/ml, ($n = 3$, $87.94\% \pm 9.76\%$, $p < 0.05$) 10ng/ml, and ($n = 3$, $101.09\% \pm 20.78\%$, $p < 0.05$) 100ng/ml. (* $p < 0.05$ vs. 0 growth factor, Mann-Whitney U test; † $p < 0.05$ vs. 0 heparin).

14.3.2.8. Figure 46H presents the effect of trophoblast invasion in the presence of HT1080 ($45.67\% \pm 2.88$, $m = 41.7$, $p < 0.05$), and PTN was significantly higher compared to cells with no heparin ($75.65\% \pm 12.83$, $m = 63.0$, $p < 0.05$), ($78.09\% \pm 3.03$, $m = 69.5$, $p < 0.05$), ($85.94\% \pm 8.02$, $m = 77.5$, $p < 0.05$) and ($99.84\% \pm 6.35$, $m = 98.7$, $p < 0.05$) at concentrations of 0, 1, 10, and 100nmol/L respectively. The effect of trophoblast invasion was stimulated in the presence of HBGFs with no heparin HT1080 ($45.96\% \pm 9.87$, $m = 49.2$, $p < 0.05$), demonstrated that increasing concentrations of PTN produced a small increase. In the presence of heparin at 0ng/ml, ($81.38\% \pm 13.39$, $m = 96.6$, $p < 0.05$) 1ng/ml, ($83.25\% \pm 6.73$, $m = 77.5$, $p < 0.05$) 10ng/ml, ($87.94\% \pm 9.76$, $m = 88.4$, $p < 0.05$) and 100ng/ml ($101.09\% \pm 20.78$, $m = 96.6$, $p < 0.05$) there was a further increase. PTN stimulated trophoblast invasion in the presence of heparin and showed a further significant increase for increasing concentrations. There was no significant difference between untreated and

heparin treated cells for each dose of PTN. The medians were increased in absence and presence of heparin.

Thus, HBGFs yielding the greatest change and those that showed the greatest response to the presence of heparin were FGF2, PDGF, HGF, and MK. However, little is known about MK and PTN expression and their roles in the human placenta. The present results which are the first of the actions of MK and PTN show different effects of heparin. Namely large increases were observed at the higher dose for MK, but little effect was seen on the PTN response. HBGFs caused villous trophoblast cells to invade, but only in the presence of heparin. This demonstrates that the *in vitro* trophoblast invasion model provides a useful method to determine the effects of heparin and these HBGFs on placental function.

Chapter 5

Expression

5.1. Quantify mRNA expression of HBGFs using real time polymerase chain reaction (RT-PCR).

Since immunohistochemistry can only give semiquantitative information, this study was extended to quantify mRNA expression with HBGFs using RT-PCR. The progress of the reaction is monitored in “real-time”. I performed the stabilisation of RNA later reagent, in protocol 1 and extraction and purification in protocol 2 with homogenization of the total RNA production from tissues-RNAeasy RNA mini purification, in human placentae samples (n=7) from placental normal development and in pre-eclampsia in this first study. The quantification of RNA, concentration and purity (A260/280nm ratio) was determined in a spectrophotometer. The amount of total quantitative RNA was evaluated by measuring its integrity and stability using Agilent 2100 bioanalyzer. Reverse-transcription with elimination of genomic DNA for quantitative RT-PCR procedure in protocol 3 for cDNA synthesis. The RNA was converted to cDNA by quantification of mRNA expression with HBGFs using Real Time-Polymerase Chain Reaction (RT-PCR). Its amplification were monitored during two-step RT-PCR cycling and relative expression of RNA per sample were quantified by Quantitect SYBER Green rPCR as in protocol 4. The PLGF, FGF2, MK, and PTN were selected because they showed significant alterations of placental expression in pre-eclampsia. I performed mRNA extraction from 7 placental tissue samples at a concentration of 100ng/ul that were used for the following primer assays: PLGF, FGF2, MK, PTN, and Succinate dehydrogenase complex subunit A (SDHA) an endogenous control, a housekeeping gene for standardization between tissue samples in Table 26 and 27. The results of specificity of PCR products were observed by RNA agarose gel electrophoresis and gel electrophoresis representation analysis on Multina.

Group	Maternal age (Years)	Gestational age at delivery (weeks)	Birth weight (g)
PE	30 (16-39)	34 (24-41)	3025 (1960-3430)
PTL	28 (18-37)	31(28-35)	2865 (1860-2350)
TL	27 (16-38)	39 (37-42)	3415 (2250-5026)
TNL	28 (17-38)	39 (37-41) *	3250 (2100-3936)

Table 25. The clinical characteristics of pregnancies are represented for this cohort study of 87 women. The values are expressed as mean \pm IQR (interquartile range). PE-Pre-eclampsia, PTL-Pre-term Labour, TNL-Term-non-Labour,* and TL-term labour.

The clinical characteristics of pregnancies are represented for this cohort study of 87 women represented in Table 25. There was no significance at maternal age and gestational delivery when occurred. However, the BMI and placental weight were not available for this study.

Labour:

The birth weight in pre-eclampsia women reflected the early delivery. For, maternal age 30 (interquartile range 16-39) years, gestational age at delivery of 34 (interquartile range 24-41) weeks, the birth weight was of 3025 (interquartile range 1960-3430)g.

Pre-term Labour (PTL) women at birth weight delivered infants. For, maternal age 28 (interquartile range 18-37) years, gestational age at delivery of 31 (interquartile range 28-35) weeks, the birth weight was of 2865 (interquartile range 1860-2350)g.

Non-Labour:

Non-labour group (TL) women delivered infants at birth weight. For, maternal age 27 (interquartile range 16-38) years, gestational age at delivery of 39 (interquartile range 37-42) weeks, the birth weight was of 3415 (interquartile range 2250-5026)g.

Term-non-labour (TNL) women at birth weight delivered infants but term-non-labour TNL women underwent Caesarean section as gestational delivery occurred. For, maternal age 28 (interquartile range 17-38) years, gestational age at delivery of 39 (interquartile range 37-41)* weeks, the birth weight was of 3250 (interquartile range 2100-3936)g.

The present results of this study cohort of 87 women showed that there was no significance at maternal age and gestational delivery when occurred. The labour group results showed that the birth weight in pre-eclampsia women reflected the early delivery. The Pre-term Labour (PTL) women at birth weight delivered infants. The non-labour group results for (TL) women delivered infants at birth weight, but term-non-labour (TNL) women underwent Caesarean section as gestational delivery occurred. The TNL women delivered infants at birth weight.

Sample Number of 7 different placental tissues	Concentration (ng/ul)				
	Placental Growth factor	Fibroblast Growth factor 2	Pleiotrophin	Midkine	Succinate dehydrogenase complex subunit A
1. PE	4.52	26.58	4.09	2.42	7.26
2. PE	5.55	27.05	2.97	6.01	5.13
3. Control*	12.38	29.73	10.38	3.03	20.71
4. PE	6.91	30.23	8.52	2.37	0.42
5. Control	5.89	25.12	8.65	3.13	8.84
6. Control	5.84	24.76	6.46	1.62	10.24
7. Control	8.54	22.69	10.24	2.00	12.43

Table 26. The 7 different placental tissue mRNA samples of control and pre-eclampsia (PE) at a concentration of 100ng/ul for placental growth factor (PLGF), Fibroblast growth factor 2 (FGF2), Pleiotrophin (PTN), Midkine (MK), and Succinate dehydrogenase complex subunit A (SDHA) primer assays.* higher protein mRNA value for control 3.

The 7 different placental mRNA samples of control and pre-eclampsia (PE) were determined by RT-PCR of heparin binding growth factor primer assays: for fibroblast growth factor 2 (FGF2), placental growth factor (PLGF), midkine (MK), pleiotrophin (PTN) and to endogenous house keeping gene Succinate dehydrogenase complex subunit A (SDHA) as represented in Table 26. Pre-eclampsia (PE-1, 2, and 4) at a concentration of 100ng/ul for PLGF, of 4.52ng/ul, FGF2, of 26.58ng/ul, PTN, of 4.09

ng/ul, MK of 2.42ng/ul, and SDHA of 7.26ng/ul primer assays. PE-2 at a concentration of 100ng/ul for PLGF, of 5.55ng/ul, FGF2, of 27.05ng/ul, PTN, of 2.97ng/ul, MK, of 6.01ng/ul, and SDHA of 5.13ng/ul primer assays. Control-3 at a concentration of 100ng/ul for PLGF, of 12.38ng/ul, FGF2, of 29.73ng/ul, PTN, of 10.38ng/ul, MK of 3.03ng/ul, and SDHA of 7.26ng/ul primer assays.* higher protein mRNA value for control 3. PE-4 at a concentration of 100ng/ul for PLGF, of 6.91ng/ul, FGF2 of 30.23ng/ul, PTN of 8.52ng/ul, MK of 2.37ng/ul, and SDHA of 0.42 ng/ul primer assays. Control-5 at a concentration of 100ng/ul for PLGF of 5.89ng/ul, FGF2 of 25.12ng/ul, PTN of 8.65ng/ul, MK of 3.13ng/ul, and SDHA of 8.84ng/ul primer assays. Control-6 at a concentration of 100ng/ul for PLGF of 5.84ng/ul, FGF2 of 24.76ng/ul, PTN of 6.46ng/ul, MK of 1.62ng/ul, and SDHA of 10.24ng/ul primer assays. Control-7 at a concentration of 100ng/ul for PLGF of 8.54ng/ul, FGF2 of 22.69ng/ul, PTN of 10.24 ng/ul, MK of 2.00ng/ul and SDHA of 12.43ng/ul primer assays.

Growth factors	Control versus PE P= value; median= value
FGF2	Con m=2.12 PE m=5.27 P=0.03*
PLGF	Con m=0.63 PE m= 1.08 P=0.11
MK	Con m=0.160 PE m=1.17 P=0.06
PTN	Con m=0.73 PE m= 0.58 P= 0.57
SDHA	Con m= 11.34 PE m= 5.13 P=0.03*

Table 27. Comparison of 7 different placental tissue mRNA expression at a concentration of 100ng/ul for the following heparin binding growth factor primer assays using the Mann-Whitney *U* test. Abbreviations for growth factors: fibroblast growth factor 2 (FGF2), placental growth factor (PLGF), midkine (MK), pleiotrophin (PTN) and Succinate dehydrogenase complex subunit A (SDHA). Control (Term (TL + TNL) not in labour) and pre-eclampsia (PE) were compared. The median is given above and p-values are shown. The p-values are indicated $p < 0.05^*$ and $p < 0.01^{**}$.

The comparison of 7 different placental mRNA levels were expressed as the ratio of heparin binding growth factor primer assays at a concentration of 100ng/ul: for fibroblast growth factor 2 (FGF2), placental growth factor (PLGF), midkine (MK), pleiotrophin (PTN) and to endogenous control, a housekeeping gene Succinate dehydrogenase complex subunit A (SDHA) required for normalization between placental tissue samples using the Mann Whitney *U* test. The comparison between control (Term (TL + TNL) not in labour) versus pre-eclampsia of placental tissue mRNA expression for PLGF of control, $m = 0.63$, PE $m = 1.08$, ($p = 0.11$), FGF2 of control $m = 2$, PE $m = 5.27$, ($p=0.03^*$), MK of control, $m = 0.16$, PE $m = 1.17$, ($p=$

0.06), PTN of control, $m=0.73$, PE $m=0.58$, ($p=0.57$), and SDHA of control $m=11.34$, PE $m=5.13$, $p=0.03^*$) primer assays. Both FGF2 of control $m=2$, PE $m=5.27$, ($p=0.03^*$), and SDHA of control $m=11.34$, PE $m=5.13$, ($p=0.03^*$) in the placenta was confirmed at the mRNA level by RT-PCR, showed significant differences as shown in Table 26 and 27 using Mann-Whitney U test ($*p<0.05$).

5.2. Specificity of PCR products by RNA agarose gel electrophoresis:

The third technique of RNA quality was determined by RNA agarose gel electrophoresis. I performed on 7 different placental tissue mRNA samples at a concentration of 100ng/ul that were pipetted into the wells as in Figure 47 for Fibroblast growth factor 2 (FGF2) a, b, c, d and e for primer assay. Figure 47e was photographed using the portable UV-Trans illuminator for visible DNA ladder on agarose gel electrophoresis at the time the bands were gradually moving from upper to lower marker 2072 to 100 base pairs (bp) in size. These results used were reproduced for Fibroblast growth factor 2 (FGF2) and Midkine (MK) in 7 further experiments.

5.2.1. An agarose gel electrophoresis for 7 different placental tissue mRNA samples with a concentration of 100ng/ul for Fibroblast growth factor 2 (FGF2) primer assay.

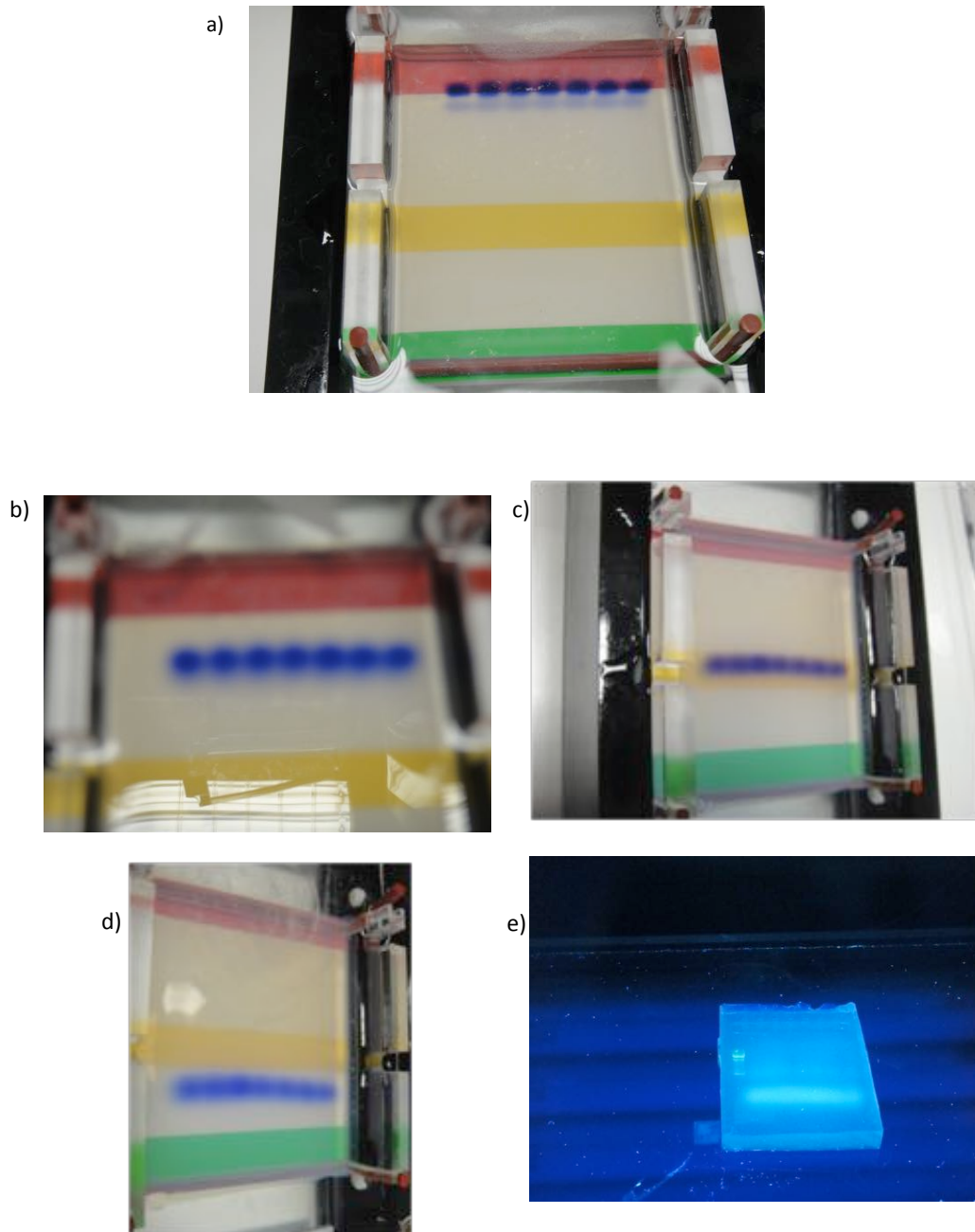


Figure 47. An agarose gel electrophoresis for 7 different placental tissue mRNA samples with a concentration of 100ng/ul for Fibroblast growth factor 2 (FGF2) primer assay a, b, c, d and e. This analysis required the DNA ladder and 2 internal standard markers (LM and UM).

DNA samples are pipetted into the sample wells, seen as dark slots at the top of the picture. Application of an electric current at the top (anodal, negative) end causes the

negatively charged DNA to migrate (electrophorese) towards the bottom (cathodal, positive) end. Smaller DNA fragments move more quickly, and finish at the end of the gel. Although, each of the fragments of a single class of molecule is present in equimolar proportions, the smaller fragments have a less mass of DNA. The length of a DNA segment is accurately determined by running it on an agarose gel alongside a DNA ladder.

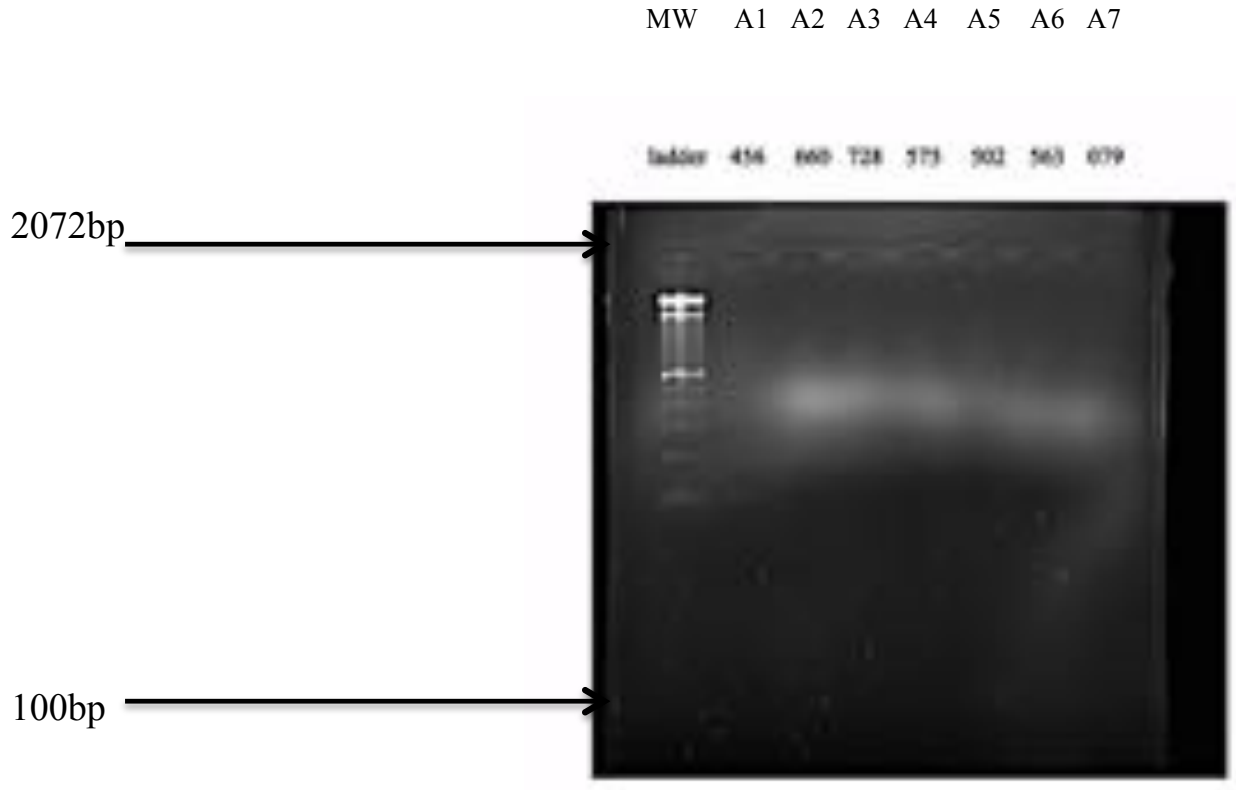
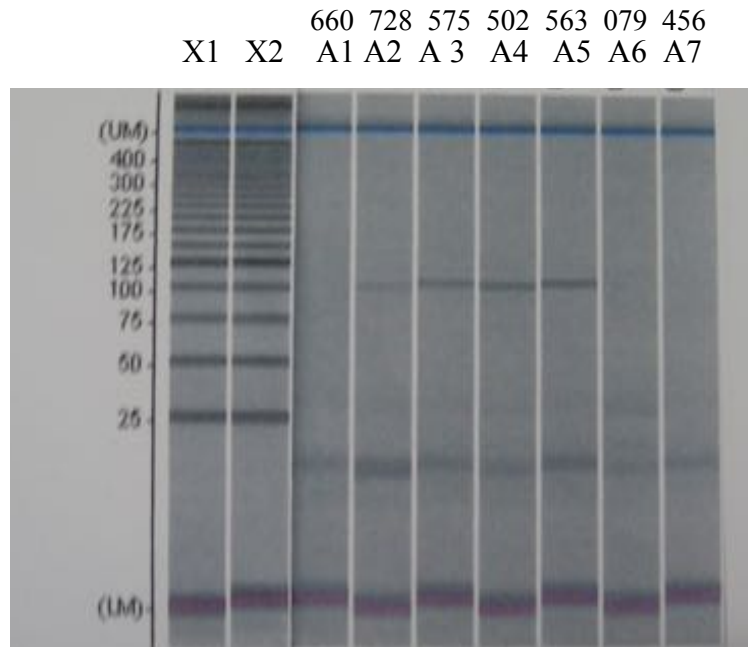


Figure 48. RT-PCR analysis for 7 different placental tissue mRNA samples from control and pre-eclampsia (100bp respectively) with their concentration (100ng/ul) for Fibroblast growth factor 2 (FGF2) primer assay. This analysis required the DNA ladder 100bp ladder (2072-100bp), lanes A1-7 by agarose gel electrophoresis, MW Lane: Molecular weight and 2 internal standard markers (LM and UM).

