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Placental Expression of Matrix Metalloproteinases MMP-2 and MMP-9 in Pregnancy:

The use of villous explants and high altitude pregnancy studies to explore the role of oxygen in the pathogenesis of pre-eclampsia.

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Abstract

Placental expression of matrix metalloproteinases MMP-2 and MMP-9 in pregnancy:
The use of villous explants and high altitude pregnancy studies to explore the role of oxygen in the pathogenesis of pre-eclampsia.

Successful placental development is essential for the normal progression of pregnancy. Pre-eclampsia is a leading complication of human pregnancy. The condition is characterised by a lack of remodelling of the maternal spiral arteries, thought to result from a failure of cytotrophoblast (CTB) invasion during the early stages of pregnancy. The subsequent reduced placental perfusion may cause relative placental hypoxia and lead to endothelial activation in the maternal circulation through the action of several factors including placental debris. In order to understand this condition it is necessary to establish the mechanisms which control normal placentation. Studies on placentae as well as *in vitro* models have proved useful in helping to understand these processes.

The matrix metalloproteinases MMP-2 and MMP-9 have been implicated in trophoblast invasion in several studies but the *in vivo* expression patterns of these proteins and their inhibitors (tissue inhibitors of matrix metalloproteinases - TIMPs) and regulators of their transcription (including the transcription factor HIF-1α) are unclear. The first aim of this study was to investigate levels of MMP-2, MMP-9, TIMP-1, TIMP-2 and HIF-1α in placentae throughout pregnancy using various techniques.

The study used immunohistochemistry to detect and quantify MMP-2 and MMP-9 expression in placentae from 7-19 and at 40 weeks of gestation. A reduction was found in extravillous trophoblast (EVT) expression of both MMPs as pregnancy progressed. In contrast endothelial expression of both MMPs increased. MMP-2 expression in villous CTB (vCTB) was highest in early pregnancy but decreased with gestation. MMP-9 expression in vCTB was very low throughout pregnancy.

Zymography studies revealed a significant decrease in pro-MMP-2 but not pro-MMP-9 activity through gestation although the latter did decrease during the first trimester.

The results of the study suggest a role for MMP-2 and MMP-9 not only in early placental development but also in vascular remodelling in the later stages of gestation. Moreover it also suggests that the role of MMP-2, often regarded as less important than MMP-9 in placental development, warrants further study.

Immunohistochemical studies on the same placentae showed that TIMP-1 expression in muscle surrounding the villous endothelium and in the villous stroma increased with gestation while vCTB expression did not alter and EVT expression increased during the second trimester. TIMP-2 staining in vCTB decreased with gestation while staining on endothelium, muscle and stroma increased. EVT expression of TIMP-2 was negatively correlated with gestation. Thus TIMP-2 appeared to have a high degree of co-distribution with MMP-2 both spatially and temporally. HIF-1α staining on vCTB and EVT decreased with gestation while expression on the endothelium increased.

These results suggest both paracrine and autocrine regulation of MMPs may occur early in pregnancy. Furthermore TIMPs and HIF-1α may have a role in regulating the processes in which MMPs are involved in later on in pregnancy.

Pregnant women living at high altitude are exposed to chronic hypoxia throughout gestation. It has been reported that high altitude pregnancies show physiology intermediate between normal pregnancy and pre-eclampsia and thus may provide a useful *in vivo* model of the disease. This study compared MMP-9 and MMP-2 expression in placentae from high, moderate and low altitude. Endothelial expression of MMP-9 was lower at high altitude than low altitude while MMP-9 expression in the villous stroma was higher. This may implicate MMP-9 in adaptive responses to hypoxia and villous remodelling in high altitude placentae. There were no differences in MMP-2 expression between high and low altitude placentae supporting previous studies, which have reported that MMP-2 expression may not be subject to regulation by oxygen.

Maternal serum markers of endothelial cell activation were measured throughout pregnancy at high and moderate altitude. It was hypothesised that since

circulating VCAM-1 and E-Selectin are increased in pre-eclampsia, they might also be elevated in high altitude pregnancy. This hypothesis was not supported and another marker of endothelial activation, ICAM-1, was not found to be increased either. On the contrary, levels of VCAM-1 showed an overall tendency to be lower at high altitude than at moderate altitude. Thus while some aspects of high altitude pregnancy may be suitable for modelling pre-eclampsia, clearly this is not reflected in endothelial cell activation. High altitude pregnancy is useful for determining which aspects of pre-eclampsia may result from hypoxia alone and which are due to more complex factors.

Explant studies provide a model to study aspects of trophoblast invasion including MMP secretion. Therefore we set up and validated a model to allow examination of MMP expression during trophoblast invasion at different oxygen tensions. A semi-quantitative evaluation of trophoblast proliferation and invasion at different oxygen tensions was carried out. Explants of human villous tissue (6–9 weeks of gestation) cultured on matrigel in both standard culture conditions (18% O_2) and in a low oxygen environment (2% O_2) produced regions of outgrowth of cytotrophoblast cells from villous tips and migration of cells into the matrigel. The number of sites of outgrowth and migration, area of outgrowth and extent of migration of cells into the matrigel tended to increase throughout the culture period but varied between explants from the same placenta and those from different placentae. There were no significant differences in the number of sites of outgrowth or migration scores in explants cultured in a low oxygen environment compared to those cultured in standard conditions. This study highlights the importance of careful validation, design and interpretation of experiments using *in vitro* culture systems, particularly those investigating the regulatory role of oxygen.

Understanding normal placentation and developing adequate models for failed pregnancy may provide a key to elucidating the fundamental principles of conditions such as pre-eclampsia. This may ultimately lead to improved therapeutic interventions.

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Declaration

Excerpts from the results of this thesis have been published as detailed on pages 25 and 26. I certify that this thesis does not contain any other material published or written by another person except where due reference is made in the text. The results presented in this thesis have not been submitted for any other degree or diploma.

Leah Marks

Excerpts and preliminary results of this thesis have been published and presented as detailed below.

Publications

(1) **Dissolved Oxygen Concentration in Culture Medium: Assumptions, Pitfalls and Solutions.**
D. Newby, BSc, PhD; L. K. Marks, BSc; F. Lyall, BSc, PhD. (submitted)

(2) **Villous Explant Culture: Characterisation and Evaluation of a Model to Study Trophoblast Invasion.**
Deborah Newby, BSc, PhD; Leah Marks, BSc; Frances Cousins; F. Lyall, BSc, PhD. (submitted)

(3) **Endothelial activation and cell adhesion molecule concentrations in pregnant women living at high altitude.**
Leah Marks BSc, Stacy Zamudio PhD, Frances Cousins, Elizabeth Duffie, and Fiona Lyall PhD. (submitted)

(4) **MMP-2 and MMP-9 expression is temporally and spatially regulated throughout pregnancy.**
Leah Marks, BSc; Frances Cousins; Elizabeth Duffie, and Fiona Lyall, PhD. (manuscript in preparation)

Oral Presentations

(1) British meeting of the ISSHP Glasgow, July, 2003
Maternal cell adhesion molecule concentrations in high altitude pregnancy.
L. Marks, S. Zamudio, E. Duffie, F. Cousins & F. Lyall.

(2) McDonald Club Meeting, Glasgow, April 2004
High altitude pregnancy: a model for understanding endothelial dysfunction in pre-eclampsia.
L. Marks, S. Zamudio, E. Duffie, F. Cousins & F. Lyall.

Poster Presentations

Meeting of the ISSHP, Toronto, 2002

(1) **MMP-9 Expression is Abnormal in Placentae at High Altitude : A Link to Chronic Hypoxia?**

Marks, L. (BSc), Zamudio, S. (PhD), and Lyall, F. (PhD).