I performed RT-PCR analysis on 7 different placental tissue mRNA samples at a concentration of 100ng/ul that were pipetted into the wells for Fibroblast growth factor 2 (FGF2) primer assay as in Figure 48. The visible DNA ladder on agarose gel electrophoresis was photographed at the time the bands were gradually moving from upper to lower marker 2072 to 100base pairs (bp) in size using the UV-Trans illuminator. The placental sample 456 bands in lanes A1 were bettered visualised with 100bp. But the other placental samples 660, 728, 575, 502, 563, 079 bands in lanes A2-7 were all in the region of ≈ 1000 bp to 800bp and travelled down to 100bp.

≈ 123 bp, C2 lane of ≈ 127 bp, C3 lane of ≈ 123 bp, C4 lane of ≈ 127 bp, C5 lane of ≈ 123 bp, C6 lane of ≈ 127 bp and C7 lane of ≈ 123 bp. There were significant differences in mRNA expression for FGF2 and SDHA in placentae from pre-eclampsia compared to the controls except for PLGF, MK, and PTN, using the Mann-Whitney *U* test.

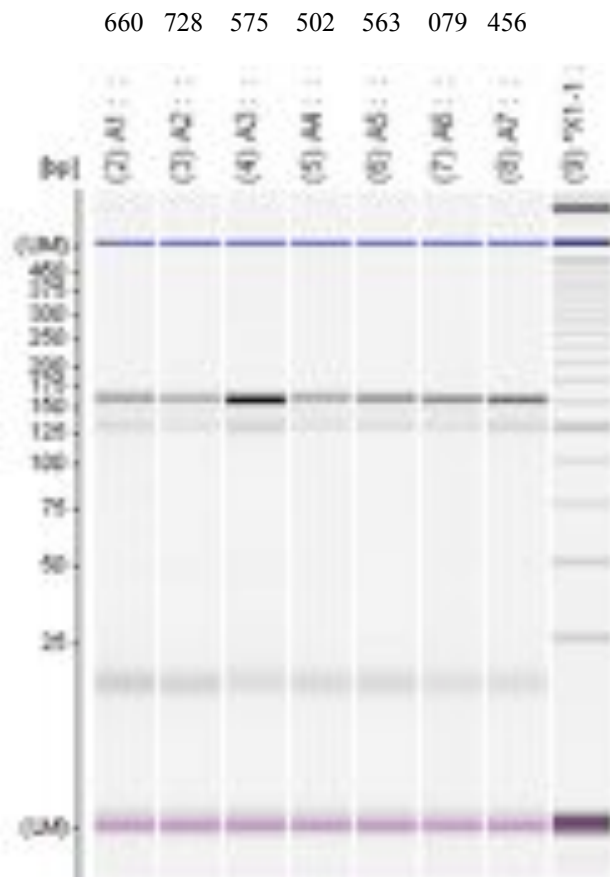


↑

A3 ≈ 105 bp

Figure 50. A gel electrophoresis representation analysis on Multina of 7 placental tissue mRNA samples at a concentration of 100ng/ul for lanes (A1-7) Midkine (MK) primer assay using the DNA sizing ladder on lanes (X1-2) and 2 internal standard markers (LM and UM).

5.4. The Figure 50. is a gel electrophoresis representation analysis that I performed on 7 placental tissue mRNA samples at a concentration of 100ng/ul represents the positions of DNA as a distinct bands for MK with A (3,5 of ≈ 105 bp) and A 2 of ≈ 97 bp) and A4 of ≈ 102 bp. The lane showed peaks for MK at a concentration of 100ng/ul, but A1, A6 and A7 lanes showed no DNA base pairs.



↑

A7 lane of ≈ 162 bp

Figure 51. A gel electrophoresis representation analysis on Multina of 7 placental tissue mRNA samples at a concentration of 100ng/ul for Succinate dehydrogenase complex subunit A (SDHA) primer assay (A1-7) analysis using the DNA sizing ladder on lanes (X1) and 2 internal standard markers (LM and UM).

5.5. Figure 51 and table 25 and 26 is a gel electrophoresis representation analysis of the time course degradation that I performed on 7 different placental tissue mRNA samples at a concentration of 100ng/ul for endogenous control, a housekeeping gene SDHA primer assays analysis that remains stable under pathophysiological and experimental conditions by qRT-PCR was measured for normalization between tissue samples. The positions of DNA as a distinct band for A1 lane of ≈ 162 base pairs (bp), A2 lane of ≈ 162 bp, A3 lane of ≈ 165 bp, A4 lane of ≈ 162 bp, A5 lane of ≈ 162 bp, A6 lane of ≈ 162 bp and A7 lane of ≈ 162 bp.

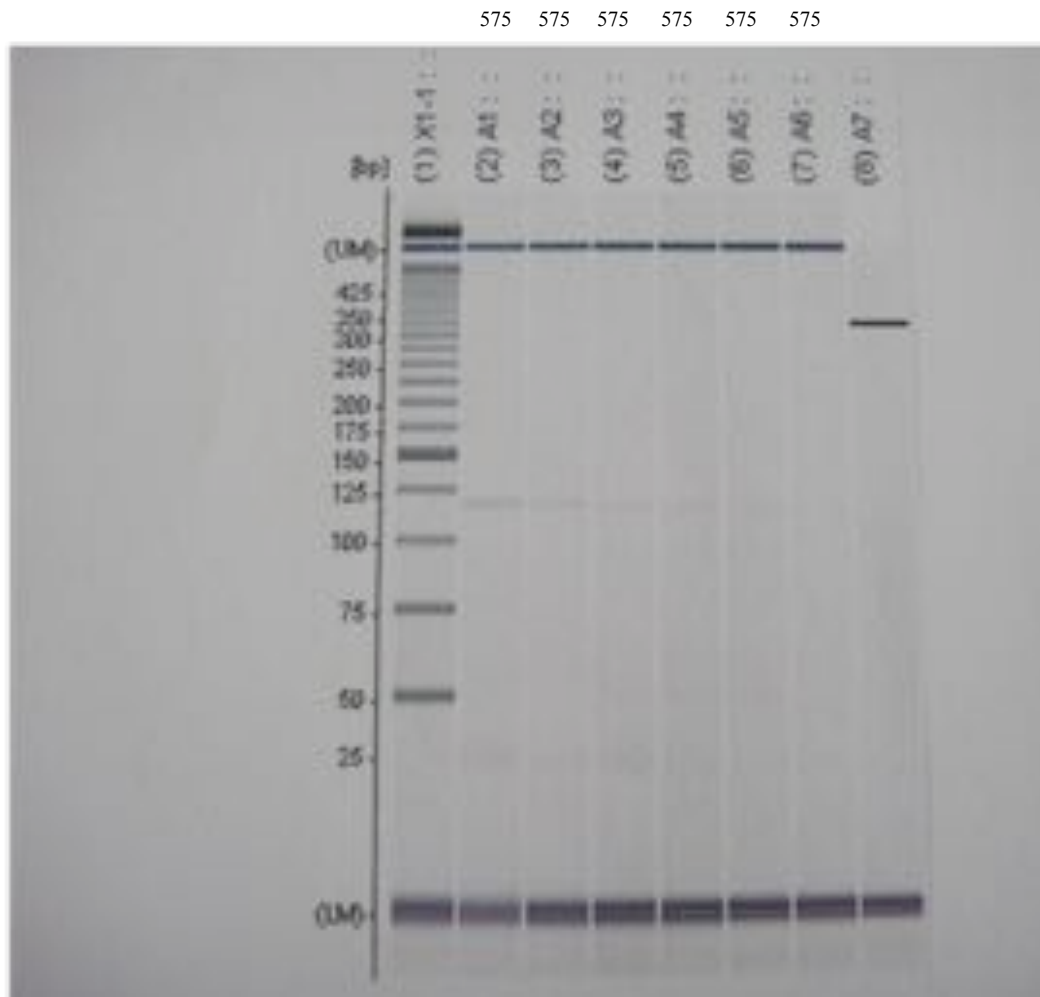


Figure 52. A gel electrophoresis representation analysis on Multina of 7 placental tissue mRNA samples with their sensitivity detection for dilutions at a concentration of 100ng/ul for Fibroblast growth factor 2 (FGF2 primer assay: analysis using the X1 and X2 DNA ladder and 2 internal standard markers (LM and UM)).

5.6. In Figure 52 and Table 28 is a gel electrophoresis representation analysis that I performed on 7 different placental tissue mRNA samples. I demonstrated their sensitivity detection for dilutions at a concentration of 100ng/ul for FGF2 primer assay. This showed that the visible bands are obtained at highest lane A (1, 2, 3,). Lane A (4, 5, 6 and 7) represents decreasing dilutions using neat FGF2. However, A7 showed no DNA bp.

Sample Number of 7 different placental tissues	Concentration (100ng/ul)			
	Placental Growth factor	Fibroblast Growth factor 2	Pleiotrophin	Midkine
1. PE	0.62	3.66	0.56	0.33
2. PE	1.08	5.27	0.58	1.17
3. Control	0.60	1.44	0.50	0.15
4. PE	16.45	71.98	20.28	5.64
5. Control	0.67	2.84	0.98	0.35
6. Control	0.57	2.42	0.63	0.16
7. Control	0.69	1.83	0.82	0.16

Table 28. The 7 different placental tissue mRNA samples with their sensitivity dilutions at a concentration of 100ng/ul for Placental growth factor (PLGF), Fibroblast growth factor 2 (FGF2), Pleiotrophin (PTN), and Midkine (MK) primer assays.

The 7 different placental mRNA samples of control and pre-eclampsia (PE) were determined by RT-PCR of heparin binding growth factor primer assays: for fibroblast growth factor 2 (FGF2), placental growth factor (PLGF), midkine (MK), pleiotrophin (PTN) and to endogenous housekeeping gene Succinate dehydrogenase complex subunit A (SDHA) as presented in Table 28. Pre-eclampsia (PE-1, 2, and 4) with

their sensitivity dilutions at a concentration of 100ng/ul for PLGF of 0.62ng/ul, FGF2 of 3.66ng/ul, PTN of 0.56ng/ul and MK of 0.33ng/ul, primer assays.

PE-2 at a concentration of 100ng/ul for PLGF of 1.08ng/ul, FGF2 of 5.27ng/ul, PTN, of 0.58ng/ul and MK of 1.17ng/ul, primer assays. Control-3 at a concentration of 100ng/ul for PLGF of 0.60ng/ul, FGF2, of 1.44ng/ul, PTN of 0.50ng/ul and MK, of 0.15ng/ul, primer assays. PE-4 at a concentration of 100ng/ul for PLGF of 16.45ng/ul, FGF2 of 71.98ng/ul, PTN of 20.28ng/ul and MK of 5.64ng/ul, primer assays. Control-5 at a concentration of 100ng/ul for PLGF of 0.67ng/ul, FGF2 of 2.84ng/ul, PTN of 0.98ng/ul and MK of 0.35ng/ul, primer assays. Control-6 at a concentration of 100ng/ul for PLGF of 0.57ng/ul, FGF2 of 2.42ng/ul, PTN of 0.63ng/ul, MK of 0.16ng/ul, primer assays. Control-7 at a concentration of 100ng/ul for PLGF of 0.69ng/ul, FGF2 of 1.83ng/ul, PTN of 0.82ng/ul, and MK of 0.16ng/ul primer assays.

Growth factors	Control versus PE P= value; median= value
FGF2	Con m=2.13 PE m= 5.27 P= 0.03*
PLGF	Con m= 0.64 PE m= 1.08 P=0.11
MK	Con m= 0.16 PE m=1.17 P= 0.06
PTN	Con m= 0.73 PE m= 0.58 P=0.57

Table 29. Comparison of 7 placental tissue mRNA expression with their sensitivity at concentration (100ng/ul) for the following Heparin-binding growth factors primer assays using the Mann Whitney *U* test. Abbreviations: fibroblast growth factor 2 (FGF2), placental growth factor (PLGF), midkine (MK), and pleiotrophin (PTN). The control (Term (TL + TNL) not in labour) was compared versus pre-eclampsia (PE). The median is given above and p-values are shown. The p-values are indicated $p < 0.05^*$ and $p < 0.01$. **

As shown in Table 29. the comparison of 7 different placental mRNA levels were expressed as the ratio of heparin-binding growth factor primer assays at a concentration of 100ng/ul: for fibroblast growth factor 2 (FGF2), placental growth factor (PLGF), midkine (MK), pleiotrophin (PTN) and to endogenous house keeping gene as a control Succinate dehydrogenase complex subunit A (SDHA) required for normalization between placental tissue samples using the Mann Whitney *U* test.

This comparison was between control (Term (TL + TNL) not in labour) versus pre-eclampsia with their sensitivity at concentration 100ng/ul for PLGF of control $m = 0.64$, PE $m = 1.08$, ($p = 0.11$), FGF2 of control, $m=2.13$, PE $m = 5.27$, ($p = 0.03^*$), MK of control, $m = 0.16$, PE $m=1.17$, ($p= 0.06$), and PTN of control, $m=0.73$, PE $m= 0.58$, ($p = 0.57$) primer assays. FGF2 of control, $m = 2.13$, PE $m = 5.27$, and ($p= 0.03^*$) in the placenta was confirmed at the mRNA level by RT-PCR, only showed significant difference using Mann Whitney U test.

Chapter 6

Discussion

The main findings of these studies that tested the novel hypothesis for this cohort study of 87 women is that the expression of heparin binding growth factor (HBGFs) in normal placental development was altered in the specific disorder of pre-eclampsia. This was the first study at Middlesex University London in United Kingdom of a cohort of 87 women of normal pregnancies including placenta from women with pre-

eclampsia, pre-term labour, term labour and term non-labour. This first technique I performed on immunohistochemical localization showed the following growth factors VEGF, PLGF, FGF2, HGF, PDGF-BB, HB-EGF, CD105, PTN, and MK that were expressed in the human placenta. This present study has identified these growth factors that have been extensively studied as previously reported by (Duff *et al*, 2003; Tseng *et al*, 2006; Clark *et al*, 1998; Leach *et al*, 2002; Zhong *et al*, 2006; Gurski *et al*, 1990; Kilby *et al*, 1996; Milner *et al*, 1992; and Shulte *et al*, 1996). The purpose of observation that MK and PTN are present is a potential novel finding because little is known. The present study identified the localisation of receptors for VEGF, PLGF, and FGF2 in the human placenta.

Many studies have investigated the potential of growth factors in early pregnancy as to act as predictors of pre-eclampsia. This study's important discovery was devoted to demonstrating how several of heparin binding growth factors in normal pregnancy have altered the expression in association with the specific disorder known as pre-eclampsia. The significance our immunohistochemical findings for VEGF, PLGF, FGF2, HGF, PDGF-BB, HB-EGF, CD105, PTN, and MK expression, is that they have an important role in regulation of villous trophoblast development, promoting endothelial cell proliferation, vasculogenesis, and angiogenesis (Benirschke, 2006; Kay *et al*, 2010; Kingdom *et al*, 2000; Lyall and Belfort, 2007; Pijnenborg, 2010). However, the significance of our immunohistochemical findings for HB-EGF, CD105, PTN, and MK is that they have an important role in regulation of villous development, vasculogenesis, and angiogenesis (Benirschke, 2006; Kay *et al*, 2010; Lyall and Belfort, 2007; and Pijnenborg, 2010).

6.1. FGF2 expression, localisation of FGF2R-1 receptors in the human placenta and pre-eclampsia.

I found that the FGF2 expression was confined to villous trophoblast and endothelial cells as shown in Figure 28a and negative control for pre-eclampsia as in Figure 28j.

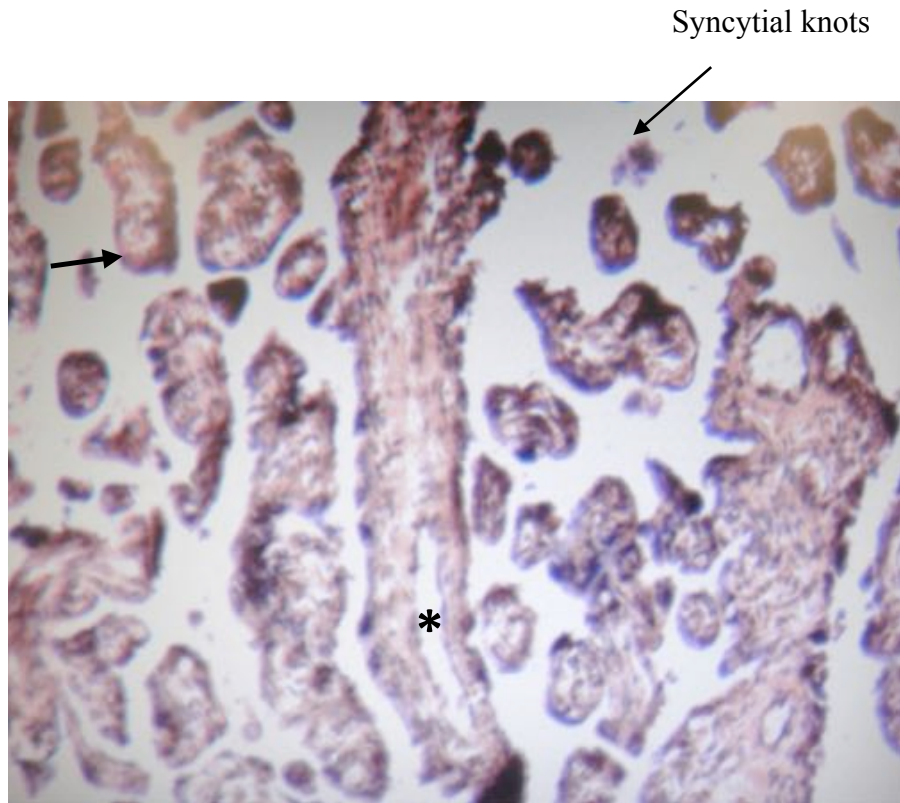


Figure 28a.

Immunohistochemical positive staining in villous trophoblast (both syncytiotrophoblast and cytotrophoblast), in Figure 28a. for fibroblast growth factor 2 (FGF2) expression, 3 = moderate to strong, Magnification of original (x101) distributed in placental tissues for Pre-eclampsia (PE), (n = 19). Magnification of original (x401). The arrow indicates the trophoblast and (*) the endothelial cells.

Syncytial knots

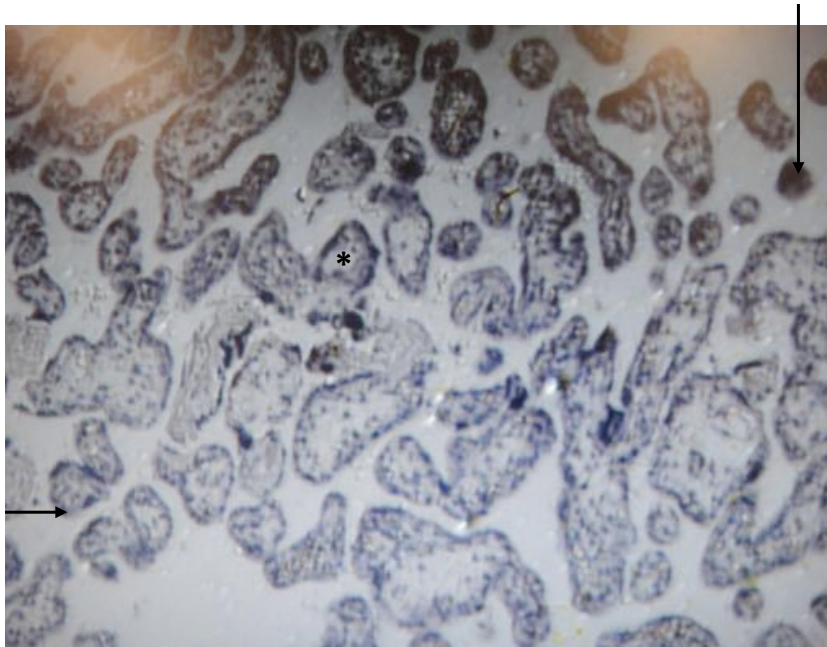


Figure 28j.

Immunohistochemical representative negative control, 0 = none, without the antibody distributed in placental tissues in Figure 28j. for Pre-eclampsia (PE), (n = 19) in the absence of citrate buffer. Magnification of original (x401). The arrows indicate the trophoblast and the endothelial cells. The arrow indicates the trophoblast and (*) the endothelial cells.

The FGF2 expression was significantly lower at term labour compared to the other placenta groups studied. There was no significant difference in staining in pre-eclampsia when compared to any of the other placenta groups studied. The fibroblast growth factor 2 (FGF2) is a potent angiogenic factor and promotes endothelial cell proliferation and vasculogenesis. Shams and Ahmed, (1994) demonstrated FGF2 gene expression in the syncytiotrophoblasts and foetal membranes at term human placenta by *in situ* hybridization. Hill *et al's*, (1995) studies in human pregnancy revealed that maternal and cord serum levels of FGF2 correlated with foetal weight. The effect on foetal growth was also accompanied by alterations in the placenta growth suggesting that FGF2 may exert its effects by influencing placental development. Luo *et al*, (2011) investigated FGF2 release by endothelial cells of uterine spiral arteries. They showed decreasing MMP9 and increasing TIMP1 production in extravillous trophoblasts (EVT) in response to stress, resulting in reduced EVT invasion and shallow implantation of the placenta. Wang *et al*, (2009) showed that hypoxia promotes FGF2- and VEGF-stimulated cell proliferation in human placenta.

I identified FGF2R-1 receptor expression as shown in Figure 32 and found it to be localised to villous, extra villous trophoblasts, syncytiotrophoblast, villous mesenchyme and Hofbauer cells.

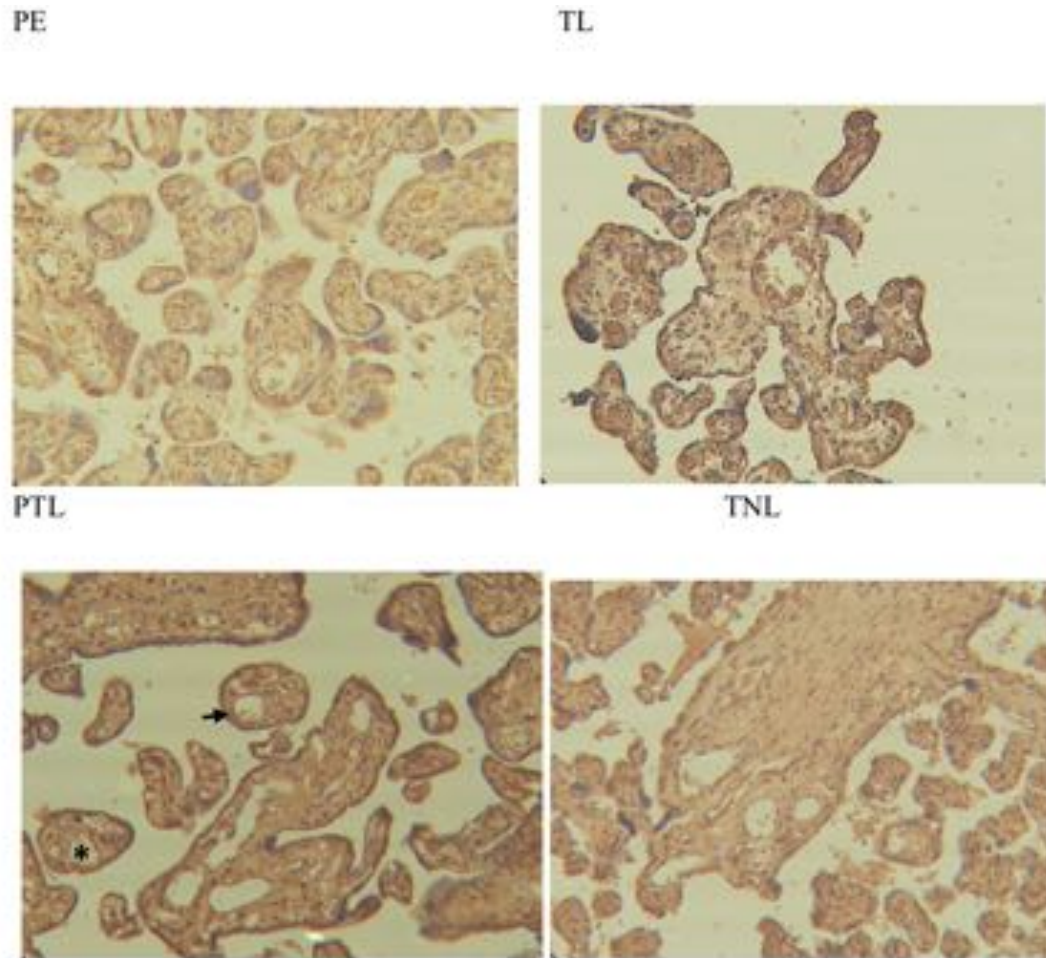


Figure 32. Immunohistochemical moderate to strong positive staining in villous trophoblast (both syncytiotrophoblast and cytotrophoblast), weaker staining in the mesenchyme, and in the core foetal vessels for fibroblast growth factor 2 (FGF2) receptor expression distributed in placental tissues for the four groups PE-Pre-eclampsia, (n = 12), and PTL-Pre-term Labour, (n = 6), TL-term labour, (n = 22), and TNL-Term-non-Labour, (n = 21), 3=moderate to strong with a magnification of x401. The arrow indicates the trophoblast and (*) the endothelial cells.

Arany and Hill, (1998) observed this, but they found FGF2R-1 expression in placenta from diabetic women elevated compared to those of normal women. Anteby *et al*, (2005) found FGF2R1 was expressed only within the villous stroma and FGF2R1-4

expression was abundant in the Hofbauer cells. The mesenchyme trophoblast interactions are important for regulation of villous development (Anteby *et al*, 2005).

There are 23 members of the FGF family (Haque *et al*, (2007). The human FGF gene produces FGF-1, FGF-2, FGF-3, FGF-4, FGF-5, FGF-6, FGF-7, FGF-8, FGF-9, FGF-10, FGF-11, FGF-12, FGF-13, FGF-14, FGF-15, FGF-16, FGF-17, FGF-18, FGF-19, FGF-20, FGF-21, FGF-22, FGF-23 respectively by alternative exon splicing processes (Haque *et al*, 2007). Some FGFs exert their effects by interacting with 4 different (FGFR-1-4) receptors (Anteby *et al*, 2005; Folkman and Shing, 1992; Li *et al*, 1999; and Haque *et al*, 2007).

It's the interaction of VEGF and FGF2 with specific, high affinity cognate receptors located in plasma membrane of target cells that triggers events inside the cells promoting DNA synthesis (Anteby *et al*, 2005; Folkman and Shing, 1992; Li *et al*, 1999; and Haque *et al*, 2007). The FGF2R-1, VEGF receptor-1 (VEGFR-1, FLt-1) and VEGF receptor-2 (VEGFR-2, Flk-1/KDR) consists of protein-tyrosine kinases. The significance of our immunocytochemical localization of FGF2R-1, VEGF R1 and 2 placental expression is that these receptors have an important role in regulation of vasculogenesis, angiogenesis and villous trophoblasts development. Alterations of FGF2R-1, VEGF receptor-1 (VEGFR-1, FLt-1) and VEGF receptor-2 (VEGFR-2, Flk-1/KDR) expression of which might be associated with the etiology of pre-eclampsia (Anteby *et al*, 2005; Folkman and Shing, 1992; Li *et al*, 1999; and Haque *et al*, 2007). These receptor findings suggest a possible physiological mechanism involved in pre-eclampsia (Anteby *et al*, 2005; Folkman and Shing, 1992; Li *et al*, 1999; and Haque *et al*, 2007).

Previous evidence for hCG, VEGF and FGF2 during blastocyst implantation *in vivo* was reported. The cohort study of Paiva *et al*, (2011) provided a role for human chorionic gonadotrophin (hCG) in selectively modulating endometrial production of cytokines and growth factors such as VEGF and FGF2 that are important for human

endometrial receptivity during blastocyst implantation *in vivo*. They examined the effects of FGF2 from primary human endometrial epithelial cells hEEC line (ECC-1) associated with endometrial receptivity by adhesion assays. Their results showed that FGF2 stimulated phosphorylation of ERKI/2 in hEEC with no effect on ERKI/2 abundance and stimulated hEEC adhesion to fibronectin and collagen IV.

6.1.2. VEGF expression, localisation of VEGFR1 and VEGFR-2 receptors in the human placenta, and pre-eclampsia.

I found that the expression of VEGF was localised to villous trophoblasts and endothelial cells as shown in Figure 28c.



Figure 28c.

Immunohistochemical positive staining in villous trophoblast (both syncytiotrophoblast and cytotrophoblast), in Figure 28c. for vascular endothelial growth factor, placental growth factor (VEGF) expression, 3 = moderate to strong, Magnification of original (x401), distributed in placental tissues for Pre-eclampsia (PE), (n = 19). Magnification of original (x101). The arrow indicates the trophoblast and (*) the endothelial cells.

There was no significant difference in staining in pre-eclampsia, but expression showed a significant difference in term Labour when compared to any of the other placenta groups studied. There was no significant difference in VEGF expression in term compared to preterm and in the presence or absence of labour.

Previous studies by Demir *et al*, (2004) demonstrated that VEGF is strongly expressed in the villous cytotrophoblast cells and Hofbauer cells. VEGF is an endothelial-specific mitogen with a role in promoting angiogenesis and its receptors in the human placental villi are found in early pregnancy because placental vasculogenesis and angiogenesis are sequential events. Vuorela *et al*, (1997) in addition studied VEGF mRNA in the term human placenta by *in situ* hybridization in the chorionic mesenchyme and villous trophoblasts. They found that the immunostaining was co-localised with expression of VEGF protein in the vascular endothelium. Khaliq *et al*, (1996) demonstrated localisation of PLGF in human term placenta. Vuorela *et al*, (1997) showed the expression of PLGF mRNA in the term human placenta by *in situ* hybridization in the chorionic mesenchyme and villous trophoblasts. They found that the immunostaining was co-localized the expression of PLGF protein in the vascular endothelium. These studies by (Khaliq *et al*, 1996; and Vuorela *et al*, 1997) examined the VEGF mRNA levels.

Demir *et al*, (2004) suggested that VEGF-A known as VEGF is most abundant form in the VEGF family The VEGF gene produces 5 different VEGF isoforms: VEGF121, VEGF145, VEGF165, VEGF189 and VEGF206 respectively by alternative exon splicing processes. VEGF-A function depends on their interactions with heparin-binding growth factors to extracellular matrix and their receptors. However, the solubility of each isoform is different because of the presence or absence of heparin-binding domains (Demir *et al*, 2004).

VEGF is said to have a very important role in the pathogenesis of pre-eclampsia. Many studies have investigated the potential of growth factors in early pregnancy to act as predictors of pre-eclampsia (Anderson *et al*, 2012; and Lyall and Belfort, 2007).

There are many studies that have examined VEGF expression in pre-eclampsia with conflicting results. Some studies reported reduced levels of VEGF-A in maternal circulation (Cheng *et al*, 2001; Lyall *et al*, 1997; Reuvekamp *et al*, 1999; Sgambati *et al*, 2004; Sharkey *et al*, 1996; Simmons *et al*, 2000; and Vuorela *et al*, 1999). Other studies have shown increased levels of VEGF-A in maternal circulation, compared with normal pregnancies (Baker *et al*, 1995, Hefler *et al*, 2000; and Kupfermanc *et al*,

1997). Rainheim *et al*, (2005) study found that VEGF expression was unaltered at the mRNA level.

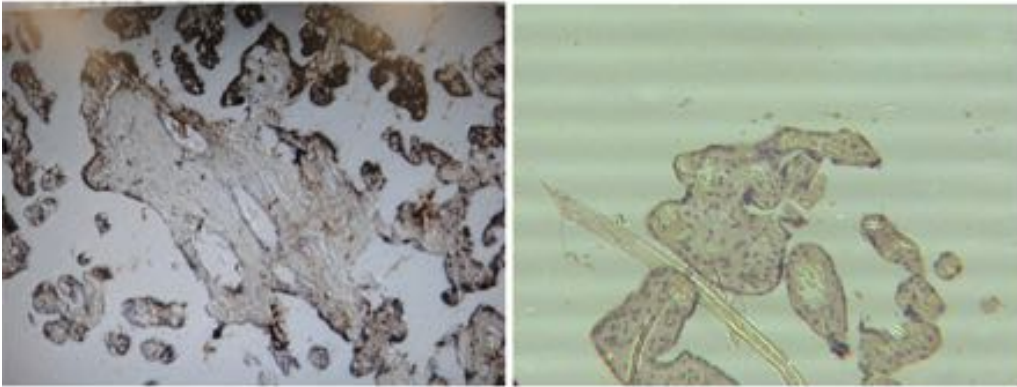
VEGF and their receptors are essential for embryonic vascular development because loss of a single VEGF allele results in embryonic death. Shalaby *et al*, (1995) study demonstrated that lack of VEGFR-2 results in absence of endothelial cells with severe impairment for the development of cardiovascular system. Ahmed *et al*, (1995) and Clark *et al*, (1996) study confirmed VEGF and VEGFR-1 expression in the placenta throughout gestation. Lash *et al*, (1999) study suggested that the possible effect of VEGF on trophoblast invasion and motility were mediated by functional receptors. Ribatti *et al*, (2008) suggested several mechanisms that are involved in angiogenic function of PLGF. As, PLGF binds to VEGFR-1 in the endothelial cells which facilitates VEGF binding and activation of VEGFR-2. Li *et al*, (2005) study demonstrated that Hypoxia promotes VEGF and VEGFR-1(Flt-1), but not that of VEGFR-2 and down regulates PLGF expression and production.

In the present study, I identified (VEGFR-1, sflt-1)/VEGFR-2 receptor expression as shown in Figure 33 and 34 was localized to villous and extra villous trophoblasts, villous mesenchyme, maternal decidua cells, and Hofbauer cells.

Sharkey *et al*, (1993); Jackson *et al*, (1994); Clark *et al*, (1996); Khaliq *et al*, (1996); and Schiessl *et al*, (2009); and Andraweera *et al*, (2012) observations throughout gestation were consistent with this. They were found on blood vessels that are consistent with the observations of (Demir *et al*, 2004) where showed that VEGF expression was localised to villous cytotrophoblast cells, Hofbauer cells in the human placental villi during early pregnancy. The VEGFR-1, (sFlt-1) receptors during placental development are found on vasculogenic and angiogenic precursors cells.

PE

TL



PTL

TNL

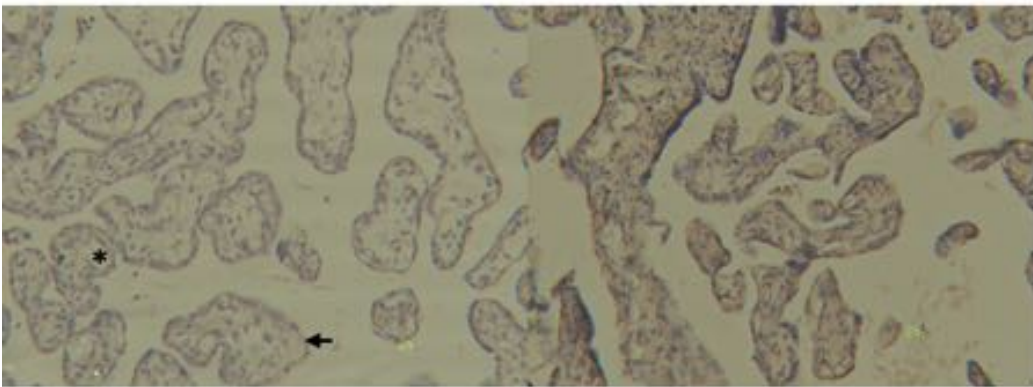


Figure 33. Immunohistochemical moderate to strong positive staining in villous trophoblast (both syncytiotrophoblast and cytotrophoblast), weaker staining in the mesenchyme, and in the core foetal vessels for vascular endothelial growth factor (VEGF) receptor I expression distributed in placental tissues for the four groups PE-Pre-eclampsia, (n = 12), 4=strong, of magnification of original x 101, and TL-term labour, (n = 22), 2=moderate, PTL-Pre-term Labour, (n = 6), and TNL-Term-non-Labour, (n = 21), 3=moderate to strong with a magnification of original x401. The arrow indicates the trophoblast and (*) the endothelial cells.