British Meeting of the ISSHP, Glasgow, 2003

(1) **MMP-2 is temporally and Spatially expressed in placentae throughout gestation.**

L. Marks (BSc), S. Robson (MD), E. Duffie, F. Cousins & F. Lyall (PhD).

(2) **MMP-9 is temporally and Spatially expressed in placentae throughout gestation.**

L. Marks (BSc), S. Robson (MD), E. Duffie, F. Cousins & F. Lyall (PhD).

(3) **Villous Explant Culture: Characterisation and evaluation of a model to study trophoblast invasion.**

D. Newby (PhD), L. Marks (BSc), F. Cousins, E. Duffie & F. Lyall (PhD).

(4) **E-Selectin concentrations in the maternal circulation are unchanged in high altitude pregnancies.**

L. Marks (BSc), S. Zamudio (PhD), E. Duffie, F. Cousins & F. Lyall (PhD).

Meeting of the SGI, Houston, 2004

(1) **MMP-9 is temporally and spatially expressed in placentae throughout pregnancy. Possible roles in invasion & tissue & vascular remodelling.**

L. Marks (BSc), F. Cousins, E. Duffie, & F. Lyall (PhD).

(2) **MMP-2 is temporally and spatially expressed in placentae throughout pregnancy.**

L. Marks (BSc), F. Cousins, E. Duffie, & F. Lyall (PhD).

(3) **Endothelial activation and cell adhesion molecule concentration in the maternal circulation of high altitude pregnancies.**

L. Marks (BSc), S. Zamudio (PhD), F. Cousins, E. Duffie, & F. Lyall (PhD).

(4) **Villous Explant Studies: A quantitative analysis of outgrowth in low and high oxygen environments**

D. Newby (PhD), L. Marks (BSc), F. Cousins, & F. Lyall (PhD).

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List of Abbreviations used

ABC	Avidin Biotin Complex
Abs	Absorbance
ANOVA	Analysis of Variance
APMA	4-Aminophenylmercuric Acetate
APS	Ammonium Persulfate
BSA	Bovine Serum Albumin
°C	Degrees Centrigrade
CAM	Cell Adhesion Molecule
CO	Carbon Monoxide
CO_2	Carbon Dioxide
CTB	Cytotrophoblast
CV	Coefficient of Variance
CVS	Chorionic Villous Sample
DAB	3', 3'-Diaminobenzidine
dH_20	Distilled Water
DTT	Dithiolthreitol
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulphoxide
ECL	Enhanced Chemiluminescence
ECM	Extracellular Matrix
ELCS	Elective Caesarian Section
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme Linked Immunosorbant Assay
eNOS	Endothelial nitric oxide synthase
E-Selectin	Endothelial Selectin
EVT	Extra-villous cytotrophoblast
FBS	Fetal Bovine Serum
g	Gravitational Acceleration
HA	High Altitude
hCG	Human Chorionic Gonadotrophin
HELLP	Hemolysis Elevated Liver Enzymes Low Platelets
HIF	Hypoxia Inducible Factor

HLA	Human Leukocyte Antigen
hPL	Human Placental Lactogen
hr	Hour
HO	Hemeoxygenase
ICAM-1	Intracellular Cell Adhesion Molecule-1
Ig	Immunoglobulin
IHC	Immunohistochemistry
iNOS	Inducible Nitric Oxide synthase
IUGR	Intra Uterine Growth Restriction
kDa	KiloDaltons
LA	Low Altitude
MA	Moderate Altitude
min	Minutes
mmHg	Millimeters of Mercury
MMP	Matrix Metalloproteinase
mRNA	Messenger Ribonucleic Acid
MT-MMP	Membrane Type Matrix Metalloproteinase
Mwt	Molecular Weight
N/A	Not Applicable
N/P	Not Present
N_2	Nitrogen
NO	Nitric Oxide
O_2	Oxygen
OD	Optical Density
p	Probability
PA	Plasminogen Activator
PAGE	Polyacrylamide Gel Electrophoresis
PAI	Plasminogen Activator Inhibitor
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PP	Post Partum
PO_2	Partial Oxygen Pressure
ROS	Reactive Oxygen Species
SD	Standard Deviation

SDS	Sodium-Dodecyl Sulphate
SE	Standard Error
Sec	Second
STB	Syncytiotrophoblast
TBS	Tris Buffered Saline
TEMED	N,N,N',N' tetramethylethylenediamine
TGF	Transforming Growth Factor
TIMP	Tissue Inhibitor of Metalloproteinase
Tween 20	Polyoxycthylene Sorbiton Monolaurate
TOP	Termination of pregnancy
uPA	Urokinase Type Plasminogen Activator
VCAM-1	Vascular Cell Adhesion Molecule-1
vCTB	Villous Cytotrophoblast
VEGF	Vascular Endothelial Growth Factor
Vol	Volume
v:w	Volume to weight ratio
w/v	Weight per unit volume
mA	Milliamperes
g	Grams
mg	Milligrams
µg	Micrograms
l	Litres
ml	Millilitres
µl	Microlitres
m	Metres
mm	Micrometres
µm	Micrometers
M	Molar
mM	Millimolar
µM	Micromolar
nM	Nanomolar
V	Volts
mV	Millivolts

Chapter 1: Introduction

1.1 The Placenta

1.1.1 The term placenta

The placenta is a unique organ that forms the connection between the mother and fetus during pregnancy. The placenta has two main roles – the physical attachment of the embryo to the uterus, and the bringing of the fetal and maternal circulations into close proximity, allowing exchange of gases, nutrients and waste.

The full term human placenta is a circular disc-like organ of, on average, 22cm in diameter and 2.5cm thick, weighing around 470g. The umbilical cord implants slightly off-centre on the fetal (chorionic) side (figure 1a). The chorionic plate is covered with the avascular glossy amnion, a protective membrane. The vessels that can be seen on the surface of this plate are the chorionic vessels. These are continuous with those of the umbilical cord and branch repeatedly. The maternal portion of the placenta is called the basal plate (figure 1b) and the top layer of this surface adheres to the placenta after separation from the uterus. It consists of endometrial and trophoblast cells and fibrinoid (Kaufmann, 1971). The basal surface is separated into a series of lobes or placentones, which roughly mark the underlying villous trees. The placental bed consists of endometrial tissue and trophoblast cells (Robertson and Warner, 1974).

Fetal growth and development is dependent on successful placentation, a process which begins almost immediately after fertilisation and continues throughout pregnancy. Many of the vital stages in the development of the placenta occur in the early weeks of gestation.

1.1.2 Early development of the blastocyst

After fertilization has occurred the nuclei of the ovum and the sperm combine to form a zygote, which is subsequently transported through the fallopian tube to the cavity of the uterus, a process taking 3-4 days. The zygote undergoes rapid mitotic division to form a ball of cells called a morula, which develops into a blastocyst soon after entry into the uterus. The blastocyst consists of a fluid filled cavity surrounded by a single layer of trophoblast cells (figure 2). At one end of the blastocyst there lies a cell mass that is the precursor of the embryo, while the cytotrophoblast (CTB) layer is the

precursor of the placenta and the membranes surrounding the embryo (Boyd and Hamilton, 1970; Benirschke and Kaufmann, 2000; Barnea et al. 1992).