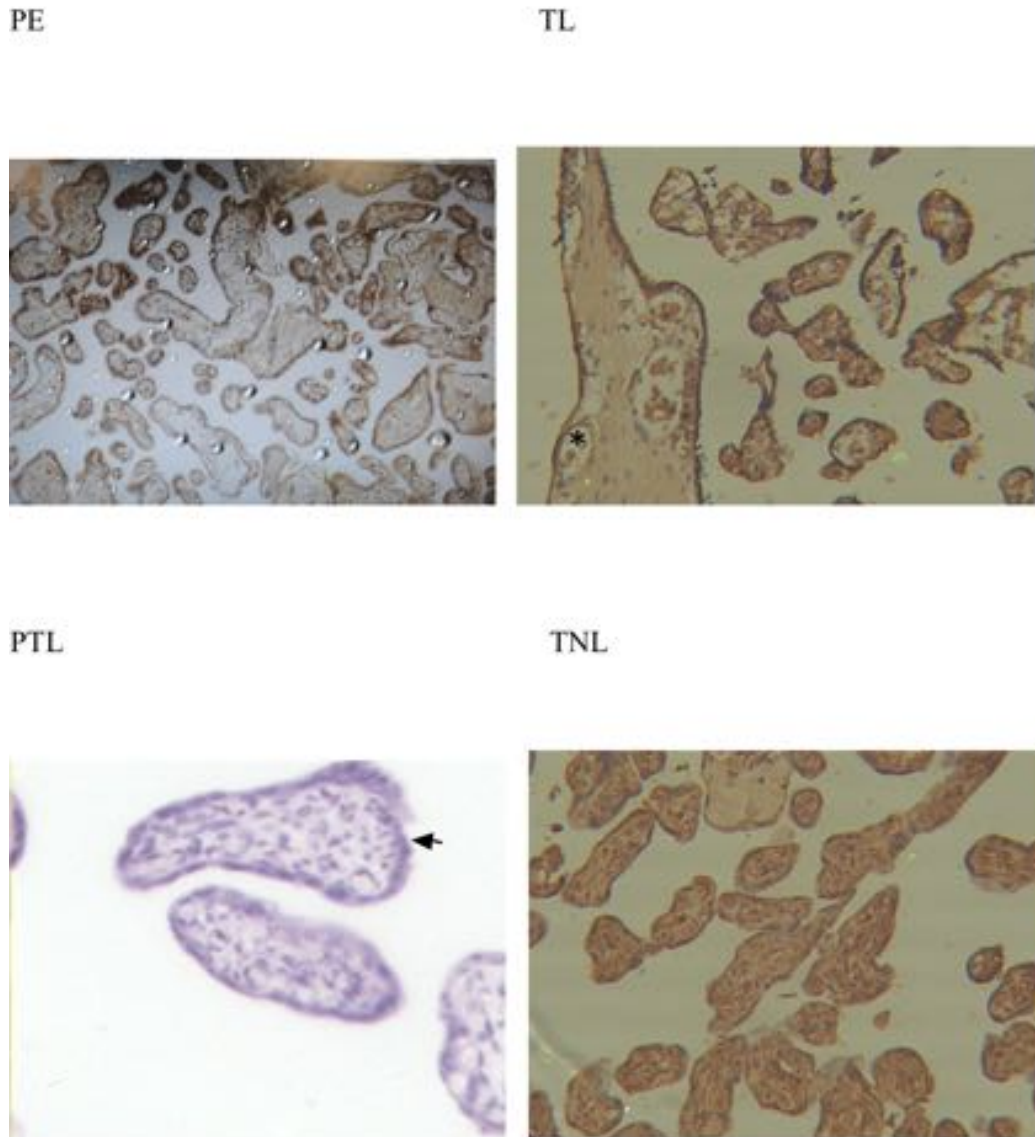


Figure 34. Immunohistochemical moderate to strong positive staining prominent in villous trophoblast (both syncytiotrophoblast and cytotrophoblast), weaker staining in the mesenchyme, and in the core foetal vessels for vascular endothelial growth factor (VEGF) receptor II expression in placental tissues for the four groups PE-Pre-eclampsia, (n = 12), 4=strong, magnification of original x 101, TL-term labour (n = 22), and TNL-Term-non-Labour (n = 21), and PTL-Pre-term Labour, (n = 6), 3=moderate to strong with a Magnification of x 401 The arrow indicates the trophoblast and (*) the endothelial cells.

Lala *et al*, (2012) reported that the actions of decorin, a transforming growth factor (TGF)-B were mediated by its binding to multiple tyrosine kinase receptors, including VEGFR-2. They examined the effect of decorin and their EVT cells were treated with VEGF121 for the migration assay. Their results demonstrated that EVT cell migration was enhanced in the presence of VEGF121 and suggested that decorin inhibits VEGFA stimulation of trophoblast migration and endovascular differentiation of EVT. Thus, angiogenesis may provide insights for pathogenesis of pre-eclampsia.

Sundrani *et al*, (2013) employs tissue from pre-eclamptic women, and examining their genes expression of the promoter CpG methylation of VEGF and its receptors, VEGFR-1, /Flt-1 and VEGFR-2 in human term and preterm placentae. Their results indicated that alterations of DNA methylation patterns of VEGF and Flt-1 in pre-eclampsia as compared to the normotensive group may be involved in pathophysiology of pre-eclampsia. G-protein receptors play an important role in control of cellular function. Two subunits are involved in modulation of hypertension and endothelial, protein subunit α -11 (GNA11) and subunit α -14 (GNA14). Using immunocytochemistry Zhao *et al*, (2014) found that these subunits were co-localised in primary trophoblasts, villous stromal cells and endothelial cells in placentas and that GNA 14 elevated expression was observed in severe pre-eclampsia placentae.

G-protein linked receptors are distinct from the tyrosine kinases, but both families appear to activate, directly or indirectly to cellular processes. The significance of these findings is the confirmation that genes expression of these VEGFR-1, /Flt-1 and VEGFR-2 receptor have an important role in pathophysiology of pre-eclampsia.

6.1.3. PLGF expression and localisation of receptors in the human placenta and pre-eclampsia.

The PLGF receptor expression has been described as abundant in villous trophoblasts (Khaliq *et al*, 1996; Vuorela *et al*, 1997; and Andraweera *et al*, 2012) interestingly as present data show. Andraweera *et al*, (2012) demonstrated that the PLGFR-1 expression for the FNL group was localised in the extravillous trophoblasts within the maternal decidua. Several other authors have made similar findings (Khaliq *et al*, 1996; Vuorela *et al*, 1997; and Andraweera *et al*, 2012). The PLGFR-1 expression for

term is abundant and localised to villous trophoblast (Khaliq *et al*, 1996; and Vuorela *et al*, 1997).

Maglione *et al*, (1991) study identified PLGF in a placental cDNA library. PLGF was structurally related to the different VEGF molecules. The PLGF gene produces 2 different PLGF isoforms: PLGF-1 known as (PLGF131), and PLGF-2 known as (PLGF152) respectively by alternative exon splicing processes (Maglione *et al*, 1991)

I found that the expression of PLGF was localised to villous trophoblasts and endothelial cells as shown in Figure 28b.

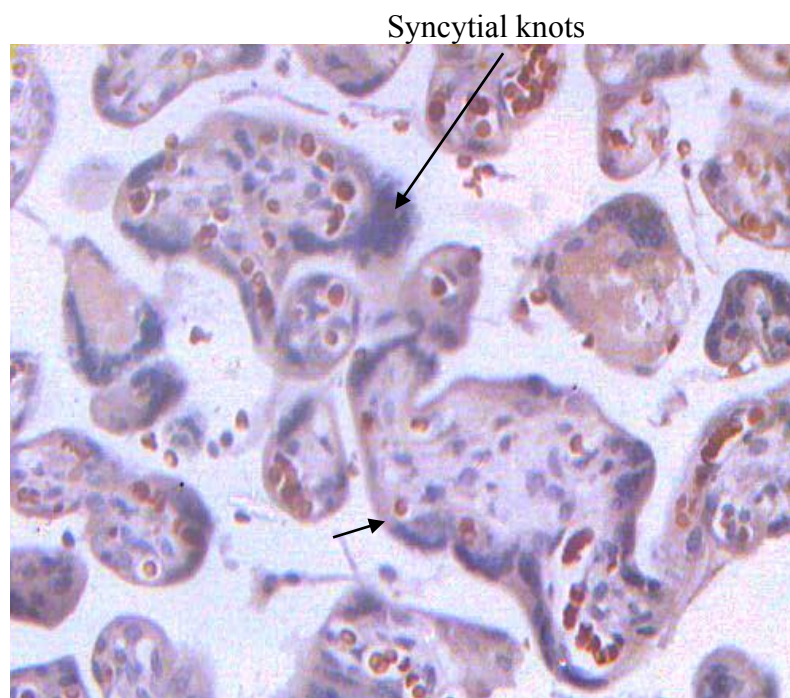


Figure 28b.

Immunohistochemical positive staining in villous trophoblast (both syncytiotrophoblast and cytotrophoblast), in Figure 28b. for placental growth factor (PLGF) expression, 2 = moderate distributed in placental tissues for pre-eclampsia (PE), (n = 19). Magnification of original (x401). The arrows indicate the trophoblast and (*) the endothelial cells.

The expression of PLGF score was significantly lower after labour onset. PLGF levels were significantly higher in pre-term compared to term placentas. Rainheim *et al*, (2005) showed that there is no evidence of abnormal VEGF expression (VEGF mRNA levels) at delivery in the decidua's basalis or the placenta in pregnancies

complicated by pre-eclampsia compared with the controls. Sgambati *et al*, (2004) showed that the levels of VEGF mRNA were higher in the gestational hypertension cases and lower in the cases of pre-eclampsia. PLGF showed a significant increase in pre-eclampsia compared to other group studied. Maynard *et al*, (2003) study of *in vivo* animal model of the rat and mouse established that overexpression of VEGFR-1, sFlt-1 using recombinant adenoviral vector developed hypertension, proteinuria, and glomerular endotheliosis except for soluble VEGF receptor-2 (sFlk-1), because these conditions were similar to pre-eclampsia. Maynard *et al*, (2003), suggested that development of pre-eclampsia resulted from the imbalance of VEGF and PLGF. This effect was prevented in pregnant rats by exogenous administration of VEGF and PLGF. Maynard *et al*, (2003), hypothesized that excess circulating soluble fms-like tyrosine kinase 1 (sFlt-1) was associated with decreased free VEGF and PLGF in the serum and are secreted by the placenta in pre-eclampsia women. This may contribute to endothelial dysfunction, hypertension, and proteinuria due to an imbalance between the circulating VEGF and PLGF (Maynard *et al*, (2003). Several studies on expression of anti-angiogenic growth factors, soluble fms-like tyrosine kinase 1 (sFlt-1), transforming growth factor β (TGF- β) and endoglin are upregulated in pregnancies affected with pre-eclampsia. However, pro-angiogenic growth factors such as VEGF, and PLGF are downregulated in these pregnancies inadequate cytotrophoblast invasion, endothelial dysfunction and impaired placental angiogenesis in pre-eclampsia women (Young *et al*, 2010; Maynard *et al*, 2003; and Ahmad and Ahmed, 2004).

Numerous studies have documented that beginning in the early second trimester and as early as 10 to 11 weeks of gestation, PLGF concentrations in women who go on to develop pre-eclampsia are lower than those of normotensive controls (Bersinger *et al*, 2004; Krauss *et al*, 2004; Levine *et al*, 2004; Livingston *et al*, 2000; Taylor *et al*, 2003; Thadhani *et al*, 2004; Tidwell *et al*, 2001; Torry *et al*, 2003; Polliotti *et al*, 2003; and Su *et al*, 2001). Athanassiades and Lala, (1998) showed that PLGF promote EVT cell proliferation and suggest that PLGF and VEGF receptor-1 (VEGFR-1, Flt-1) interactions may be regulated by heparan sulphate proteoglycans (HSPGs) *in situ*. In pre-eclampsia, PLGF concentrations begin to decrease before the appearance of hypertension and proteinuria, during the 5 weeks before the onset of disease. More than 5 weeks preceding occurrence of the maternal syndrome, the difference in PLGF

levels between normotensive controls and those who later developed pre-eclampsia was less marked (Levine, 2004). Various studies have investigated PLGF in the first or second trimester as a possible predictor of pre-eclampsia (Levine, 2004; Thadhani *et al*, 2004; Taylor *et al*, 2003; Tidwell *et al*, 2001; Tjoa *et al*, 2001; Polliotti *et al*, 2003; and Su *et al*, 2001).

6.1.4. CD105 expression in the human placenta and pre-eclampsia.

I found that the CD105 expression was confined to villous trophoblast, and endothelial cells as shown in Figure 28f.

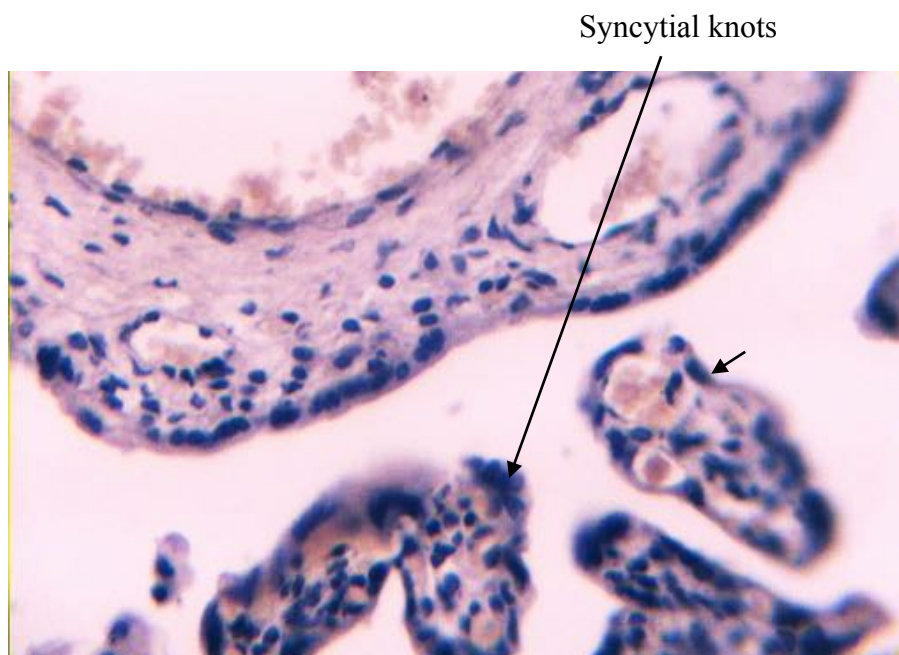


Figure 28f.

Immunohistochemical positive staining in villous trophoblast (both syncytiotrophoblast and cytotrophoblast), in Figure 28f. for cluster differentiation (CD105) expression, 2 = moderate, distributed in placental tissues for Pre-eclampsia (PE), (n = 19). Magnification of original (x401 and x402). The arrows indicate the trophoblast and (*) the endothelial cells.

CD105 expression in staining intensity was significantly higher in term Labour compared to any of the other placenta groups studied. Gougos *et al*, (1992) showed that the syncytiotrophoblasts of the term placenta contained high levels of CD105. There was no significant difference in staining score in pre-eclampsia when compared to any of the other placenta groups studied. Many of the studies have looked

statistically at CD105, an endoglin, as a potential predictor of pre-eclampsia. Our findings are consistent with those of Duff *et al*, (2003) because the staining expressions for CD105 were positive. Duff *et al*, (2003) examined staining intensity results for syncytiotrophoblast of term placenta and found 3 out of 4 antibodies used for CD105 produced positive results. CD105 can be categorised as a marker of endothelial cells activation and proliferation, marker of hypoxic stress, undefined endothelial cell antigens and proteins that are expressed in newly formed or remodelled basement membrane. CD105 may be involved in TGF- β signal transduction. It is soluble endoglin known as another anti-angiogenic factor. Studies by Venkatesha *et al*, (2006) suggest that the soluble endoglin derived from syncytiotrophoblast, whose circulating levels are increased in pre-eclampsia. Yinon *et al*'s, (2008) study shows that CD105 is upregulated by hypoxia.

6.1.5. HGF expression in the human placenta and pre-eclampsia.

I found that the expression of HGF was confined to villous trophoblast and endothelial cells as shown in Figure 28e.

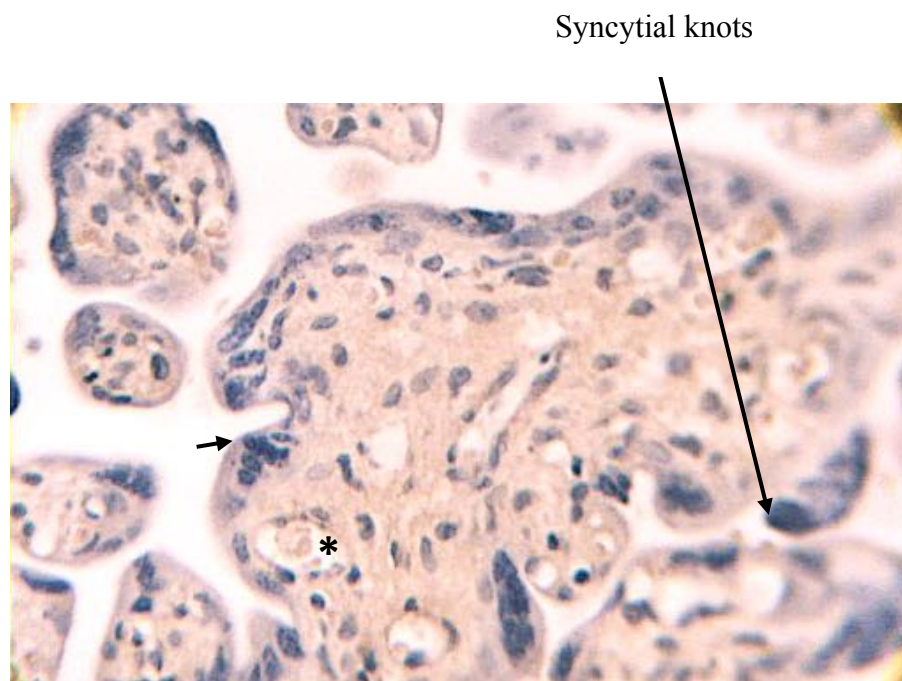


Figure 28e.

Immunohistochemical positive staining in villous trophoblast (both syncytiotrophoblast and cytotrophoblast), in Figure 28e. for hepatocyte growth factor (HGF) expression, distributed in placental tissues for Pre-eclampsia (PE), (n = 19). Magnification of original (x401 and x402). The arrows indicate the trophoblast and (*) the endothelial cells.

HGF expression was significantly higher in pre-term Labour compared to term Labour placentae. The median of HGF was significantly higher for pre-term Labour than term Labour. There was no significant difference in staining in pre-eclampsia when compared to any of the other placenta groups studied. Hepatocyte growth factor is a potent mitogen. It has been shown to stimulate dissociation and mobility of the epithelial cells. In the placenta, HGF promotes trophoblast migration and invasion but does not affect cellular proliferation. The study by Wolf *et al*, (1991), suggested a role for HGF during pregnancy. They demonstrated high levels of HGF mRNA expression and HGF protein was extracted from the human placentae. Kilby *et al*, (1996) showed mRNA for HGF and its receptor c-met in the human term placenta using *in situ* hybridisation. The study of Liu *et al*, (2012) demonstrated in the placental trophoblast cells up regulation by both small interfering RNA (siRNA) gene targeting and protein levels because of mRNA expression of HLX1 in response to HGF.

Kauma *et al*, (1996) examined HGF in the placenta and determined the expression of a tyrosine kinase receptor Met for the ligand HGF. The immunocytochemistry of HGF was localised to the villous core but not to the trophoblast. Met was localised to cytotrophoblast. Somerset *et al's*, (2000) study suggested that HGF-activator (HGF-A) is a circulating serine protease known to be responsible for activation of HGF and is produced via proteolytic cleavage of its zymogen by thrombin. An important regulator of trophoblast growth is the active HGF. The study demonstrated the anti-HGF-A/zymogen antibody immunostaining in placental villi and membranes throughout gestation.

6.1.6. PDGF-BB expression in the human placenta and pre-eclampsia.

I found that the PDGF-BB expression was confined to villous trophoblast and endothelial cells as shown in Figure 28d.



Figure 28d.

Immunohistochemical positive staining in villous trophoblast (both syncytiotrophoblast and cytotrophoblast), in Figure 28d. platelet-derived growth factor (PDGF-BB) expression, 4 = strong distributed in placental tissues for Pre-eclampsia (PE), (n = 19). Magnification of original (x101). The arrows indicate the trophoblast and (*) the endothelial cells.

PDGF-BB expression showed no significant difference in pre-eclampsia when compared to any of the other placenta groups studied. The lack of altered expression in pre-eclampsia suggests that other HBGFs may be more critical for this condition. The platelet-derived growth factor (PDGF) functions are proliferation, survival, movement, deposition of extracellular matrix (ECM) and tissue remodelling. Holmgren *et al*, (1992) demonstrated the expression of PDGF alpha and beta-receptors in different subpopulations of the human placental cytotrophoblast while PDGF-BB was confined to the cytotrophoblast primary cultures. Rolny *et al*, (2002)

showed that heparin did not potentiate the effect on PDGF-BB stimulation of its β -receptor. Gurski *et al*, (1990) and Lash *et al*, (2003) showed that in the human placental PDGFR α / β -receptors are both expressed within the syncytiotrophoblast and villous cytotrophoblast.

6.1.7. HB-EGF expression in the human placenta and pre-eclampsia.

I found that the expression of HB-EGF was localised to villous trophoblast as shown in Figure 28g.

Syncytial knots

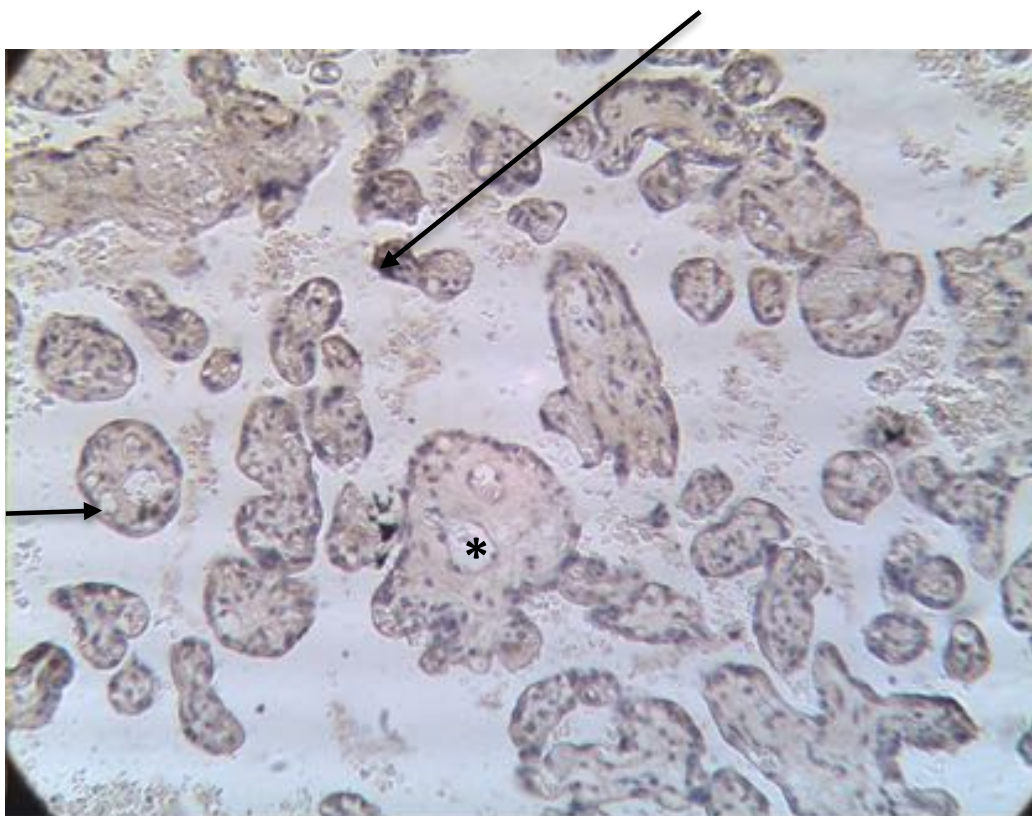


Figure 28g.

Immunohistochemical moderate to strong positive staining in villous trophoblast (both syncytiotrophoblast and cytotrophoblast), for heparin-binding epidermal growth factor (HB-EGF) expression in Figure 28g. 4 = strong, distributed in placental tissues for Pre-eclampsia (PE), (n = 21). Magnification of original (x401). The arrow indicate the trophoblast and (*) the endothelial cells.

The HB-EGF expressions were significantly higher in preterm compared to term placentae. A significant difference was observed in term Labour and term non-Labour of HB-EGF expression. There was no significant difference in pre-eclampsia, when compared to any of the other placenta groups studied. Heparin-binding epidermal-growth-factor (HB-EGF) has strong cytoprotective activity and is an important signalling protein that regulates trophoblast invasion during early placentation. In addition to the cytoprotective effect there is evidence that HB-EGF regulates trophoblast invasion during early placentation. For example in pre-eclampsia, deficient HB-EGF signalling during placental development could impair trophoblast survival, differentiation, and invasion, leading to poor placental perfusion and hypertension. Leach *et al*, (2002) showed that the HB-EGF expression was found in pre-eclamptic pregnancies. Thus, heparin and some associated HBGFs appear to have cytoprotective effects in the placenta. HB-EGF is induced by low oxygen tension and is expressed in both villous and extravillous trophoblasts at high levels in the first trimester and throughout gestation (Yoo *et al*, 1997). Furthermore, HB-EGF expression is downregulated following hypoxia/reperfusion in an *in vitro* model of oxidative stress (Leach *et al*, 2008).

6.1.8 MK expression in the human placenta and pre-eclampsia.

I found that MK was localised to villous trophoblast as shown in Figure 28h.



Figure 28h.

Immunohistochemical positive staining in villous trophoblast (both syncytiotrophoblast and cytotrophoblast), as in Figure 28h. for midkine (MK) expression distributed in placental tissues for Pre-eclampsia (PE), (n = 19). Magnification of original (x401). The arrows indicate the trophoblast and (*) the endothelial cells.

MK functions are involved in neural survival, carcinogenesis, and tissue repair. Qi-Wen Fan *et al*, (2001) showed that MK was present in the chorion, the foetal component of the placenta, whereas pleiotrophin was found in the decidua basalis, the maternal component of the placenta. The distinct expression of midkine and pleiotrophin suggests their differential role in early development in mouse. Midkine (MK) and pleiotrophin (PTN) are implicated in neuronal survival, differentiation and tissue repair, (Muramatsu, 2010). MK expression was significantly lower in pre-eclampsia other group studied.

6.1.9. PTN expression in the human placenta and pre-eclampsia.

I found that PTN was localised to villous trophoblast as shown in Figure 28i.

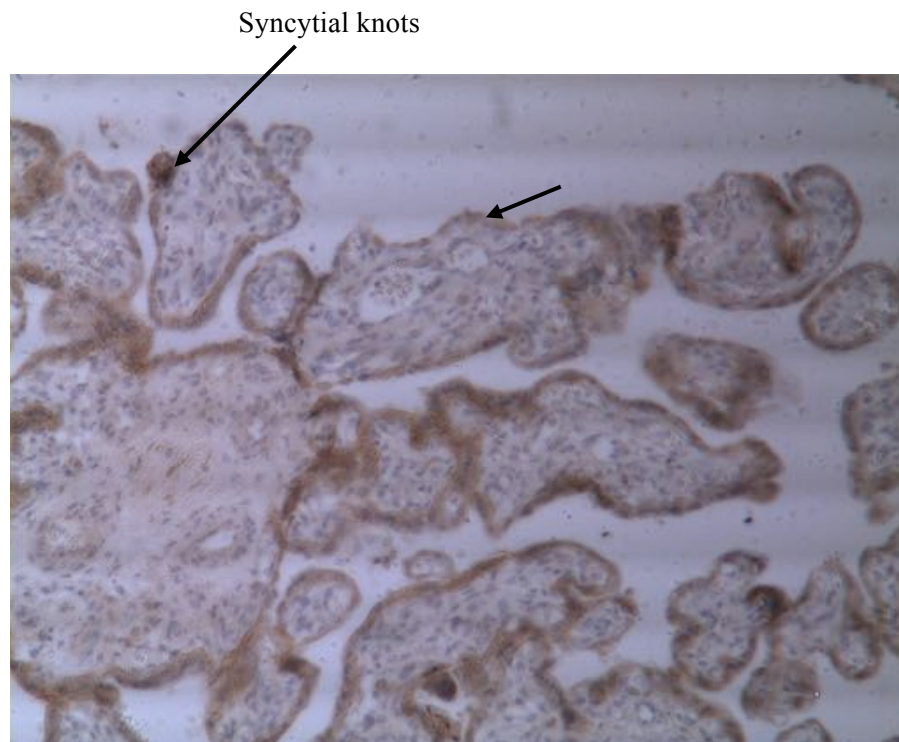


Figure 28i.

Immunohistochemical positive staining in villous trophoblast (both syncytiotrophoblast and cytotrophoblast), as in Figure 28i. for pleiotrophin (PTN) expression, 4 = strong, distributed in placental tissues for Pre-eclampsia (PE), (n = 19). Magnification of original (x401). The arrows indicate the trophoblast and (*) the endothelial cells.

The PTN expression was significantly higher in pre-term labour compared to term placentae. PTN is a heparin-binding protein with mitogenic and transforming effects on the fibroblasts and growth factor activity on the epithelial and endothelial cells. PTN is involved in cell growth, migration and differentiation effects mediated through multiple receptors. Expression of PTN in the trophoblast is found in the human (Schulte *et al*, 1996: and Ball *et al*, 2009). Schulte *et al*, (1996) showed that PTN was detected in normal human trophoblasts cell cultures as early as 9 weeks after gestation and in term placenta. Ball *et al*, (2009) found PTN in foetal macrophages and microvasculature in the villous mesenchymal core. They showed that the villus staining of the cytotrophoblasts suggests that PTN expression commences at

a pre-fusion stage of differentiation. Ball *et al*, (2009) found that different patterns of overlapping expression of PTN and its receptors occur in different trophoblast subtypes. The receptor anaplastic lymphoma kinase (ALK) had a similar staining pattern to PTN in the trophoblasts, suggesting an autocrine action. PTN staining of extravillous cytotrophoblasts and syncytial microvillous membrane was consistent with increasing levels of PTN measured by ELISA in the maternal bloodstream for pregnancy (Ball *et al*, 2009). PTN expression was significantly lower in pre-eclampsia other group studied. There have been reported studies on changes on PTN in the human placenta in patients presenting with pre-eclampsia. The lack of altered expression in pre-eclampsia suggests that other HBGFs may be more critical for this condition.

6.2. Heparin-binding growth factors and *in vitro* villous trophoblast invasion and migration.

The third technique in this study employed BD Fluoroblok was focused on VEGF, PLGF, FGF2, HB-EGF, PDGF, HGF, MK and PTN induced interestingly villous trophoblast invasion and migration studies using the immortalized human villous trophoblast invasive cell-line TCL-1. This thesis essentially uses the technique described by Partridge and Flaherty, (2009) who showed that calcein AM detects invaded cells in their model of Fluoroblok tumour invasion assay. They used human fibrosarcoma HT-1080 invasive cells as a positive control, and NIH 3T3 mouse embryonic fibroblast, a non-invasive choriocarcinoma cells as a negative control. I found that 3T3/NIH and TCL-1 were significantly different using the Mann-Whitney *U* test from HT1080 cell lines of invasion and migration studies. There are no previously reported studies using a similar approach to that employed in this first present study using the immortalized human villous trophoblast invasive cell-line TCL-1. This first study suggests an important novel role for some of the heparin binding growth factors expressed in placenta on migration and invasion through extracellular matrix *in vitro*. This *in vitro* model, using Matrigel are representative of *in vivo* invasion. Invasion and migration are important events in placental development and cellular processes such as angiogenesis, embryonic development, immune response, metastasis, and invasion of cancer cells. Published findings suggest

that VEGF, PLGF, HB-EGF, FGF2, PDGF, and HGF regulate placental function (Anteby *et al*, 2004; Dessai *et al*, 1995; and Hills *et al*, 2006). The studies of others using Matrigel invasion and migration assays found that HBGFs regulation of the process of trophoblast invasion were not clarified.

The effect of villous trophoblast invasion in the presence of HBGFs with no heparin showed increasing concentrations of HB-EGF and PTN stimulated a significant small increase in trophoblast invasion. The increase in trophoblast invasion was stimulated in the presence of VEGF, PLGF, and FGF2 at increasing concentrations was significantly small. The increase in trophoblast invasion in the presence of PDGF was significantly higher, than in cells with no heparin. The trophoblast invasion in the presence of HGF stimulated a significant dose dependent rise for increasing concentrations. The trophoblast invasion was stimulated and its values were similar to the control for increasing concentrations of MK.

6.2.1. *In vitro* villous trophoblast invasion and HBGFs following co-incubation in the presence of heparin.

This study has identified heparin-binding growth factors (HBGFs) that are important *in vivo* regulating trophoblast invasion and are potentially amenable to alteration by heparin. Evidence that the effect of villous trophoblast invasion was stimulated by HBGFs following co-incubation in the presence of heparin. The effect of villous trophoblast invasion was stimulated by HBGFs following co-incubation in the presence of heparin. As shown in Figure 39C. for FGF2, Figure 41E. for PDGF-BB, Figure 42F. for HGF and Figure 43G. for MK produced the greatest stimulation of increase in villous trophoblast invasion with co-incubation of heparin enhanced each of these responses except for PTN. A major strength in this present study is evident from the interesting results from *in vitro* villous trophoblast invasion model demonstrating that heparin has a potential therapeutic use.

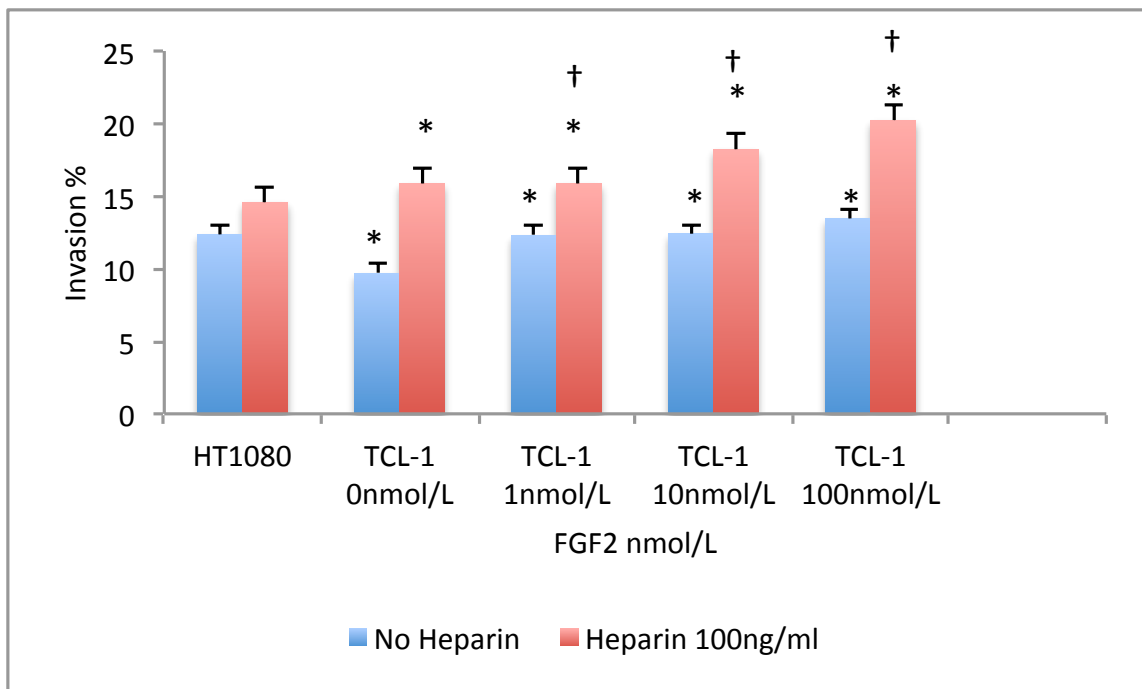


Figure 41C. Represents the percentage of invasion presented as mean \pm SEM for the following cell lines HT 1080 of ($n = 3$, 24.7% \pm 2.08%, and 29.17% \pm 1.56%, $p < 0.05$), and TCL-1 with the presence of fibroblast growth factor 2 (FGF2) expression at ($n = 3$, 9.69% \pm 1.54%, $p < 0.05$) 0nmol/l, ($n = 3$, 12.28% \pm 1.65%, $p < 0.05$) 1nmol/l, ($n = 3$, 12.41% \pm 0.58%, $p < 0.05$) 10nmol/l and ($n = 3$, 13.47% \pm 0.98%, $p < 0.05$) 100nmol/l. In the presence of heparin at ($n = 3$, 15.87% \pm 1.05%, $p < 0.05$) 0ng/ml, ($n = 3$, 15.88% \pm 0.91%, $p < 0.05$) 1ng/ml, ($n = 3$, 18.22% \pm 2.15%, $p < 0.05$) 10ng/ml, and ($n = 3$, 20.24% \pm 3.63%, $p < 0.05$) 100ng/ml. (* $p < 0.05$ vs. 0 growth factor, Mann-Whitney U test; † $p < 0.05$ vs. 0 heparin).

FGF2 stimulated trophoblast invasion in the presence of heparin, at increasing concentrations produced values that were again significantly higher. Therefore, FGF2 response was enhanced following co-incubation in presence of heparin as in Figure 41C.

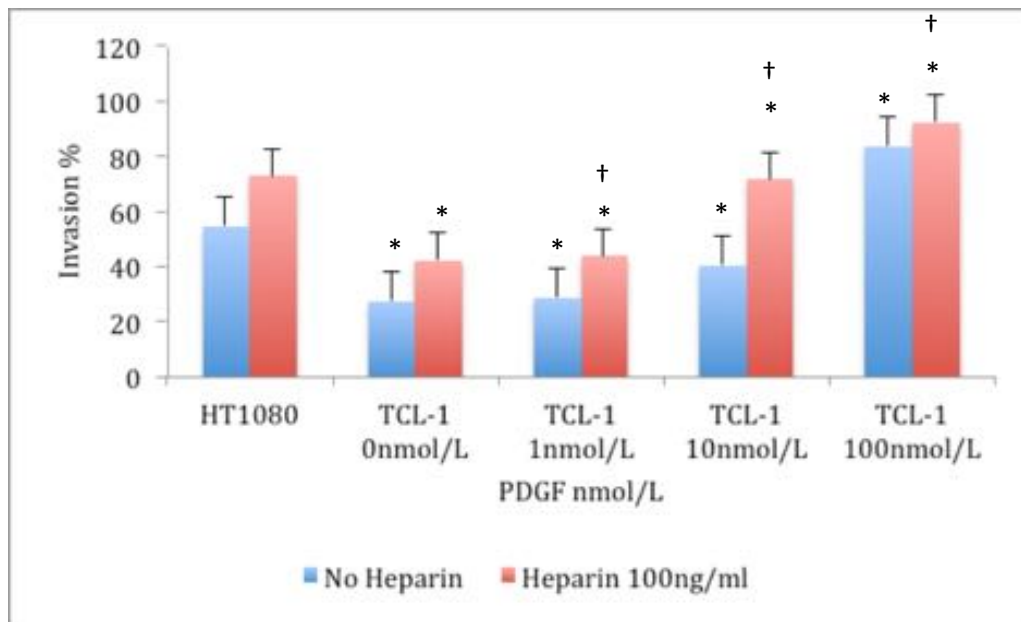


Figure 43E. Represents the percentage of invasion presented as mean \pm SEM for the following cell lines HT 1080 of ($n = 3$, $54.99\% \pm 3.65\%$ and $72.68\% \pm 5.22\%$, $p < 0.05$), and TCL-1 with the presence of platelet-derived growth factor (PDGF) expression at ($n = 3$, $27.31\% \pm 4.50\%$, $p < 0.05$) 0nmol/l, ($n = 3$, $28.18\% \pm 6.91\%$, $p < 0.05$) 1nmol/l, ($n = 3$, $40.22\% \pm 18.74\%$, $p < 0.05$) 10nmol/l and ($n = 3$, $83.51\% \pm 3.80\%$, $p < 0.05$) 100nmol/l. In the presence of heparin at ($n = 3$, $42.03\% \pm 6.79\%$, $p < 0.05$) 0ng/ml, ($n = 3$, $43.87\% \pm 6.91\%$, $p < 0.05$) 1ng/ml, ($n = 3$, $71.50\% \pm 18.74\%$, $p < 0.05$) 10ng/ml, and ($n = 3$, $92.1\% \pm 8.85\%$, $p < 0.05$) 100ng/ml. (* $p < 0.05$ vs. 0 growth factor, Mann-Whitney U test; † $p < 0.05$ vs. 0 heparin).

PDGF stimulated trophoblast invasion in with a slight change in the presence of heparin for lower concentrations, but its values were pronounced for significantly higher concentrations. Thus, the PDGF response was enhanced with only higher doses of heparin as in Figure 43E.