Figure 1a The term placenta – chorionic plate

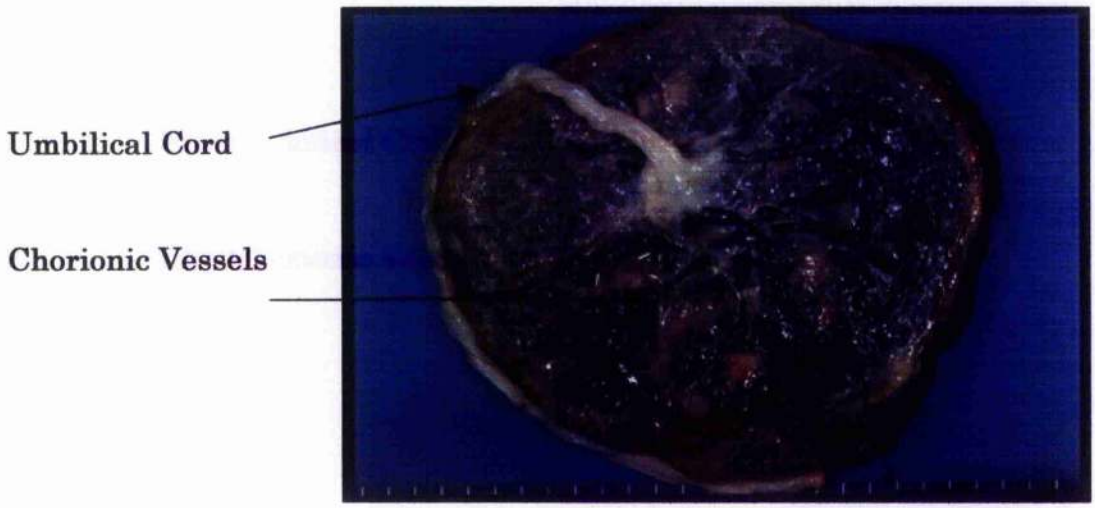

Figure 1b The term placenta – basal plate

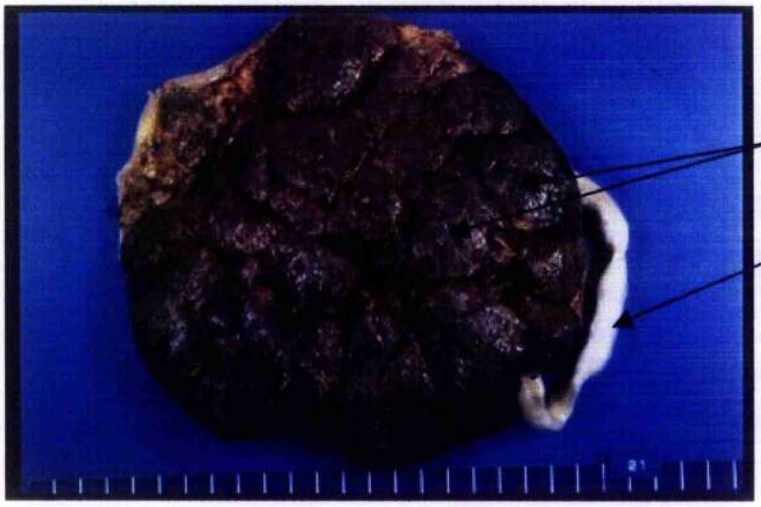

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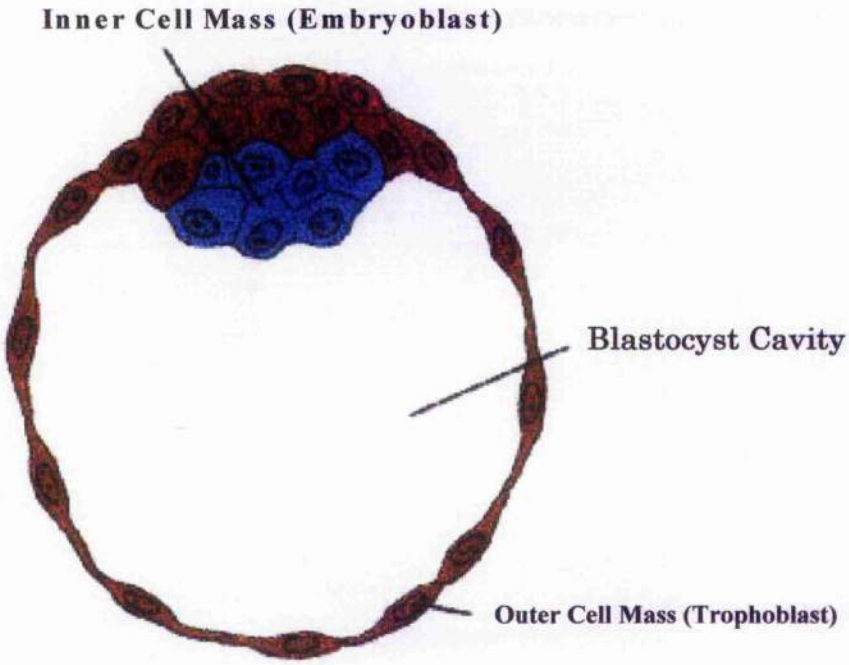
Figure 2 Blastocyst structure



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1.1.3 Implantation of the blastocyst

For the successful continuation of pregnancy, implantation of the blastocyst into the uterine wall must occur. Implantation can be divided into several stages. Firstly the blastocyst must attach itself to the epithelial surface of the uterus, this can be further divided into an apposition stage and an adhesion stage (Schlafke and Enders, 1975). The penetration of the uterine luminal epithelium then occurs as trophoblast extensions penetrate between uterine epithelial cells by a mechanism called intrusion penetration (Schlafke and Enders, 1975). By day 6-7 the embryo is completely embedded in the uterus and uterine epithelial cells grow over the implantation site (Hertig et al. 1956).

1.1.4 Early development of the placenta

As implantation proceeds, the trophoblast cell layer surrounding the blastocyst differentiates into two layers, the proliferating inner villous cytotrophoblast (vCTB) and the non-dividing outer layer called the syncytiotrophoblast (STB). vCTB cells are the precursor cells that differentiate into the STB layer (Carter, 1964). By day 8 post conception, a series of fluid filled spaces known as lacunae begin to develop within the STB mass (figure 3). These lacunae are the early intervillous space. Cords of CTB that surround the spaces start to penetrate into the lacunae. Side branches of these finger-like projections appear by day 13 (Kingdom et al. 2000).

During this early stage of placental formation, some trophoblasts migrate into the endometrial stroma, between the uterine glands and around the uterine spiral arteries (Pijnenborg, 1990). Initially maternal blood escaping from the spiral arteries passes into the lacunae (figure 4) before the mouths of the spiral arteries become blocked with trophoblast plugs. These prevent maternal blood from entering the lacunae or intervillous space for the first 10 weeks of pregnancy (Schaaps and Hustin, 1988; Hustin et al. 1988).

As a result of the prevention of blood flow into the lacunae, the oxygen concentration in the intervillous space remains relatively low (Rodesch et al. 1992) until the trophoblast plugs are loosened allowing blood to flow freely. When there is no blood flow in the intervillous space the placenta and fetus consequentially experience no oxidative stress. As the plugs dissolve oxygen tension increases and

hence oxidative stress increase, as does the production of protective anti-oxidant enzymes by the placenta (Jaffe et al. 1997; Burton et al. 1999). During the low oxygen period, nutrients necessary for fetal development are obtained from glandular secretions and plasma filtrate in the intervillous space. Important changes also occur in the endometrial lining of the uterus during these early stages of pregnancy, including cellular growth and accumulation of glycogen in a process termed decidualisation (reviewed in Dunn et al. 2003).