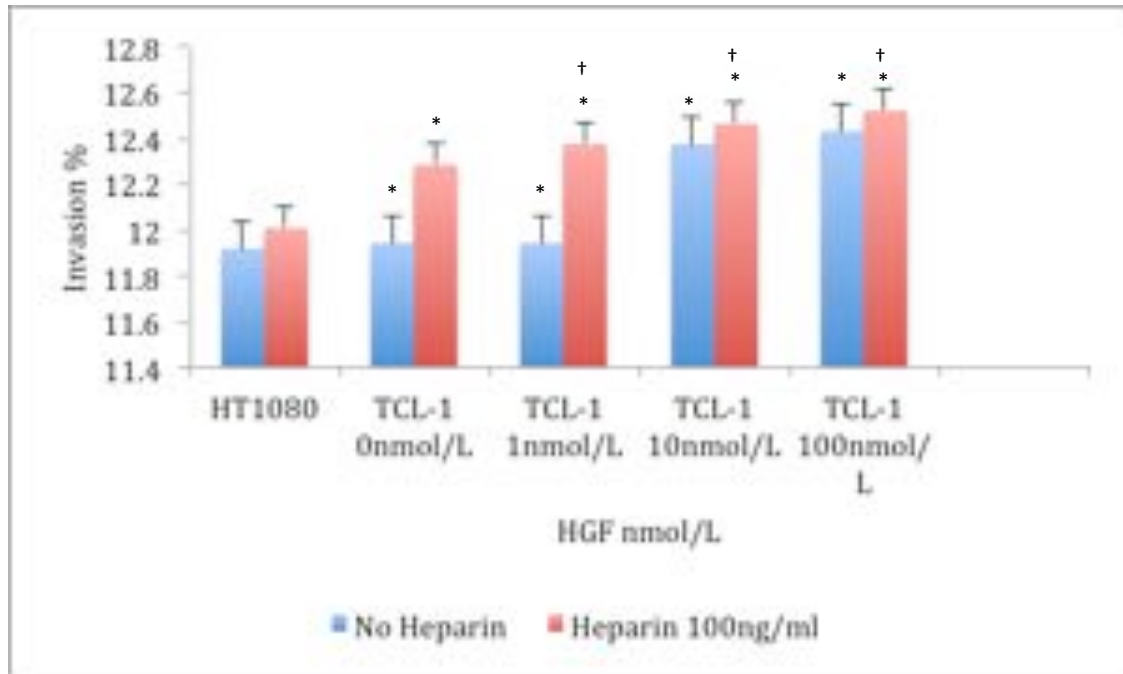


Figure 44F. Represents the percentage of invasion presented as mean \pm SEM for the following cell lines HT 1080 of ($n = 3$, $11.92\% \pm 0.58\%$, $12.01\% \pm 0.133\%$ and, $p < 0.05$), and TCL-1 with the presence of hepatocyte growth factor (HGF) expression at ($n = 3$, $11.94\% \pm 0.61\%$, $p < 0.05$) 0nmol/l, ($n = 3$, $11.94\% \pm 0.055\%$, $p < 0.05$) 1nmol/l, ($n = 3$, $12.37\% \pm 0.10\%$, $p < 0.05$) 10nmol/l and ($n = 3$, $12.43\% \pm 0.20\%$, $p < 0.05$) 100nmol/l. In the presence of heparin at ($n = 3$, $12.28\% \pm 0.15\%$, $p < 0.05$) 0ng/ml, ($n = 3$, $12.37\% \pm 0.18\%$, $p < 0.05$) 1ng/ml, ($n = 3$, $12.46\% \pm 0.02\%$, $p < 0.05$) 10ng/ml, and ($n = 3$, $12.52\% \pm 0.14\%$, $p < 0.05$) 100ng/ml. (* $p < 0.05$ vs. 0 growth factor, Mann-Whitney U test; † $p < 0.05$ vs. 0 heparin).

HGF stimulated trophoblast invasion in the presence of heparin produced a significant dose dependent rise for increasing concentrations. The effect of heparin was only observed at higher concentrations. Thus, heparin modifies the HGF response as in Figure 44F.

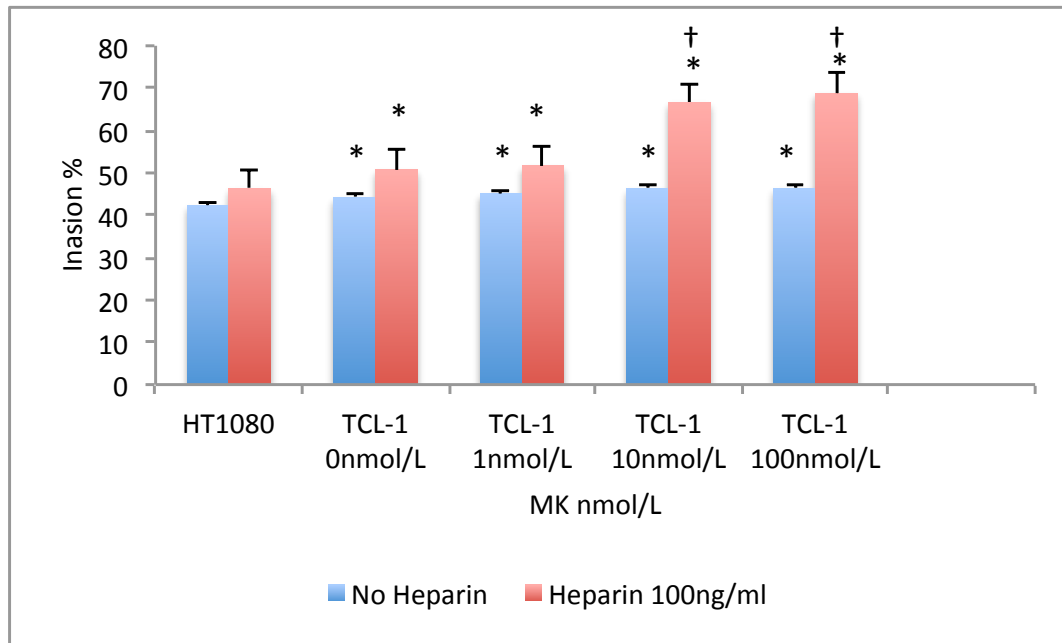


Figure 45G. Represents the percentage of invasion presented as mean \pm SEM for the following cell lines HT 1080 of ($n = 3$, 42.38% \pm 1.54%, and 46.27% \pm 4.97%, $p < 0.05$), and TCL-1 with the presence of Midkine (MK) expression at ($n = 3$, 44.16% \pm 5.80%, $p < 0.05$) 0nmol/l, ($n = 3$, 45.11% \pm 4.09%, $p < 0.05$) 1nmol/l, ($n = 3$, 46.23% \pm 4.95%, $p < 0.05$) 10nmol/l and ($n = 3$, 46.30% \pm 5.57%, $p < 0.05$) 100nmol/l. In the presence of heparin at ($n = 3$, 50.69% \pm 1.84%, $p < 0.05$) 0ng/ml, ($n = 3$, 51.63% \pm 2.58%, $p < 0.05$) 1ng/ml, ($n = 3$, 66.58% \pm 5.72%, $p < 0.05$) 10ng/ml, and ($n = 3$, 68.82% \pm 5.11%, $p < 0.05$) 100ng/ml. (* $p < 0.05$ vs. 0 growth factor, Mann-Whitney U test; † $p < 0.05$ vs. 0 heparin).

MK stimulated trophoblast invasion in the presence of heparin showed similar values for the lower concentrations, but were significant for the higher concentrations. Therefore, MK effect was striking as the greatest dose response was enhanced with only of higher doses of heparin as in Figure 45G. However, little is known about MK and PTN expression and their roles in the human placenta.

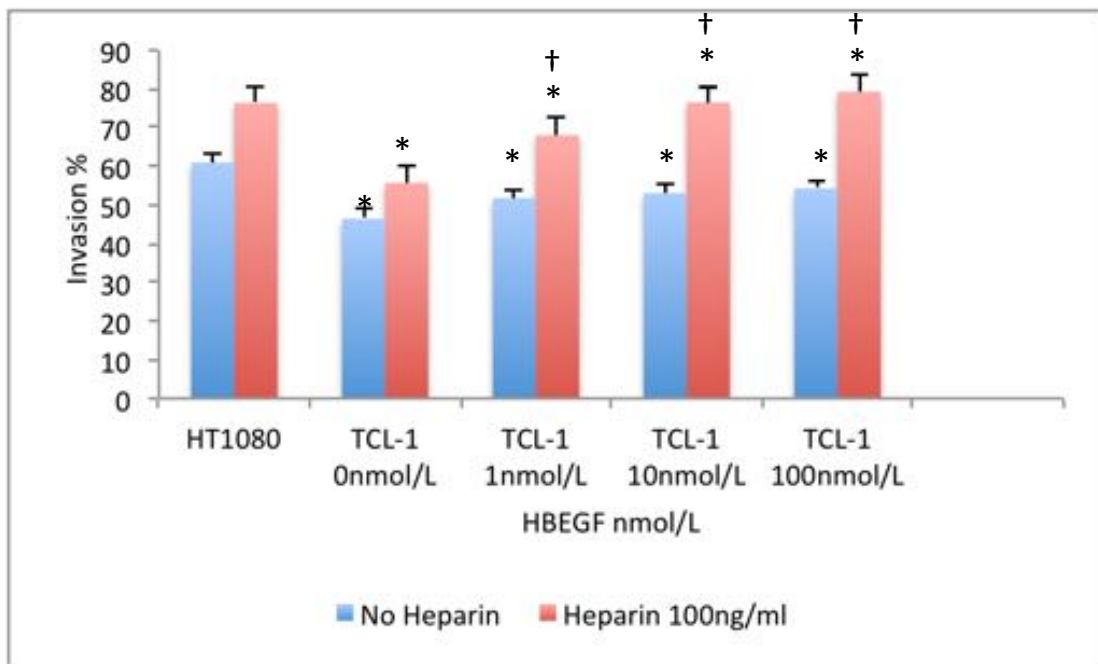


Figure 39A. Represents the percentage of invasion presented as mean \pm SEM for the following cell lines HT 1080 of ($n = 3$, $60.95\% \pm 15.70\%$, and $76.9\% \pm 6.26\%$, $p < 0.05$), and TCL-1 with the presence of heparin-binding-epidermal growth factor (HB-EGF) expression at ($n = 3$, $46.43\% \pm 4.40\%$, $p < 0.05$) 0nmol/l, ($n = 3$, $51.794.39\%$, $p < 0.05$) 1nmol/l, ($n = 3$, $53.06\% \pm 2.58\%$ $p < 0.05$) 10nmol/l and ($n = 3$, $54.28\% \pm 2.26\%$, $p < 0.05$) 100nmol/l. In the presence of heparin at ($n = 3$, $55.72\% \pm 6.38\%$, $p < 0.05$) 0ng/ml, ($n = 3$, $68.06\% \pm 4.45\%$, $p < 0.05$) 1ng/ml, ($n = 3$, $76.51\% \pm 6.80\%$, $p < 0.05$) 10ng/ml, and ($n = 3$, $79.30\% \pm 6.84\%$, $p < 0.05$) 100ng/ml. (* $p < 0.05$ vs. 0 growth factor, Mann-Whitney U test; † $p < 0.05$ vs. 0 heparin).

HB-EGF as in Figure 39A. and VEGF as in Figure 40B. stimulated trophoblast invasion in the presence of heparin produced significantly higher values. There was small increase with increasing dose of HB-EGF which was significantly enhanced into heparin as VEGF.

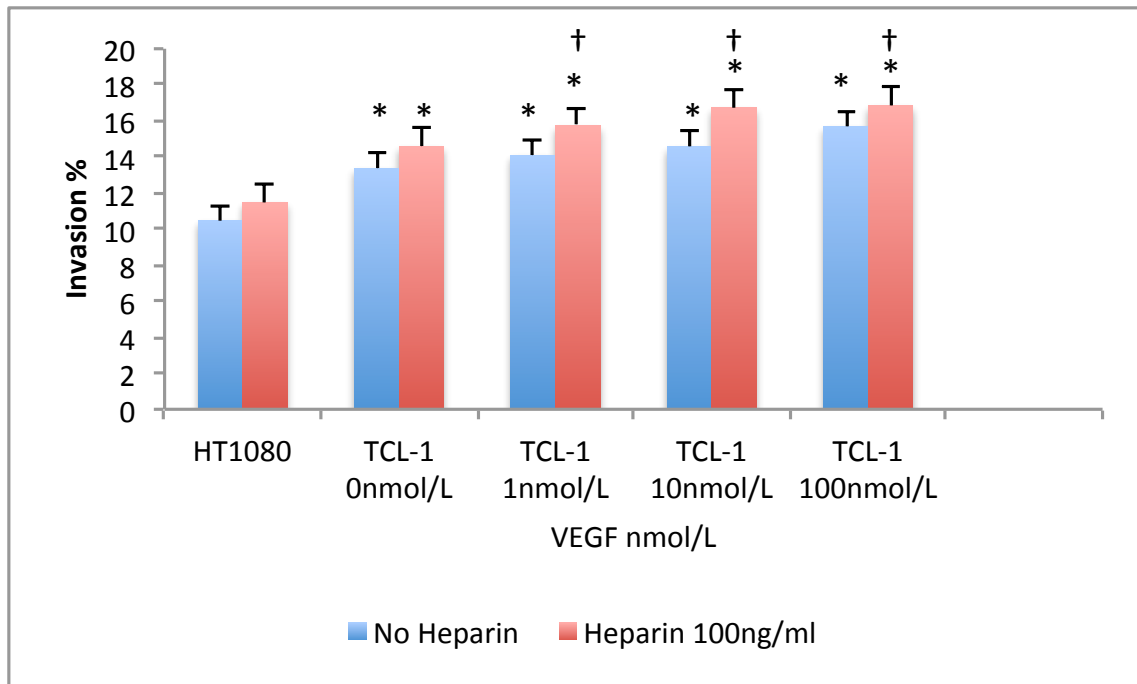


Figure 40B. Represents the percentage of invasion presented as mean \pm SEM for the following cell lines HT 1080 of ($n = 3$, $10.46\% \pm 18.68$; and $11.48\% \pm 19.56\%$, $p < 0.05$), and TCL-1 with the presence of vascular endothelial growth factor (VEGF) expression at ($n = 3$, $13.38\% \pm 0.39\%$, $p < 0.05$) 0nmol/l, ($n = 3$, $14.10\% \pm 0.99\%$, $p < 0.05$) 1nmol/l, ($n = 3$, $14.56\% \pm 1.69\%$, $p < 0.05$) 10nmol/l and ($n = 3$, $15.70\% \pm 2.20\%$, $p < 0.05$) 100nmol/l. In the presence of heparin at ($n = 3$, $14.60\% \pm 1.03\%$, $p < 0.05$) 0ng/ml, ($n = 3$, $15.73\% \pm 1.55\%$, $p < 0.05$) 1ng/ml, ($n = 3$, $16.73\% \pm 2.18\%$, $p < 0.05$) 10ng/ml, and ($n = 3$, $16.85\% \pm 1.67\%$, $p < 0.05$) 100ng/ml. (* $p < 0.05$ vs. 0 growth factor, Mann-Whitney U test; † $p < 0.05$ vs. 0 heparin).

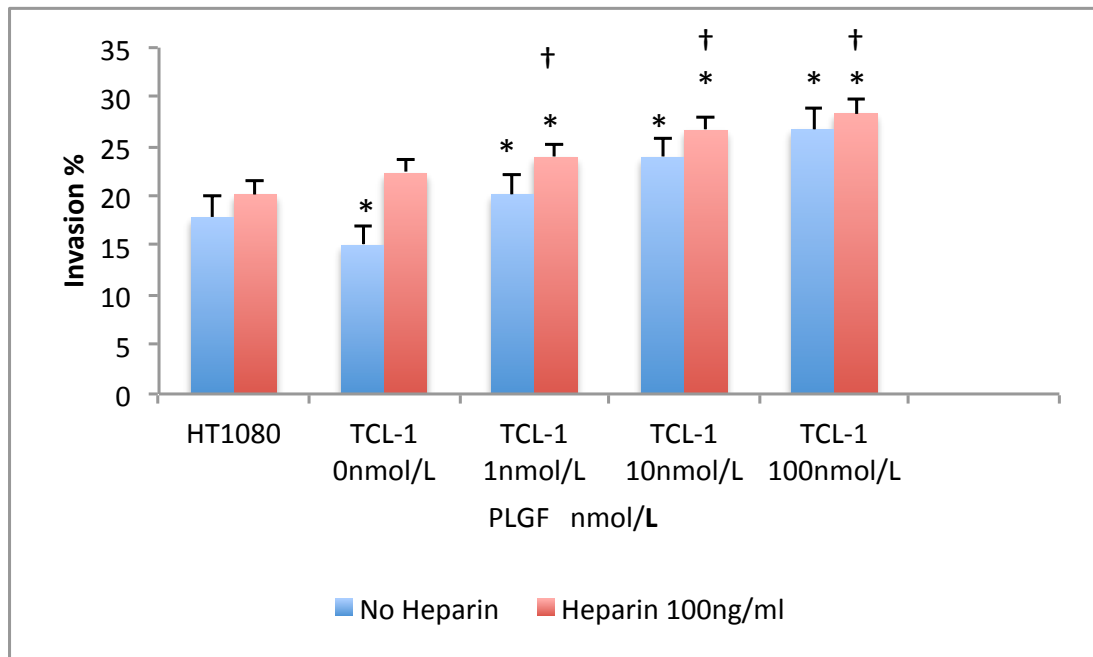


Figure 42D. Represents the percentage of invasion presented as mean \pm SEM for the following cell lines HT 1080 of (n = 3, 17.84% \pm 2.88%; and 20.16% \pm 9.87%, $p < 0.05$), and TCL-1 with the presence of placental growth factor (PLGF) expression at (n = 3, 15.02% \pm 2.70%, $p < 0.05$) 0nmol/l, (n = 3, 20.18% \pm 2.88%, $p < 0.05$) 1nmol/l, (n = 3, 23.88% \pm 7.19%, $p < 0.05$) 10nmol/l and (n=3, 26.72% \pm 10.18%, $p < 0.05$) 100nmol/l. In the presence of heparin at (n = 3, 22.33% \pm 4.03%, $p < 0.05$) 0ng/ml, (n = 3, 23.89% \pm 6.51%, $p < 0.05$) 1ng/ml, (n = 3, 26.64% \pm 7.80%, $p < 0.05$) 10ng/ml, and (n = 3, 28.30% \pm 8.82%, $p < 0.05$) 100ng/ml. (* $p < 0.05$ vs. 0 growth factor, Mann-Whitney *U* test; † $p < 0.05$ vs. 0 heparin).

PLGF stimulated trophoblast invasion in Figure 42D. in the presence of heparin only showed significant small increases because its response was little affected by heparin.

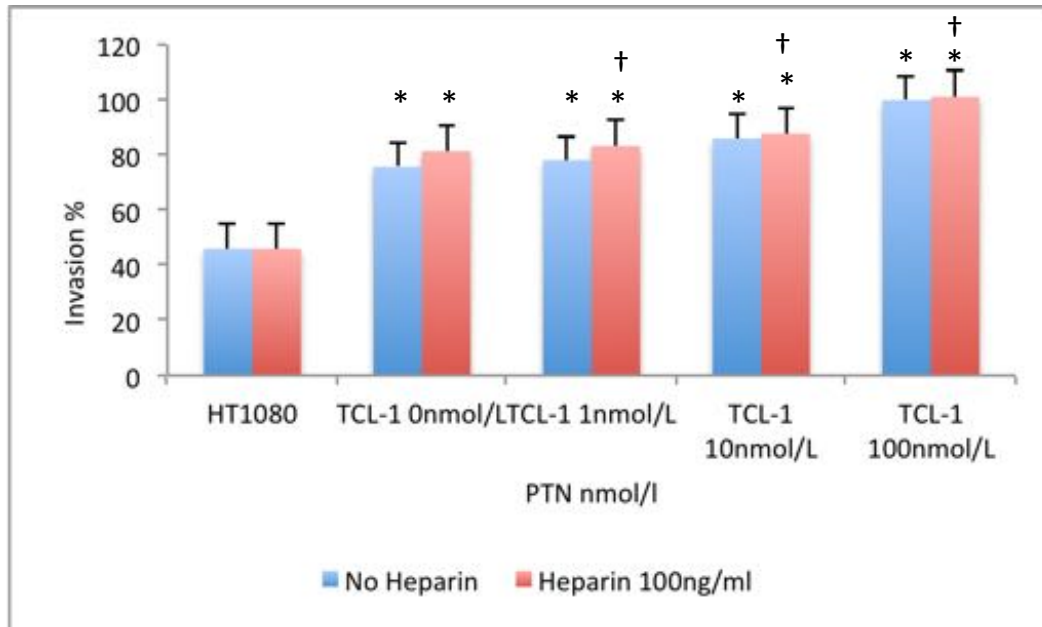


Figure 46H. Represents the percentage of invasion presented as mean \pm SEM for the following cell lines HT 1080 of ($n = 3$, $45.67\% \pm 2.88\%$, and $45.96\% \pm 9.87\%$, $p < 0.05$), and TCL-1 with the presence of Pleiotrophin (PTN) expression at ($n = 3$, $75.65\% \pm 12.83\%$, $p < 0.05$) 0nmol/l, ($n = 3$, $78.09\% \pm 3.03\%$, $p < 0.05$) 1nmol/l, ($n = 3$, $85.94\% \pm 8.02\%$, $p < 0.05$) 10nmol/l and ($n = 3$, $99.84\% \pm 6.35\%$, $p < 0.05$) 100nmol/l. In the presence of heparin at ($n = 3$, $81.38\% \pm 13.39\%$, $p < 0.05$) 0ng/ml, ($n = 3$, $83.25\% \pm 6.73\%$, $p < 0.05$) 1ng/ml, ($n = 3$, $87.94\% \pm 9.76\%$, $p < 0.05$) 10ng/ml, and ($n = 3$, $101.09\% \pm 20.78\%$, $p < 0.05$) 100ng/ml. (* $p < 0.05$ vs. 0 growth factor, Mann-Whitney U test; † $p < 0.05$ vs. 0 heparin).