Figure 3 Differentiation of trophoblasts and formation of lacunae

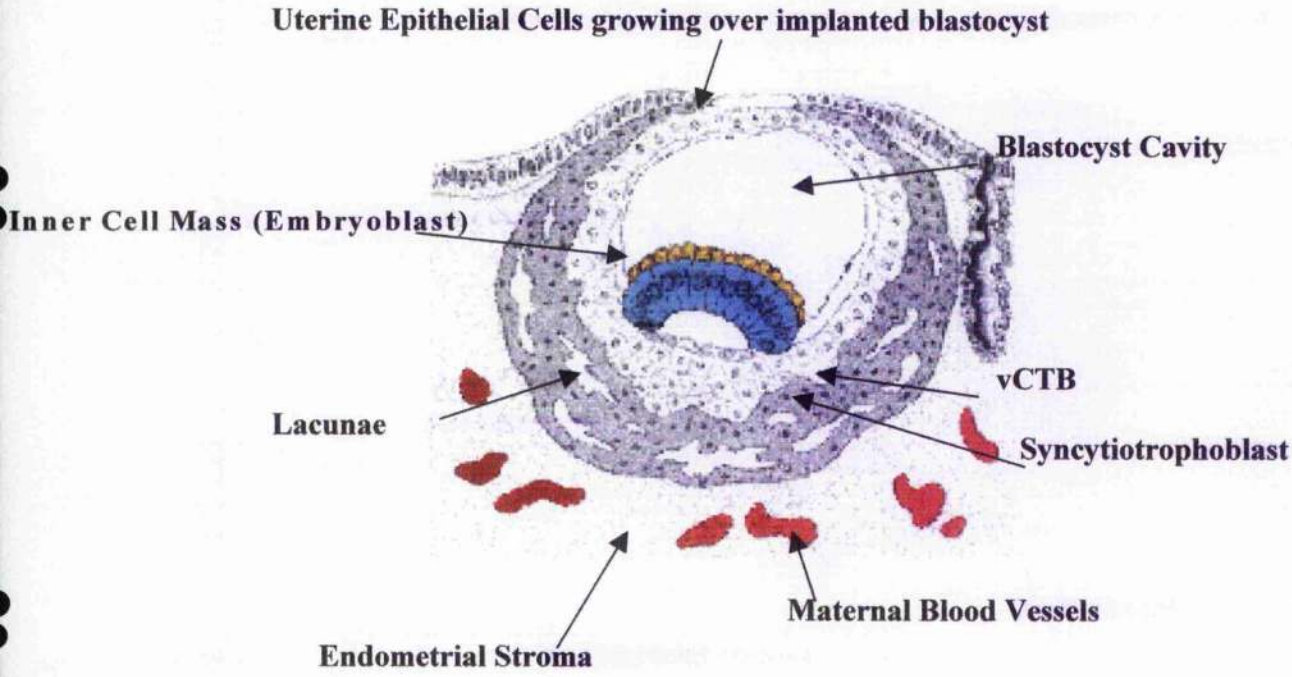

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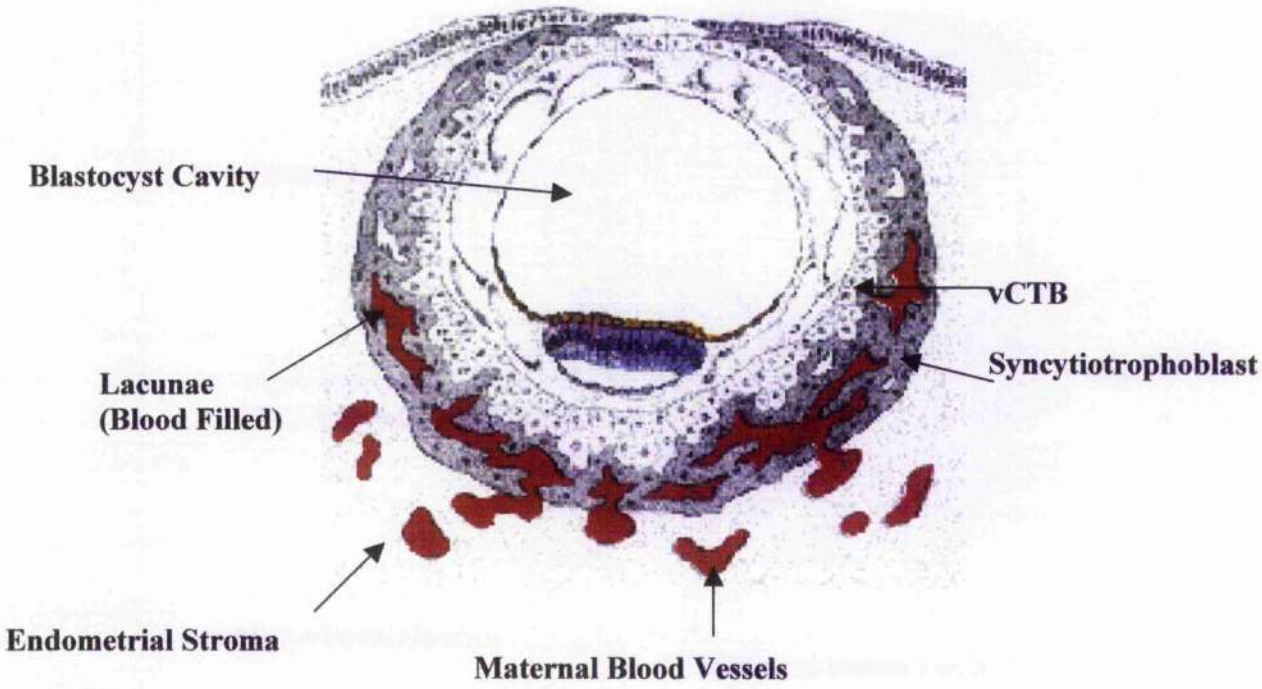
Figure 4 Blood flow into lacunae



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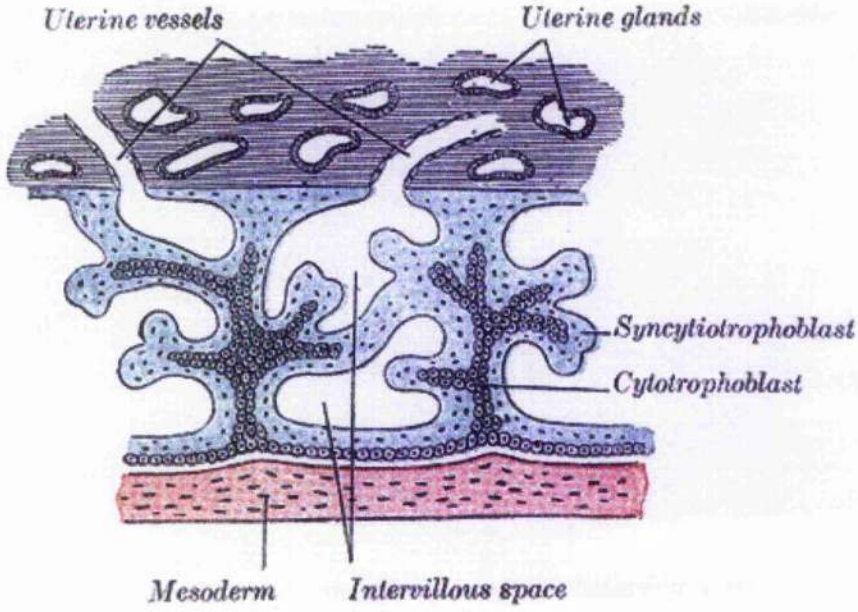
1.2 Development of the placenta and the utero-placental circulation

1.2.1 Villous structure and development

As placentation continues, the cords of trophoblasts (trabeculae) that had begun to penetrate the lacunae become increasingly branched as the trophoblasts proliferate. The lacunae are the precursors of the intervillous space, the site of exchange between maternal and fetal circulations, and the trabeculae are the precursors of the chorionic villi. At this early stage they are known as primary villi. The primary villi become secondary villi as extraembryonic mesoderm from the primary chorionic plate invades the trabeculae. The villous core is known as the stroma and is composed of a loose network of fibroblast cells with Hofbauer cells (fetal macrophages) dispersed throughout (Kaufmann et al. 1977). At day 19 of development fetal capillaries begin to develop within the villi by the differentiation of hemangioblastic progenitor cells (Demir et al. 1989). This transforms the secondary villi into tertiary villi. During the second and third trimesters tertiary villi are predominant and branches of the villi continue to form until term (Moore and Persaud, 1998). In early pregnancy, the vCTB form a continual layer below the STB surrounding the villi whereas at term very few vCTB are observed. Thus at term only the syncytial layer separates the endothelium of the villous capillaries from the maternal circulation.

As villi develop throughout the placenta a complex network called the villous tree, is established (figure 5) allowing the growing fetus to be supported by the maternal circulation. The placenta contains 60-70 main stem villi each of which is at the centre of a villous tree. These main stem villi subdivide into 2-5 branches, which further subdivide (Page, 1993). Final stem villi are 80-100 μm in diameter, have a single arteriole and venule and contain up to ten capillaries. The area of exchange of the villi is about $14m^2$ by term and the capillaries within the villi have a length of 50km (Martal and Cedard, 1993).

Figure 5 Development of the villous tree



a) Primary chorionic villi

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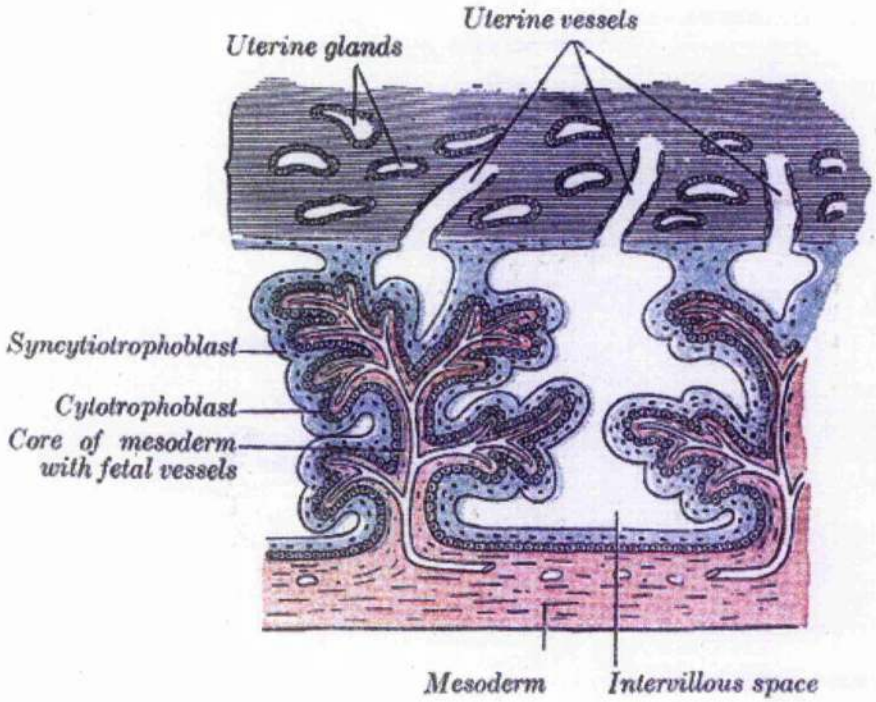

b) Secondary chorionic villi

1.2.2 Trophoblast invasion

Two types of villi are present in the placenta, known as floating and anchoring villi. Floating villi are not connected to the maternal uterus and float freely in the intervillous space allowing exchange between maternal and fetal circulations to occur. Anchoring villi attach the placenta to the uterus. Cell columns form at the tips of these villi where CTB cells proliferate and then break through the syncytium and invade the uterus and its spiral arteries. These migratory invasive cells are extra-villous CTB (EVT). Their invasion is one of the most important processes in placentation. EVT that migrate into the spiral arteries cause an important physiological change in these vessels - the loss of the endothelial and muscular layer (Brosens et al. 1967; DeWolf et al. 1973; Sheppard and Bonnar, 1974), the endothelial layer being restored later in pregnancy. As a result, the blood flow through the spiral arteries changes from a low flow-high resistance to a high flow-low resistance circuit, allowing the needs of the growing fetus to be met. Maternal vasomotor control over the intervillous circulation is also lost through the loss of contractility of the vessels (Moll et al. 1988).

Invasion by metastatic tumour cells has been defined as "the disruption of extracellular matrix boundaries (ECM, basement membrane, interstitial stroma), followed by mixing of the tumour cells with cell types of a different origin to those in the primary tumour" (Liotta and Rao, 1986). This definition is also appropriate in describing trophoblast invasion of the uterus (figure 6).

Although some invasion of trophoblasts into the spiral arteries is observed very early in gestation as described previously (section 1.1.4), the principal wave of trophoblast invasion does not occur until several weeks later. Two pathways of invasion have been described, the interstitial and the endovascular (Pijnenborg et al. 1980; Pijnenborg et al. 1981a; Pijnenborg et al. 1981b; Pijnenborg et al. 1983; Pijnenborg, 1990; Pijnenborg et al. 1996; Benirschke and Kaufmann, 2000). In the interstitial pathway the cells invade the placental bed to the endometrium and the first third of the myometrium. In the endovascular pathway, EVT also invade the lumen of arteries and the distal parts of some veins. *In vitro* studies have never been able to differentiate between the two cell populations (Lyall and Kaufmann, 2000).

There is some debate regarding the origin of endovascular trophoblast and about the "two wave theory" – the concept that endovascular invasion occurs in two waves, the first at 8-10 weeks and the second at 16-18 weeks (Pijnenborg et al. 1980).

In this model, the first wave of invasion is into the decidual segments of spiral arteries and the second wave extends into the myometrial segments.

Figure 6 Trophoblast Invasion

Figure 6 (a) **Schematic representation of trophoblast invasion**

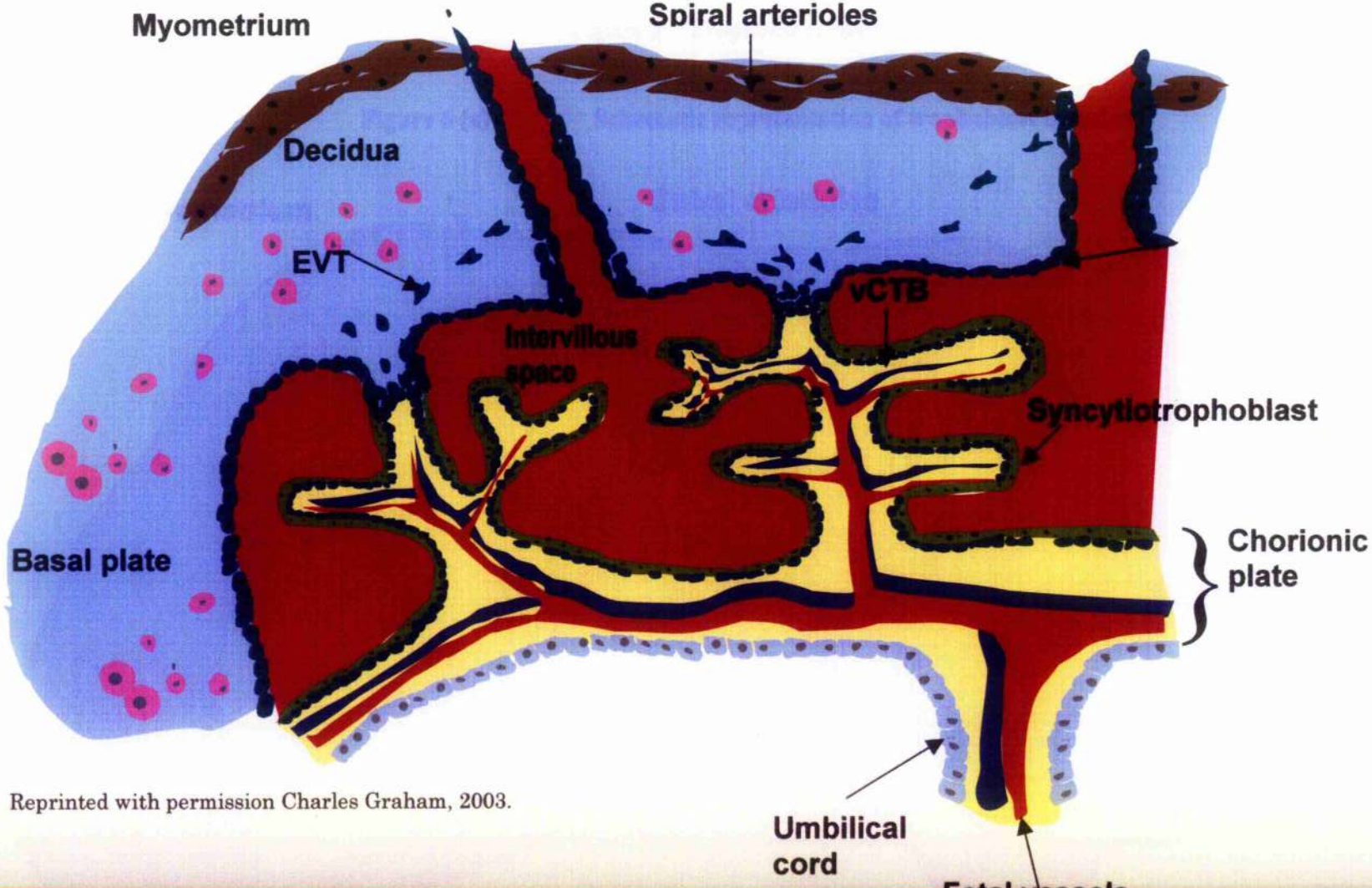

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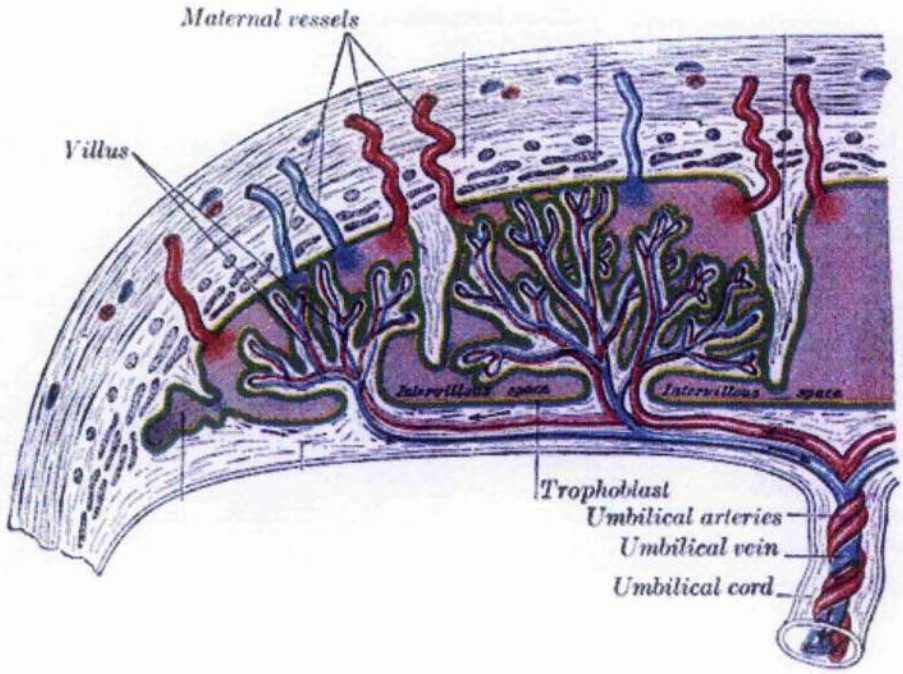
Figure 6 (b) **The utero–placental circulation**



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1.2.3 Mechanisms of control of trophoblast invasion

1.2.3.1 Control of invading trophoblast

The invasive phenotype acquired by the migrating EVT clearly requires dramatic molecular changes in the cells. Immunohistochemical studies on placental bed biopsies from 1st trimester placentae which contain both basal plate decidua and parts of the underlying myometrium and its spiral arteries, in conjunction with *in vitro* studies have suggested that changes in various factors may be involved. These factors include cell adhesion molecules (CAMs), Matrix Metalloproteinases (MMPs) and their inhibitors Tissue Inhibitors of Metalloproteinases (TIMPs), nitric oxide (NO), the carbon monoxide (CO)/hemeoxygenase (HO) pathway, transforming growth factor-βs (TGF-βs) and Human leukocyte antigen-G (HLA-G). In addition, it is thought that oxygen may have a regulatory role. Some of these factors are outlined below while MMPs and TIMPs are discussed separately in section 1.4 and oxygen tension is considered in section 1.5.

It is thought that trophoblast invasion is compromised in pre-eclampsia (section 1.7). Much remains unknown about both normal and abnormal trophoblast invasion but it is likely that some of the regulators of invasion discussed below are aberrant in pre-eclampsia (section 1.7).