PTN stimulated trophoblast invasion in Figure 46H. in the presence of heparin showed a further significant increase for increasing concentrations.

Although, the mechanisms responsible for heparin effects in the pathophysiology of pre-eclampsia have yet to be fully elucidated. Many of these HBGFs bind to heparin, a glycosaminoglycan (GAG) affecting activity. The interesting present data indicates a role and a possible physiological mechanism for the effect of heparin in promoting placental development. This suggests that dysregulation of HBGFs, and possibly changes in heparin may contribute to pre-eclampsia. Thus, our finding suggests a role for heparin or other glycosaminoglycans in mediating effect in placental tissue and a potential therapeutic use of heparin in treatment of pre-eclampsia. I identified growth factors produced the greatest change and those that showed the greatest response with heparin were FGF2, PDGF, HGF, and MK. Trophoblast invasion was augmented with increasing doses of HBGF. For example, the MK effect was strikingly augmented

then for VEGF, PLGF, FGF2, HB-EGF, PDGF, and HGF as the greatest response was enhanced with following co-incubation in the presence of higher doses for heparin except for PTN. However, little is known about MK and PTN expression and their roles in the human placenta. The present results are the first of the actions of MK and PTN and show different effects of heparin. Whether, MK and PTN plays a role in villous trophoblast invasion during placental development has never been examined. Namely large increases were seen at the higher dose for MK, but little effect was seen in response to PTN.

HBGFs caused trophoblast cells to invade, but only in the presence of heparin. A major strength in this present study is evident from the results demonstrating that through extracellular matrix *in vitro* villous trophoblast invasion model using human fibrosarcoma HT1080 as a positive control, mouse fibroblast NIH/3T3 as a negative control, and immortalised human primary villous trophoblastic cell lines provides a useful method to determine the effects of heparin and these HBGFs on trophoblast placental function. This technique is unique as it closely mimics the normal processes of placental development of early pregnancy. This demonstrated that heparin has a potential therapeutic use in treatment of pre-eclampsia.

6.2.2. Previous findings of Transwell invasion or migration of Matrigel assays.

Several authors previously have employed different methods and used transwell invasion (in presence of Matrigel), or migration in absence of Matrigel assays. Hemberger *et al*, (2004), demonstrated that the mouse differentiation of trophoblast cells *in vitro* into trophoblast giant cells recapitulates the invasive capacity of normal trophoblast cells *in vivo*. Kliman and Feinberg, (1990), and Librach *et al*, (1991) studied the general invasive phenotype of human trophoblast cells *in vitro*. The study of Lewis *et al*, (1996) demonstrated the presence of colony-stimulating factor-1 (CSF-1) autocrine loop found in immortalised Human trophoblast cell line TCL-1. Several authors assessed various factors affecting and regulating trophoblast invasion (Librach *et al*, 1994; Mandl *et al*, 2002; O'Brien *et al*, 2003; Sato *et al*, 2002; Xu *et al*, 2002; Janatpour *et al*, 2000; and Lala, 2000). The study of Athanassiades and Lala, (1998), suggested that PLGF -Flt-1 interactions may be regulated by the HSPGs *in situ* because PLGF promotes EVT cell proliferation. Exogenous PLGF-1 had any

effect on EVT cell proliferation migration and invasion assay. Previous studies suggested that the effects are modified by heparin for VEGF, PLGF, and HB-EGF (Lash *et al*, 2003; and Hills *et al*, 2006). However, Lash *et al*, (1999), study found that VEGF, and PLGF did not mediate heparin induced the *in vitro* invasion model of trophoblast cells into extracellular matrix. Their results suggested that addition of VEGF resulted in a significant decrease in number of trophoblasts and this effect was not influenced by heparin. Trew *et al*, (2000) re-investigated cytotrophoblast invasion using the Matrigel invasion assay for VEGF from first trimester placentae. Their results suggest that immunofluorescent cytokeratin-positive staining on Matrigel coated transwell was not for cell invasion. Harris *et al*, (2008), demonstrated that trophoblast heparanase is not required for invasion *in vitro* transwell assay. Their results showed that its expression was evident on the cell surface and the nucleus of trophoblast and decidual cells. Dubinsky *et al*, (2010) reported that the expression of VEGF in trophoblast cells are involved in the control of invasion and migration. Liu *et al*, (2012) discovered that HLX1, a homeobox gene is essential for downstream signalling because it's an important component of HGF response that leads to growth and invasiveness of trophoblasts.

It's the primary human trophoblast cells in cell culture that are valuable in research of placental physiology (Benirschke, 2006; Kingdom *et al*, 2000; and Knobil and Neil, 1994). As, isolation of human trophoblast cells is essential to investigate the early placental human trophoblast cell lineages because of their self-renewing properties. These differentiate into all trophoblast cell types of the mature placenta (Benirschke, 2006; Kay *et al*, 2010; and Kingdom *et al*, 2000). The trophoblast invasion is of physiological importance as this begins early in pregnancy and continues until the 20th week of gestation. Abnormal placental function, trophoblast differentiation, and defects in trophoblast invasion and spiral artery remodelling are associated with the placental pathology of pregnancy complications in obstetrical disorder as pre-eclampsia (Benirschke, 2006; Kay *et al*, 2010; Kingdom *et al*, 2000; Lyall and Belfort, 2007; Pijnenborg, 2010; and Williams, 2010). Trophoblast proliferation, migration and invasion are controlled by both soluble autocrine and paracrine factors, signalling pathways and regulatory transcription factors (Benirschke, 2006; Kay *et al*, 2010; Kingdom *et al*, 2000; Lyall and Belfort, 2007; and Williams, 2010). Autocrine factors are expressed in the trophoblast such as IGF-II, hCG, HB-EGF and

interleukins. Paracrine factors are EGF, interleukin-11 (IL-11), activins and chemokines that are expressed in different decidua cell types, uterine natural killer (uNK) cells and macrophages that produce growth factors, cytokines, interleukins, chemokines, prostaglandins and angiogenic growth factors (Benirschke, 2006; Kay *et al*, 2010; Kingdom *et al*, 2000; Lyall and Belfort, 2007; and Williams, 2010). *In vitro* invasion models using human trophoblast cells will provide to be beneficial because of new insights into the characteristics of human trophoblast cells (Benirschke, 2006; Kay *et al*, 2010; Kingdom *et al*, 2000; Lyall and Belfort, 2007; and Pijnenborg, 2010). Particularly, *in vitro* invasion assays which mimic the *in vivo* environment of the placental human trophoblast (Benirschke, 2006; Kay *et al*, 2010; and Pijnenborg, 2010). A comparison of *in vitro* effects of angiogenic and anti-angiogenic growth factors activities to results observed with *in vivo* assays can be assessed. This may prove valuable in the future for placental human trophoblast research and lead to a new approach for treating pre-eclampsia, eclampsia and IUGF women with trophoblast cells transfer (Benirschke, 2006; Kay *et al*, 2010; Kingdom *et al*, 2000; Lyall and Belfort, 2007; and Pijnenborg, 2010).

Heparin is important because its reported cytoprotective effect which promotes trophoblast survival and proliferation. This present results from *in vitro* studies show heparin can influence migration and invasion. The heparin-binding growth factors (HBGFs) are important in regulating trophoblast invasion *in vitro*. These observations may indicate that heparin-binding growth factors (HBGFs) are important *in vivo* regulating trophoblast invasion. There is evidence to suggest that the cytoprotective effects of heparin are mediated through binding to HB-EGF and enhancement of HB-EGF activity (Bose *et al*, 2005; and Hills *et al*, 2006). Hills *et al*, (2006) showed that the effect of HB-EGF *in vitro* and *in vivo* is modified by heparin eliciting epidermal growth factor receptor (EGFR) signalling transduction pathways in primary villous trophoblast. This is directly relevant to pathological pregnancies. They demonstrated that heparin protects human villous trophoblast against apoptosis and its effect was maximal at a concentration of 100ng/ml. Hills *et al*, (2012) showed that co-incubation with IGF-II and heparin at 100ng/ml decreased trophoblast apoptosis within the placenta. The Tunnel staining was employed to determine trophoblast apoptosis (Hills *et al*, (2012). Hills *et al*, (2012) study investigated cell line model of villous cytotrophoblast cells from first trimester (n=12) and term placental tissues (n=21)

using negative immunoselection with an antibody for HLA class 1 antigens. The present data indicate a role and a possible mechanism for the effect of heparin in promoting placental development which could suggest that dysregulation of HBGFs, and possibly heparin may contribute to pre-eclampsia.

The second technique in this study was the first devoted to extraction and purification of the total RNA production in normal placenta and in pre-eclampsia. Then, quantification of mRNA expression with HBGFs using qRT-PCR. Extraction of mRNA was from 7 placental tissue samples and it was used for the following primer assays: PLGF, FGF2, MK, PTN, and succinate dehydrogenase complex subunit A (SDHA) as a endogenous housekeeping gene. Identification of PCR products of 7 different placental tissue mRNA samples for FGF2 and MK by RNA agarose gel electrophoresis as in Figure 47 and 48 resulted fragments sizes as expected. A gel electrophoresis analysis as in Figure 49A, Figure 50A, and Figure 51B, of the time course degradation of 7 different placental tissue mRNA samples resulted as expected fragments sizes in the placenta tissue for PLGF, FGF2, MK, PTN and SDHA.

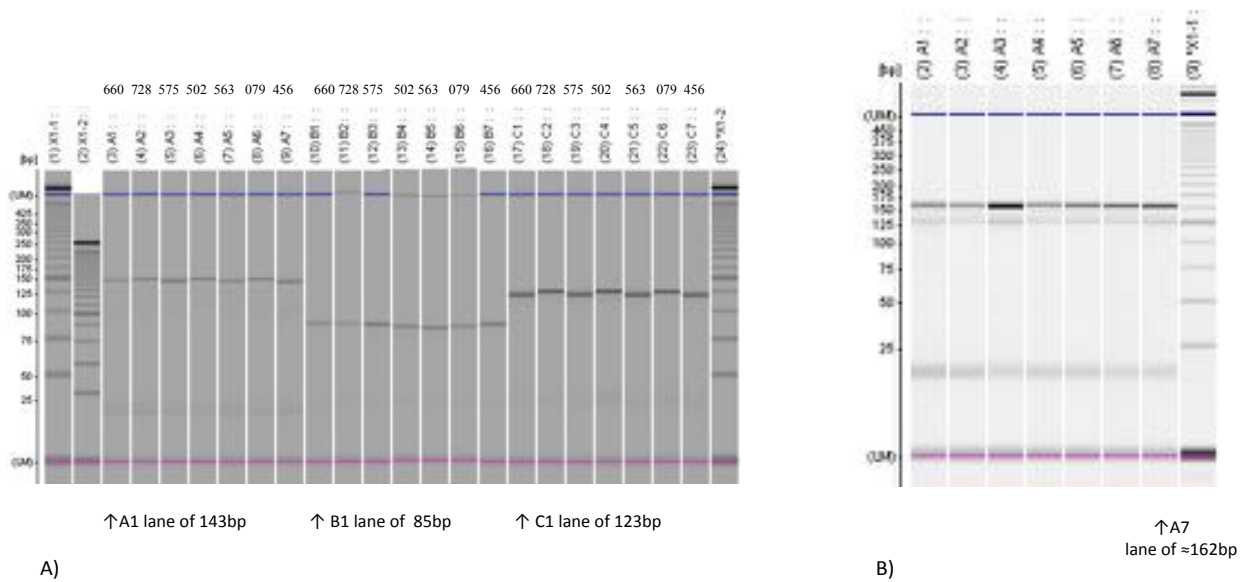


Figure 49A. Represents the positions of DNA as a distinct bands for growth factor primer assays: placental growth factor (PLGF), pleiotrophin (PTN), and fibroblast growth factor (FGF2) analysis at a concentration of 100ng/ul for lanes showed peaks for A1 of 143base pairs (bp), A2 lane of 145bp, A3 lane of 142bp, A4 lane of 146bp, A5 lane of 142bp, A6 lane of 146bp and A7 lane of 142bp. The lanes showed peaks for lane B1 lane of 85bp, B2 lane of 87bp, B3 lane of 87.5bp, B4 lane of 85.5bp, B5 of 84.5bp, B6 lane of 85.5bp and B7 lane of 87.5bp. The lanes showed peaks for lane C1 lane of 123bp, C2 lane of 127bp, C3 lane of 123bp, C4 lane of 127bp, C5 lane of 123bp, C6 lane of 127bp and C7 lane of 123bp. In Figure 51B. Succinate dehydrogenase complex A (SDHA) at a concentration of 100ng/ul showed peaks for lanes (A1-7) with A1 lane of 62base pairs (bp), A2 lane of 162bp, A3 lane of 165bp, A4 lane of 162bp, A5 lane of 162bp, A6 lane of 162bp and A7 lane of 162bp using the DNA sizing ladder and 2 internal standard markers (LM and UM).

The comparison between control (Term (TL + TNL) not in labour) versus pre-eclampsia of placental tissue mRNA expression for FGF2 and SDHA showed significant differences except for PLGF, and MK, and PTN primer assays, using the Mann-Whitney *U* test.

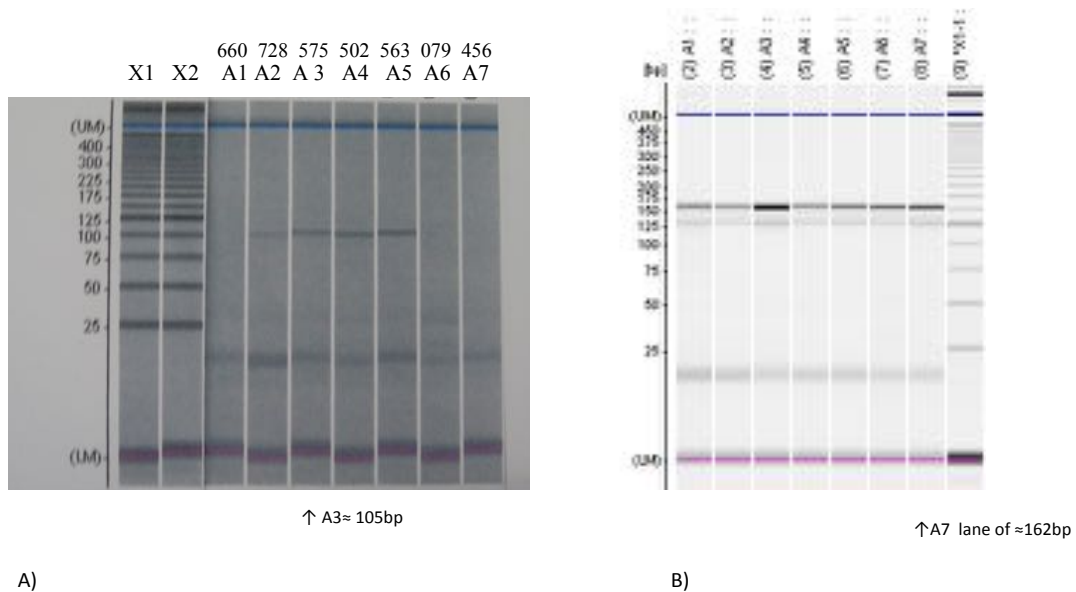


Figure 50A. Represents the positions of DNA as a distinct bands for growth factor primer assays: MK at a concentration of 100ng/ul, For lanes (A1-7) showed peaks with A (3,5 of 105bp) and A (2 of 97bp) and A(4 of 102bp), but A1, A6 and A7 lanes showed no DNA base pairs. In Figure 51B. Succinate dehydrogenase complex A (SDHA) at a concentration of 100ng/ul showed peaks for lanes (A1-7) with A1 lane of 62base pairs (bp), A2 lane of 162bp, A3 lane of 165bp, A4 lane of 162bp, A5 lane of 162bp, A6 lane of 162bp and A7 lane of 162bp using the DNA sizing ladder and 2 internal standard markers (LM and UM).

There are no previously reported qRT-PCR studies for MK and PTN using a similar approach to that employed in this current study is a novel finding.

6.2.3. Previous studies on mRNA expression in the placenta.

Poston's (2002) and Radaelli *et al*, 2003 studies on the placenta established that regulation of specific placental genes in the tissue may be related to the gestational disorder, such as pre-eclampsia. Identifying gene expression alterations may advance the knowledge of pathophysiology of disorders of the placenta. In addition, monitoring changes of gene expression may identify new biomarkers that might predict pre-eclampsia earlier in pregnancy. It is by measuring the RNA expression in

the placenta that determines certain genes that are specifically transcribed in the placenta tissue and how their regulation can lead to abnormal gestational effects. The study of Bustin, (2002) on quantification of mRNA using RT-PCR suggested that comparison of RNA samples by controlling the amount of total RNA is time consuming. This required accurate quantification of RNA samples that was complicated by many physiologically variations.

6.2.4. Other studies on Housekeeping genes in the human placenta.

As few reports are available on stability of housekeeping genes in the human placenta. Studies of Meller *et al*, (2005) and Cleal *et al*, (2009) found that GAPDH varied within placenta and unreliable reference gene. However, Murthi *et al*, (2008) employed GAPDH and found it stable and suitable for normalisation. It is an important concern in relative gene analysis to have a reliable normalisation factor, and this should be a reference gene that shows a comparative expression i.e. with high stability and low variability irrespective of experimental conditions. Molecular studies of pre-eclampsia placentae may elucidate etiologically important genomic alterations. RNA measurement by RT-PCR in comparative gene expression studies requires endogenous housekeeping genes to normalise between sample variations.

Tricarico *et al*, (2002) suggested that housekeeping genes are measured to better normalize between samples. Sample variation was avoided by reporting the amount of particular gene expression relative quantitation to that of a housekeeping gene demonstrated accurate normalisation of RT-PCR data reported that for comparison of genes in placental tissue samples, geometric averaging of housekeeping gene should be employed.

Yuen *et al*, (2002) study on RNA measured by RT-PCR and microarray analysis showed subtle changes in relative quantities for large number of genes and consume smaller amounts of each sample. This sensitivity variation between samples must be quantified, accounted for and minimized. Thus, endogenous control genes are evaluated for stability between many types of placenta samples, including samples from complicated pregnancies.

Meller *et al*, (2006) study compared 7 different housekeeping genes β -2 microglobulin (B2M), Hydroxymethyl-bilane synthase (HMBS), Hypoxanthine phosphoribosyl-transferase I (HPRT), TATA box binding protein (TBA), and Tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein, zeta polypeptide (YWHAZ) including the commonly used Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Succinate dehydrogenase complex subunit A (SDHA) expression in the placenta. Their tissue samples were taken from uncomplicated and complicated pregnancies. They examined both maternal and foetal sides of the placenta, but our study examined neither the villous nor the decidual sides of the placenta. They looked at the mRNA isolated from 20 placentae and relative overall levels of expression of 7 genes using RT-PCR. They found that GAPDH analysis resulted a large variability and that TBA, SDHA and YWHAZ produced greater expression stability.