1.2.3.2 Cell adhesion molecules

Cell adhesion molecules (CAMs) are expressed on the surface of invading CTB, and their ligands, and regulate the adhesion of cells to each other and to other cell types. They are involved in interactions with the extracellular matrix (ECM) and thus may be important in controlling invasion. There are four major families of CAMs – the immunoglobulin superfamily, integrins, selectins and cadherins, each with differing functions in cell adhesion. All highly invasive cells have alterations in CAM expression (Alexander and Werb, 1989) thus it is not surprising that for invasive CTB this also seems to occur.

Integrins bind to ECM proteins e.g. collagen, laminin and fibronectin. Villous trophoblasts express integrins found on many polarized epithelial cells,

primarily α6/β4, and to a lesser extent α3/β1 (Korhonen et al. 1991; Damsky et al. 1992; Burrows et al. 1993; Aplin, 1993; Damsky et al. 1994). The basement membrane to which the CTB are attached in the first trimester expresses collagen, laminins and heparin sulphate (Ohno et al. 1986; Levio et al. 1989; Rukosuev, 1992; Tryggvason, 1993; Castellucci et al. 1993; Church et al. 1998) but only weakly expresses fibronectin and collagen IV (Damsky et al. 1992). When trophoblasts in cell columns are studied there appears to be a gradual modulation of adhesion molecules as distance from the villi increases. α6/β4 staining remains strong on CTB close to the villi, although α3 is no longer detectable (Damsky et al. 1992). CTB furthest from the villi increase the production of α5/β1 subunits of the fibronectin receptor while α6/β4 expression deceases to very low levels (Damsky et al. 1992; Aplin, 1993). Single CTB or cell clusters within the placental bed have been shown to express α1/β1 and α5/β1 integrins (Damsky et al. 1992). During the differentiation of CTB into EVT, changes in the distribution of laminin and fibronectin isoforms have also been noted (Levio et al. 1989; Castellucci et al. 1993; Frank et al. 1994; Korhonen et al. 1997; Church et al. 1998). Several *in vitro* studies have shown similar findings (Yagel et al. 1989; Librach et al. 1991; Damsky et al. 1994; Librach et al. 1994; Irving et al. 1995; Vicovac et al. 1996; Zhou et al. 1997a). Some studies have suggested that as CTB invade spiral arteries, they undergo a switch in adhesion molecules to mimic the endothelial cells they are replacing (Zhou et al. 1997a; Damsky and Fisher, 1998). However these studies failed to take into account the fact that spiral arteries are re-endothelialised in the third trimester (DeWolf et al. 1973; Khong et al. 1992) and that the vessel lumen is not replaced with a lining of CTB. Lyall et al. (2001b) reported no expression of PECAM-1, an endothelial CAM, on EVT in either normal or abnormal pregnancy at any stage of gestation illustrating that care must be taken in interpreting previous reports.

1.2.3.3 Transforming growth factor-βs

Transforming Growth Factor-βs (TGF-βs) are part of a large family of cytokines that comprises three related 25 kilodalton (kDa) proteins TGF-β1, TGF-β2, and TGF-β3. Several studies have suggested that decidual production of TBF-βs may act as a negative regulator of trophoblast invasion (Lala and Hamilton, 1996; Caniggia et al.

1999). It was reported that TGF-β3 expression in the placenta was low until seven weeks of gestation, rose during the following two weeks then fell dramatically at nine weeks of gestation, thus corresponding with the proposed first wave of invasion. Furthermore the same study reported overexpression of TGF-β3 in pre-eclamptic pregnancies during the third trimester. Another study reported TGF-β2 but not TGF-β3 expression in the placenta and placental bed (Simpson et al. 2002). The data may be inconsistent due to use of different antibodies, and methods of reporting placental age must also be taken into consideration. There is some evidence that TGF-βs can regulate trophoblast invasion *in vitro* although the data is again inconsistent (Graham et al. 1992; Bass et al. 1994; Caniggia et al. 1998) Lyall et al. (2001a) found no difference in expression of any of the TGF-βs in the complications of pregnancy pre-eclampsia or intra-uterine growth restriction (IUGR) compared to normal pregnancy suggesting that they may not have a pathological role in either of these conditions.

1.2.3.4 Nitric Oxide Synthase pathway

The production of endothelial nitric oxide synthase (eNOS) by human placental syncytiotrophoblast and villous endothelial cells and studies in guinea pig led to the speculation that nitric oxide expression by CTB may be partly responsible for dilation of the spiral arteries during human placentation (Nanaev et al. 1995). Studies by Lyall et al. (1999b), however, showed that invasive CTB did not express eNOS or inducible nitrous oxide Synthase (iNOS) and suggested that CTB-derived nitric oxide was not responsible for dilation of spiral arteries. Furthermore no differences were observed between normal pregnancy and cases of pre-eclampsia and IUGR where trophoblast invasion was compromised.

1.2.3.5 Carbon Monoxide/Hemeoxygenase pathway

Carbon Monoxide (CO) is produced by the enzyme hemeoxygenase (HO) which exists in two forms; HO-1 (inducible) and HO-2 (constitutive). The similarities between the effects of NO and CO led to speculation that CO may contribute to spiral artery dilation during placentation. Significant differences were found in HO-2 immunostaining between 1st, 2nd and 3rd trimester placenta on villous trophoblast and

endothelium (Lyall et al. 2000a). In placental and placental bed biopsies from pregnancies complicated by pre-eclampsia endothelial HO-2 expression was significantly reduced, perhaps contributing to the reduced blood flow seen in this condition. Within the placental bed all EVT were positive for HO-2 and no differences were seen in pre-eclampsia (Lyall et al. 2000). It is thus possible that the expression of HO-2 and hence production of CO contributes to spiral artery dilation.

1.2.3.6 Human leucocyte antigen-G (HLA-G)

HLA-G is a class IB major histocompatability antigen expressed by EVT that may protect cells from natural killer cell lysis (Chumbly et al. 1994). It is upregulated in culture as cells invade and is only expressed *in vivo* on cells which invade the decidua (McMaster et al. 1995). It has also been suggested that HLA-G expression enables trophoblasts to evade damage by interleukin-2 which is a cytotoxic cytokine found in decidual tissue (Hamai et al. 1999). Other HLA's may have specific interactions with uterine natural killer cells and much current research is focussed in this area. The subject is reviewed in King et al. (2000b) and Moffett and Loke (2004).

1.2.4 Spatial and temporal regulation of trophoblast invasion

Ensuring that invasion occurs at the right stage of gestation and the limiting of invasion to the endometrium and first third of the myometrium is clearly critical. Where invasion does not occur properly blood flow to the developing fetus is compromised and this may result in loss of the pregnancy, lead to pre-eclampsia or IUGR (Kingdom, 1998). Alternatively, if invasion is unrestrained choriocarcinoma, a very metastatic cancer, may develop.

While many of the factors required for trophoblast invasion such as integrin expression modulation and the secretion of proteases to degrade the ECM have been studied, the underlying mechanisms controlling these are less well understood. It is possible that the transition from the invasive to the non-invasive phenotype results from an intrinsic control mechanism within the trophoblast cells themselves that allows invasion to cease 4-5 months after implantation. Another possibility is that exposure to the myometrium during the late stages of invasion acts as a signal to halt the process. Alternatively the maternal decidua may physically act as a barrier to

intrinsically invasive trophoblasts (Billington, 1971; Aplin, 1991; Bischof et al. 2000). Much recent work has focussed on the role of oxygen in controlling trophoblast invasion and this will be considered separately (section 1.5).

1.2.5 Syncytiotrophoblast and the consequences of the development of the maternal-fetal circulation

1.2.5.1 Syncytiotrophoblast

The syncytiotrophoblast (STB) is the continual cell layer forming the outer surface of the placental villi. It is formed by fusion of the underlying vCTB layer. The STB is bathed in maternal blood and has many microvilli on its surface increasing the surface area available for exchange of substances between the mother and fetus. The syncytial surface also expresses receptors for molecules to be transported into the placenta from maternal blood. Conversely the syncytial layer acts as a barrier against substances which would be harmful were they to pass into the fetal circulation. Lyall et al reported expression of nitric oxide synthase by the STB suggesting it may have a role in regulating blood flow and as an anticoagulant (Lyall et al. 1999a). STB is also the principal site of various hormones vital for pregnancy including human chorionic gonadotrophin (hCG) and human placental lactogen (hPL) (section 1.2.5.3). By term the underlying vCTB layer is sparse (Jones and Fox, 1991) and STB dominates.

1.2.5.2 Transport of metabolites and nutrients

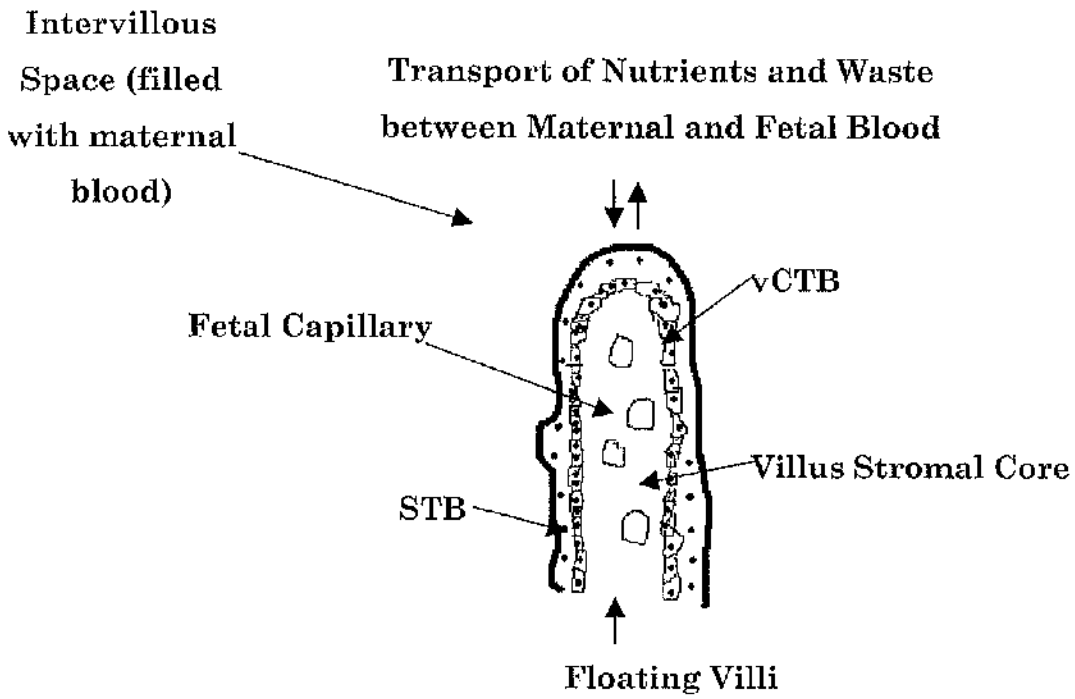
Once the utero-placental circulation is established fetal blood, which is low in oxygen and nutrients, flows through two umbilical arteries to the capillaries of the villi and then returns through the single umbilical vein to the fetus. Maternal blood (rich in oxygen and nutrients) flows from the uterine arteries into large blood sinuses surrounding the villi then back into the uterine veins of the mother. Since the villous capillaries are lined with a thin endothelium and surrounded by a layer of mesenchymal tissue, which is covered on the outside of the villi by a layer of trophoblast cells, a very short distance separates the maternal and fetal circulations. It is thought that placental exchange mainly occurs across the vasculosyncytial membranes, formed by insertion of capillary loops within the syncytiotrophoblast

layer. In such areas no mesenchymal layer is present between fetal endothelium and the very thin nucleus-free region of the syncytiotrophoblast. This allows for diffusion of various substances while ensuring that the individual circulations never actually come into direct contact (figure 7). As pregnancy progresses the permeability of the placenta increases, allowing greater diffusion. Oxygen and metabolic substrates (eg glucose, fatty acids, ionic substances) diffuse readily into fetal blood by concentration gradients, while other products such as amino acids are transferred via active transport processes. Waste products from the fetus also pass through the placenta into the maternal circulation for disposal by the liver (Moore and Persaud, 1998). Materno-fetal exchange across the placenta is reviewed in Sibley and Boyd, (1988) and Sibley et al. (1998).

1.2.5.3 Endocrine secretion

During pregnancy the placenta is an important endocrine organ which releases the steroid hormones progesterone and oestrogens into the fetal and maternal circulations (Conley and Mason, 1990). Towards the end of pregnancy the placenta produces up to 30 times the normal amount of oestrogen that the body produces in the non-pregnant state and ten times the normal levels of progesterone. These hormones are vital both in fetal development and in preparing the mother's body for parturition. The placenta also secretes peptide hormones including hCG and hPL into the maternal circulation from very early stages ensuring the persistence of the corpus luteum and preventing menstruation which would result in the loss of pregnancy (Jones, 1989). A review of placental growth hormones is found in Lacroix et al. (2002).

Figure 7 **Transport of metabolites from maternal to fetal blood**



1.3 Methods for studying trophoblast invasion

1.3.1 The study of trophoblast invasion

Morphological studies of placenta and placental bed biopsies from first and second trimester hysterectomies have provided important information on the timing of trophoblast invasion of the maternal spiral arteries. (Brosens et al. 1964; Brosens et al. 1967; Brosens et al. 1978; DeWolf et al. 1980; Pijnenborg et al. 1980; Pijnenborg et al. 1981a; Pijnenborg et al. 1981b; Pijnenborg et al. 1982; Pijnenborg et al. 1983; Robertson et al. 1986; Khong et al. 1986; Wells and Bulmer, 1988; Loke, 1990; Pijnenborg, 1990). However, because of practical and ethical considerations, dynamic studies of implantation and trophoblast invasion are generally limited to *in vitro* models. Hence the gaps in the understanding of the processes that occur during early placentation result partially from a lack of adequate experimental models although the past decade has seen significant advances in this area.

Two main *in vitro* systems have been used to study trophoblast invasion. CTB isolated from placentae have been cultured extensively to determine characteristics of the cells and to examine invasion of artificial basement membranes. More recently, culture of placental villous explants has been used to model differentiation of CTB cells whilst maintaining the tissue integrity and simulating an environment as close to the *in vivo* situation as possible.

1.3.2 Primary trophoblast cell culture

For many years the principal *in vitro* system for studying trophoblast behaviour has been the isolation and culture of dispersed mononuclear CTB from 1st, 2nd and 3rd trimester placentae. Many studies have described methods for culturing CTB (Kliman et al. 1986; Bax et al. 1989; Bloxam, 1991; Bloxam et al. 1997a; Bloxam et al. 1997b; Frank et al. 2000; Frank et al. 2001). Dissection and mincing of the placental tissue followed by enzymatic digestion provides an initial means of isolating CTB from the placentae. The digestion is followed with further purification steps using, for example, density gradient centrifugation or HLA-Class I antibody-coated beads to remove contaminating cells (Kawata et al. 1984; Butterworth and Loke, 1985). First trimester CTB always require further purification due to the high level of contamination and

term CTB may also require purification, depending on experimental requirements. Possible contaminating cells include fibroblasts, myofibroblasts, pericytes, endothelial and blood cells and remnants of the syncytium (Loke, 1982; Butterworth and Loke, 1985; Loke et al. 1988; Douglas et al. 1989; Shorter et al. 1990; Bischof et al. 1991; Douglas et al. 1993) There has been much debate over purification procedures (Blaschitz et al. 2000). The CTB obtained at the end of the purification are a mixture of non-invasive villous and invasive EVT. EVT can only be recovered from the tips of anchoring villi since the cells that have invaded will remain in the placental bed after delivery of the placenta. Care must be taken when choosing the antibody for purification as some commonly used HLA-Class I antibodies bind to EVT due to its expression of HLA-G and HLA-C (Hutter et al. 1996; King et al. 2000a).

Studies of CTB behaviour during differentiation into a continuous syncytial layer can be undertaken on cells cultured on plastic surfaces. In order to study cell behaviour during invasion, CTB are cultured on ECM-like matrices. The cells can also be co-cultured with decidual extract from human endometrial tissue, endometrial explants or endothelial cells in order to study paracrine interactions (Babawale et al. 1996; Gallery et al. 2001).

1.3.3 Invasive properties of CTB cultured on extra cellular matrix

Matrigel is an ECM material containing type IV collagen, laminin, heparan sulfate, proteoglycan derived cells and enactin. Trophoblast cells seeded onto a filter coated in this substance (or with collagen) tend to aggregate then move through the gel and the pores to the opposite side of the filter. The ability of cells to penetrate matrigel and migrate to the underside of the filter has been associated with invasive potential in several studies (Fisher et al.1985; Fisher et al. 1989; Yagel et al. 1988; Librach et al. 1991). Matrix degrading proteolytic activity has also been correlated with invasive potential (Yagel et al. 1988; Fisher et al. 1989; Feinberg et al. 1989; Emonard et al. 1990b; Librach et al. 1991).

CTB cells isolated from placentae at term have been reported to be less invasive than those isolated from early gestation placentae in several studies (Fisher et al. 1989; Librach et al. 1991) although Kliman et al. (1990) reported that both first and third trimester CTB cause degradation of matrigel. It is possible that during the

isolation protocol, the third trimester trophoblasts undergo some degree of de-differentiation that allows them to exhibit invasive properties.