Rosenberg *et al*, (2011) longitudinal study on 42 pregnant women showed that heparin did not effect circulating levels of VEGF, and PLGF using ELISA. They showed a distinct band of 100-112kDa by Western blot analysis both *in vivo* and *in vitro* as heparin induced shedding of the extracellular domain of soluble fms-like tyrosine kinase 1 (sFlt-1). Kauma *et al*, (1996) confirmed Met (a tyrosine kinase receptor for the ligand HGF) in placental villi and trophoblast cells to be 140kDa band and placental villous tissues demonstrated approximately 100-110kDa band by Western Blot analysis. Obama *et al*, (1995) identified that MK and PTN mRNA expression was present in the mouse extraembryonic membranes but only MK was found in the placenta at gestation. Their Western Blot analysis showed MK presence in amniotic fluid and cerebrospinal fluid.

Different methods of quantitative RT-PCR were employed by several authors for their studies on placentae from pre-eclampsia women. Lala and Athanassiades (1998) examined first trimester EVT cells propagation *in vitro* mRNA expression of VEGF, and for Flt-1 using RT-PCR. Their results demonstrated presence for both protein and mRNA of VEGF, Flt-1 and expressed SV40 tag-immortalised EVT cells. Interestingly, study of 30 women with gestational diabetes mellitus (GDM) and their 30 macrosomia (foetal overgrowth) babies by Grissa *et al*, (2010) demonstrated several concentrations of growth factors IGF-1, EGF, FGF2 and PDGF-B and their

receptors in women with GDM and their macrosomia babies. These results demonstrated that serum concentrations of IGF-1, IGF-BP3, EGF, FGF2 and PDGF-B were determined by ELISA were higher in GDM women and their macrosomia babies compared to their controls. The expression of mRNA for these growth factors and their receptors were quantified by qRT-PCR. The mRNA expression was upregulated for FGF2 and PDGF-B but was unaltered for IGF-1 and EGF in the placenta of GDM women. The mRNA expression of 3 growth factor receptors IGF-1R, EGFR, and PDGFR- β were upregulated in the placenta of GDM women. Therefore, growth factors might be implicated in GDM and possibly pathology of macrosomia via the materno-foeto-placental axis in regulation of foetal growth.

Andraweera *et al*, (2012a) quantified a reduction in mRNA expression when compared to control (uncomplicated term pregnancies) of both VEGF, and PLGF and Flt-1 using RT-PCR in pregnancies complicated by pre-eclampsia, gestational hypertension disorders, small for gestational age SGA and preterm births. Maebavashi *et al*, (2012) quantified mRNA expression by using RT-PCR. Their results showed a decrease of mRNA expression for PLGF, and Flt-1 increased in pre-eclampsia placentae. The effect of pre-eclampsia sera was analysed using choriocarcinoma cells (JEG3) from pre-eclampsia patients (Maebavashi *et al*, (2012).

Arany and Hill, (2012) quantified and localised the sites and expression for FGF2 and its receptor FGFR-1 in placentae from normal term pregnancies and from pregnancies complicated by type 1 diabetes. Both *in situ* hybridization and immunocytochemistry demonstrated the presence FGF2 mRNA in low abundance in foetal villous tissue, and syncytiotrophoblast in normal term placenta. The Northern blot hybridization showed an significant increase of mRNA for both FGF2 and FGFR-1 in placentae and intense staining existed in syncytiotrophoblast from diabetic women when compared to the normal women. Grissa *et al*, (2010), investigated FGF2, PDGF-B and their receptors using RT-PCR. Their results demonstrated that the placental mRNA expression of growth factors might be implicated gestational diabetes mellitus and in pathology of macrosomia. The serum concentrations of FGF2 and PDGF-B were higher in GMD women and their macrosomic babies as compared to the controls.

Chauhan *et al*, (2013) examined concentrations of glycosaminoglycans and the proteoglycan syndecan-1 within villous trophoblast. They investigated changes associated with pre-eclampsia on seventy-five placental samples from third trimester singleton pregnancies. Their results showed a decrease in sulphated glycosaminoglycans and syndecan-1 in pre-eclampsia women that were independent of labour, gestational age and birth weight centile.

Recently, a study of Escudero *et al*, (2014) in which mRNA expression was determined and which employed placentas showed that from both early onset (EOPE) and late onset pre-eclampsia (LOPE) were associated with differential activation of VEGFR-2 and hence demonstrated increased placental angiogenesis.

During normal pregnancy increases in sFlt-1 occur; but in pre-eclampsia the sFlt-1 concentrations significantly increase and exceed normal levels. Kumasawa *et al*, (2011) developed a mouse model for pre-eclampsia reproduces findings similar to those in LOPE. They demonstrated that the lipid lowering drug pravastatin induces PLGF and ameliorates pre-eclamptic changes in sFlt-1 in their model. Taguchi *et al*, (2008) study showed that pravastatin could be used in treatment of pre-eclampsia does not cross the human placenta because it is hydrophilic.

I have established the three techniques in this study by employing firstly immunocytochemistry, secondly invasion and migration assays and thirdly qRT-PCR for pre-eclampsia. I confirmed FGF2 expression significantly in 3 techniques. Although, PLGF, MK, and PTN expressions were significant for both immunocytochemistry, invasion and migration studies, they were not by qRT-PCR. This was probably due to placental tissue mRNA transcription or protein level. This present study of 3 years time did not permit a further study to clarify these results. Therefore, our findings suggest a role for heparin has potential therapeutic use in mediating effect in placental tissue and can be applied for prediction, prevention and achieve an earlier diagnosis to better treatment of pre-eclampsia in the future.

6.2.5. Findings on prediction, prevention, diagnosis and treatment of pre-eclampsia.

Pre-eclampsia is a complex multifactorial pregnancy disorder characterised by hypertension, and proteinuria after 20 weeks of gestation, followed later by oedema. Previous studies suggested that pre-eclampsia is an important threat to public health and is the leading cause of maternal and foetal morbidity and mortality in pregnancy in both developed and developing countries (Duley, 2006; Tattersall *et al*, 2008; Meads *et al*, 2008; and Munir, 2013). The histopathologic correlation is the shallow invasion and aberrant remodelling of maternal spiral arteries, which leads to decreased uteroplacental perfusion. Reduced perfusion in the pre-eclamptic placenta, inducing trophoblast cell death apoptosis, has been linked to proinflammatory cytokine processes, hypoxia, and oxidative stress. Pre-eclampsia is associated with numerous changes including: deleterious effects on the extravillous trophoblast; failure of the extravillous trophoblast to adequately transform the uterine spiral arteries; reduced flow of maternal blood into the intervillous space; hypoxia or intervals of hypoxia followed by reoxygenation of the placenta; hypoxic damage of the villous trophoblast; and release of syncytiotrophoblast membrane fragments (STBM) into the maternal blood stream. The maternal inflammatory response to the STBM results in the development of the clinical symptoms of pre-eclampsia (Huppertz, 2008).

Clinical research may contribute to developing new insights into placentology and novel therapeutic technologies for placental disorders as pre-eclampsia (Benirschke, 2006; Kay *et al*, 2010; Kingdom *et al*, 2000; Lyall and Belfort, 2007; Pijnenborg, 2010; Williams, 2010). New methodologies such as genomics, proteomics and metabolomics are widely available for research (Kay *et al*, 2010; Kingdom *et al*, 2000; Lyall and Belfort, 2007; and Pijnenborg, 2010). There are many maternal clinical characteristics such as ethnicity, age, parity, multiple pregnancy and a history of pre-eclampsia in earlier pregnancies in first-world countries that are identified as risk factors for developing pre-eclampsia (Anderson *et al*, 2012; and Lyall and Belfort, 2007). Angiogenic growth factors promote vasculogenesis and angiogenesis in the placenta and the most potent include the VEGF, FGF2, and PLGF family. There are many other growth factors for example HB-EGF, PDGF, HGF, PTN, MK, CD105, and Endoglin (Eng) (TGF)- β 1 and (TGF)- β 3 (Pijnenborg *et al*, 2010). Many

of these angiogenic and (sFlt-1) a trophoblast derived anti-angiogenic factors are promising biochemical markers for example free foetal haemoglobin (HbF), alpha-1-microglobulin (A1M), pregnancy-associated protein A (PAPP-A), placental protein 13(PP13) Eng, metabolomics, angiogenesis factors and PLGF (Anderson *et al*, 2012; and Lyall and Belfort, 2007). Alterations in circulating levels are evident with increased levels of sFlt-1 and reduced levels of VEGF and PLGF and thus prevents their availability to stimulate angiogenesis this occur weeks before the clinical onset of pre-eclampsia (Ahmed and Ramma, 2014; Luttun and Carmeliet, 2003; Maynard *et al*, 2003). The largest study to evaluate PLGF and other factors in suspected pre-eclampsia women were by Chappell *et al*, (2013). They studied the diagnostic accuracy of low plasma PLGF concentrations (<5th centile for gestation, Alere Triage Assay) in suspected pre-eclampsia women between 20 and 30 weeks of gestation, (including up to 41 weeks gestation as a secondary analysis) in determining delivery for pre-eclampsia within 14 weeks of testing. A total of 625 women were tested. There outcome was delivery for confirmed 346 pre-eclampsia. However, 287 women had enrolled before 35 weeks of gestation, and their PLGF showed high sensitivity and negative predictive value for suspected pre-eclampsia within 14 days. This technique confirmed to be better than many currently used tests for management of pre-eclampsia women.

As, the pathophysiological mechanisms are not fully understood for aetiology of pre-eclampsia (Benirschke, 2006; Kay *et al*, 2010; Kingdom *et al*, 2000; Lyall and Belfort, 2007; Pijnenborg, 2010; and Williams, 2010). There is an emphasis on the recently characterised circulating anti-angiogenic proteins (Mutter and Karumanchi, 2008). In pre-eclampsia, as in the vast majority of pregnancies complicated by preterm labour, a vascular component has an important role in the course of the disease (Hediger *et al*, 1995). Placental histology has revealed similar findings in some cases of preterm labour (Pijnenborg *et al*, 1991; and Salafia *et al*, 1995). The expression Hypoxia inducible factor-1 α (HIF-1 α) protein and mRNA in human foetal membranes and placenta was investigated in study by (Singh *et al*, 2006). VEGF expression activates HIF-1 α in the placenta. Singh *et al*, (2006) showed that VEGF protein levels were decreased in foetal membranes after preterm labour and term labour.

As, the pathophysiological mechanisms are not fully understood for aetiology of pre-eclampsia (Benirschke, 2006; Kay *et al*, 2010; Kingdom *et al*, 2000; Lyall and Belfort, 2007; Pijnenborg, 2010; Williams, 2010). Recently, VEGF, PLGF and (sFlt-1) a trophoblast derived anti-angiogenic markers are of interest particularly, relating to the potential role of biomarkers for prediction and diagnosis of pre-eclampsia (Benirschke, 2006; Kay *et al*, 2010; Kingdom *et al*, 2000; Lyall and Belfort, 2007; Pijnenborg, 2010; and Williams, 2010). Anderson *et al*, (2012), suggested that an ideal biochemical marker should show the following characteristics for both prediction and diagnosis of pre-eclampsia for routine use in clinical practice. Several important points that are considered for clinical investigations are as follows. These biochemical markers must have central role in the pathogenesis and would be specific. They should be detected and appear early or before the clinical manifestations. It is important that they are safe, easy and cost-effective approach to measure in maternal blood or urine. They should prove a high sensitivity and specificity. The severity of the condition should be correlated with the progression of disease. They should in normal pregnancies be expressed at very low levels. Lyall and Belfort, (2007); and Hadker *et al*, (2010) suggests that screening pregnant women with an effective diagnostic marker for pre-eclampsia could reduce suffering and major health care costs. Therefore, a significant impact on maternal and foetal health worldwide could occur if an intervention could prevent pre-eclampsia. Pre-eclampsia is a dominant in the third world, and diagnosed in women present with eclamptic seizures. There is clinical need for improving screening or diagnostic tests for pre-eclampsia. This would advance obstetric care by reducing maternal and foetal morbidity and mortality in third world countries. The effective treatment of pre-eclampsia is simple, early delivery of baby and the placenta, there is no known cure. It is after delivery that symptoms resolve within 48–72 h. Potential novel pharmacological treatment strategies may restore the normal angiogenic balance in the maternal circulation (Wang *et al*, 2015; Lyall and Belfort, 2007). Heparin can be applied for prediction, prevention and achieve an earlier diagnosis to better treatment of pre-eclampsia in the future. Thus, improving the development of interventions and to increase normal pregnancy outcome reducing maternal and foetal morbidity and mortality.

6.2.6.Limitations in this study

There were several limitations in three major techniques immunocytochemistry, invasion and migration assays and RT-PCR studied for pre-eclampsia. Immunocytochemistry of FGF2 expression for covering the placental specimen slides specimen (n=109) in different dilutions (1 in 100, 1 in 200, and 1 in 400), control (n=43), and a negative control IgG (n=11) in the absence of citrate buffer showed no staining with overnight incubation at 2-8°C. In this study, a total of (n=4) slides comprised first trimester prior to labour were too small number for analysis. Therefore, no scoring was performed. Thus, a larger cohort of placenta tissue samples should be used for further investigations on the placental pathologies including pre-eclampsia.

For the invasion and migration studies, the experiment for standard curve calibration was performed. These results were reproduced in 4 further experiments at relative fluorescence units (RFU) measurement of 1000 and 2000 were converted to number of cells. Its from the free calcein that fluoresces, and quantitate these number of cells that have migrated and invaded using standard curve. This invasion assay used different cell types such as the Human fibrosarcoma HT1080 invasive cells (positive control), and human villous trophoblast cell-line TCL-1 in the absence of BD FluoroBlok that has a uniform coated layer with Matrigel Matrix. The identification of PCR products by RNA agarose gel electrophoresis, of 7 different placental tissue mRNA samples with their concentration (100ng/ul) were optimised for some of growth factor primer assays. These results were reproduced in 7 further experiments.

6.9.7. In summary, these studies support the novel hypothesis tested for cohort study of 87 women is that the expression of heparin binding growth factors (HBGFs) in normal placental development was altered in the specific disorder of pre-eclampsia.

- Heparin-binding growth factors are involved in placental development and many of these bind glycosaminoglycans (GAGs) and this binding affects activity.
- It suggests that heparin-binding growth factors (HBGFs) are important in regulating trophoblast invasion.
- It suggests that dysregulation of HBGFs and possibly changes in heparin may contribute to pre-eclampsia.
- This indicates a role for heparin in mediating the effects of HBGFs.
- This indicates a possible mechanism for the effect of heparin in promoting placental development.

6.9.8. In conclusion, our main finding for this current study is that alterations in growth factor are involved in the pathophysiology of pre-eclampsia. Future research may elucidate further roles of HBGFs in placental development and pre-eclampsia. It suggests that heparin has a new potential therapeutic use in the treatment of pre-eclampsia.

6.9.9. Recommendations for future studies:

These results from experiments employing techniques as immunocytochemistry, qRT-PCR and invasion and migration assays provide evidence for future research that would provide new insights and better understanding of pathophysiology in pre-eclampsia for human and the mouse placental development. New developments in diagnosis with a focus on angiogenic growth factors as VEGF, PLGF and (sFlt-1) a trophoblast derived anti-angiogenic factors are promising biochemical markers and contribute to early diagnosis, management and treatments of obstetrical disorder complications of pregnancy for predication of disease onset in pre-eclampsia with recent management strategies for patients. Thus, reducing maternal and foetal morbidity (Benirschke *et al*, 2006; Lyall and Belfort, 2007; Kay *et al*, 2010; Pijnenborg *et al*, 2010; and Williams, 2010). As, placental abnormalities of pre-eclamptic women lead to reduce blood supply and deficient placental trophoblast invasion during implantation contribute to inadequate spiral artery remodelling (Benirschke *et al*, 2006; Lyall and Belfort, 2007; Kay *et al*, 2010; Pijnenborg *et al*, 2010; and Williams, 2010). Its through the activation of increased placental oxidative stress, and formation of reactive oxygen species (ROS) production, promoting alterations in syncytiotrophoblast apoptosis in maternal circulation via syncytial knots that are associated with increased release of syncytiotrophoblast microparticles (STBM) and initiation of inflammatory cytokine production in placental tissues that cause endothelial dysfunction in pre-eclampsia (Benirschke *et al*, 2006; Lyall and Belfort, 2007; Reddy *et al*, 2008; and Kay *et al*, 2010).

The aim of the study would be to test the hypothesis for the role of STBMs in the pathophysiology of pre-eclampsia which has not been determined. Further studies, of placental ischaemia, hypoxia, and oxidative stress due to reduced perfusion *in vitro* or *in vivo* during labour of normal pregnancies, Gestational Diabetes Mellitus, and pre-eclamptic women would be clarified (Benirschke *et al*, 2006; Lyall and Belfort, 2007; and Kay *et al*, 2010). The new therapeutic use of heparin in mediating effects of HBGFs such as on villous trophoblast, STBMs, cell survival, proliferation, invasion, migration, and differentiation of placental villi to these different O₂ tensions could be determined.

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AUTHOR'S DECLARATION:

The Laboratory experimental work described in this thesis was in Hatchcroft Laboratories at Middlesex University between January 2010 and July 2013. The completed immunohistochemistry photomicrographs were in March 2014. I am the author Farha Nazir Ahmad Mohamad and owner of the copyright in the thesis and I have the authority of authors and owners of copyright in thesis to make agreement. This thesis may not be reproduced in any format or medium, or extensive quotations taken from it, or its content changed in any way, without obtaining permission in writing from the copyright holder. I have made full, accurate, and appropriate acknowledgement where reference has been made to the work of others. This thesis is my own original work. I have not presented the work of others as my own and that no part of the thesis has been submitted for a prior degree or a degree at another University. I confirm that my thesis does not exceed 80,000 words has been prepared in accordance with Middlesex University's guidelines on the presentation of a research thesis.

Farha Nazir Ahmad Mohamad

Date: 27thMay 2016

Middlesex University London

My Biomedical research work is being published during the course of my PhD in Reproductive Science in Departments of Science and Technology/Natural Science at Middlesex University. The three major techniques studied were immunohistochemistry, invasion/migration assays and placental mRNA Expression of the following heparin binding growth factors: vascular endothelial growth factor (VEGF), placental growth factor (PLGF), heparin-binding epidermal growth factor (HB-EGF), fibroblast growth factor 2(FGF2), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), midkine (MK) and pleiotrophin (PTN) and cluster differentiation (CD105).

F.N.A. Mohamad, S.Dilworth, and F.Hills. The Role of Heparin and Heparin-binding Growth Factors in Pre-eclampsia 2015. [In preparation]

Abstract in the School of Health and Social Science Summer Conference (HSSC) at Middlesex University (2012).

F.N.A. Mohamad, R.Iles, and F.Hills. The Role of Heparin and Heparin-binding Growth Factors in Pre-eclampsia 2015. [In preparation]

My Biomedical research work is being published for my MSc in Obstetrics and Gynaecology in Departments of Natural Science at Middlesex University. The major technique studied was immunohistochemistry, for the following heparin binding growth factors: vascular endothelial growth factor (VEGF), placental growth factor (PLGF), and cluster differentiation (CD105).

F.N.A. Mohamad, R.Iles, and F.Hills. The expression of VEGFs and their receptors in normal and abnormal pregnancies 2015. [In preparation]

King's College London University

My BSc Honours degree in Physiology Reproductive Physiology research project with Professor Forsling in Departments of Physiology and Obstetrics and Gynaecology at King's, Guy's and St Thomas' Hospital titled: The effect of reproductive status on renal responsiveness to neurohypophysial hormone yielded interesting results.

Physiological Society 2 Abstracts in the *Journal of Physiology* at King's College London University

- D.E.Hartley, F.N.A.Mohammed, Y Zhou and M.L.Forsling. Are prostaglandins involved in the natriuretic response to neurohypophysial hormones? *J. Physiol.* 493, 94P (1996)
- D.E.Hartley, F.N.A.Mohammed, Y Zhou and M.L.Forsling. The influence of reproductive status on the renal response to vasopressin in the rat. *J. Physiol.* 493, 100-101P (1996)

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The International Conference on Gonadotropins and receptors from July 6-9th 2008.

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Workshops and research seminars (2010-2011):

International research and collaboration with other institutions in the UK.

Presentational skills: Writing and presenting your work at conferences.

PhD Supervision - using it, making the most of it, what to do when it goes wrong.

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