It is also important to note that the composition of the ECM can affect the profile of proteolytic enzymes produced by first trimester CTB (Emonard et al. 1990a; Kliman and Feinberg, 1990). Since matrigel contains endogenous proteolytic activity as well as growth factors, results based on models such as those described must be treated with caution (Vukicevic et al. 1992; Mackay et al. 1993; Vicovac and Aplin, 1996). Bischof et al. (1991) identified 4 enzymes secreted into the medium of cultured CTB, and showed that a 59kDa gelatinase was specific to cells cultured on matrigel.

1.3.4 Trophoblast cell lines

In addition to primary cell culture of CTB isolated from placentae, numerous trophoblast cell lines have been used for studying invasion. The most commonly used cell lines are BeWo, JEG and JAR, all three being derived from choriocarcinomas. Other cell lines have been generated from viral transformation or from fusion of normal CTB with choriocarcinoma cells (White et al. 1989; White et al. 1990; King et al. 2000c).

There are several advantages in using transformed cell lines in invasion studies. No lengthy isolation from placentae is required, there is no limit on cell yield, the cells replicate rapidly in culture, and they retain some similarities to non-transformed CTB. The main drawback is that transformed cells have little control over their invasiveness and growth, in contrast to EVT cells in which proliferation and invasive behaviour are tightly regulated. Therefore transformed cells have limited applications in modelling trophoblast invasion.

A replicating CTB cell line from 1st trimester placentae has also been produced by spontaneous detachment of cells from villous placental tissue that had been cultured for several days. The cells were able to migrate through epithelium free human amnion composed of basement membrane and connective tissue, indicating that they had retained their invasive potential (Yagel, 1989).

1.3.5 Limitations of trophoblast cell culture

Despite the numerous uses of primary cultures in modelling CTB invasion, currently available cell isolation protocols produce a heterogeneous mixture of cells and the effect of contaminants is hard to appreciate fully. The structure of the villous tissue is not maintained resulting in the removal of locally produced factors that may have a role in regulating CTB behaviour. Moreover, isolated CTB may suffer from alterations in phenotype *in vitro*, which could lead to errors in interpreting their behaviour. As an alternative, explant culture offers the potential to maintain the basic architecture of placental tissue in a culture system that can be readily manipulated.

1.3.6 Villous explant culture

Explant culture of anchoring villi from early in gestation provides a system in which to study the differentiation, proliferation and invasion of EVT cells (Genbacev et al. 1992). EVT are present in the remains of the cell columns on the tips of anchoring villi, which have detached from the uterine wall. *In vivo*, these are the sites of differentiation of invasive CTB (Fisher and Damsky, 1993).

To set up placental explant culture, placental tissue is obtained from the termination of clinically normal pregnancies, and dissected to provide villous tissue. Villous explants are cultured on the surface of matrigel, which supports EVT cell differentiation, proliferation and invasion. Cell culture chambers have been used to give a three-dimensional culture that allows access of media to both the upper and lower cell surfaces. The explants are cultured under standard conditions and remain viable for 6-8 days.

EVT differentiation, proliferation and invasion can be observed by examination of the cultures using an inverted microscope. Villous tips flatten and attach to the substrate within 12 hours, and outgrowth commonly occurs after 24 hours of culture. Cell migration into the matrix is seen after 48-72 hours. After the period of study the explants can be flash frozen and serial sections cut through the explant and underlying matrigel. Immunohistochemical characterisation of invasive cells can then be performed.

Genbacev et al. (1993a; 1993b and 1993c) evaluated metabolic activity and morphological characteristics of villous tissue explants. The study showed that villous

explants of first trimester placentas (5 to 10 weeks of gestation) readily attached to matrigel and decidual extract containing collagen 1 within 12 hours of culture. Proliferation of CTB was seen in all explants and these sites of proliferation gave rise to migrating EVT cells within 24 hours of culture. Proliferative (Ki67 and Proliferating cell nuclear antigen positive) cells were identified amongst both villous and extravillous CTB.

EVT cells that differentiated *in vitro* were cytokeratin and HLA-A, B, C class 1 antigen positive and hCG negative, correlating with EVT cell properties *in vivo*. Villous explants of 2nd trimester placentas attached to the matrix but no proliferating CTB cells or EVT cells were seen.

In several other explant studies it has been observed that the pattern of CTB differentiation *in vitro* closely resembles differentiation of the CTB cells described at the sites of invasion *in vivo* (Aplin, 1993; Vicovac et al. 1995; Aplin et al. 1999). In both *in vitro* and *in vivo* studies a decrease in lipid and glycogen stores during cell migration and a gradual expression of EVT cell proteolytic activity and hPL synthesis and accumulation were noted.

Various groups have utilised the explant culture model to study the effects of oxygen on explant behaviour in culture. These will be discussed in section 1.5

1.3.7 Summary

In summary, both primary cell culture and villous explant *in vitro* models have been important in studying CTB invasion at the molecular level. While primary culture is widely applicable for testing the invasiveness of already differentiated cells and assessing the effects of various factors on invasion itself, villous explants provide a more comprehensive system for studying the differentiation of CTB and their detachment and migration outside the villi.

1.4 Matrix Metalloproteinases

1.4.1 Introduction to Matrix Metalloproteinases

As discussed in section 1.2, trophoblast invasion is thought to involve many factors including matrix metalloproteinases. The matrix metalloproteinases (MMPs) are a family of zinc dependent endopeptidases, capable of degrading components of the ECM. They are a family of enzymes belonging to the broader class of Metalloproteinases, which comprises over 200 members. Over 25 MMPs have been discovered (table 1). Most MMPs are extracellular enzymes, secreted as inactive zymogens that require cleavage of a pro peptide sequence for activation. They can be subdivided according to their principle substrate into 4 groups; collagenases, gelatinases, stromelysins and enamelysins. Several membrane inserted MMPs (MT-MMPs) have also been described.

Of particular interest with regard to trophoblast invasion are MMP-2 and MMP-9. MMP-2 (72kDa gelatinase A) activity was first noted in rheumatoid synovial tissue (Harris and Krane, 1972) and was separated from collagenase-1 and stromelysin-1 several years later in medium from rabbit bone culture (Sellers et al. 1978). The complete sequence for the human enzyme, excluding the signal peptide, was reported by Collier et al. (1988).

MMP-9 (Gelatinase B), the largest of the known MMPs at a molecular weight (Mw) of 92kDa, was isolated from human polymorphonuclear leukocytes (Sopata and Dancewicz, 1974; Sopata and Wize, 1979) and was later purified and characterized (Rantala-Ryhanen et al. 1983). The sequencing of the cDNA followed this (Wilhelm et al. 1989).

Table 1 Matrix Metalloproteinases

MMP-No.	Name	Mw(kDa) latent/active
MMP-1	Collagenase 1	52000/43000
MMP-2	Gelatinase A	71000/62000
MMP-3	Stromelysin 1	52000/43000
MMP-7	Matrilysin	28000/19000
MMP-8	Collagenase 2	51000/42000
MMP-9	Gelatinase B	76000/67000
MMP-10	Stromelysin 2	52000/44000
MMP-11	Stromelysin 3	51000/46000
MMP-12	Macrophage Elastase	52000/20000
MMP-13	Collagenase 3	52000/42000
MMP-14	MT-MMP-1	64000/54000
MMP-15	MT MMP- 2	71000/61000
MMP-16	MT MMP-3	66000/56000
MMP-17	MT MMP-4	62000/51000
MMP-18	Collagenase 4	53000/42000
MMP-19	No trivial name	54000/45000
MMP-20	Enamelysin	54000/22000
MMP-21	MMP-23a	70000/53000
MMP-22	MMP-23b	51000/43000
MMP-23	Cysteine array MMP	44000/43000
MMP-24	MT-MMP-5	71000/61000
MMP-25	MT-MMP-6	59000/38000
MMP-26	No trivial name	28000/19000

1.4.2 Physiological roles

MMPs have a broad spectrum of involvement in biological activity since the breakdown of connective tissue collagen is important in various physiological processes. These include bone remodelling, embryogenesis, wound healing and postpartum involution of the uterus (Woessner, 1991; Woessner, 1998). They have also been implicated in several pathological processes including tissue destruction, fibrotic diseases and weakening of the matrix, with relevance to diseases such as arthritis, oral pathology, arteriosclerosis and liver/renal fibrosis. A major field of interest in MMPs is regarding their role in invasion and metastasis in cancer and many reviews have been written on this subject (Basset et al. 1997; Chambers and Matrisian. 1997).

It is difficult to elucidate the roles of individual MMPs in specific processes and pathologies and most conclusions are drawn from relative activity levels in specific situations. Individual gene knockout experiments of specific MMPs are reviewed in Shapiro, (1997).

1.4.3 MMP structure

All MMPs have an N-terminal signal peptide of variable length which is removed during transit to the extracellular environment. This is followed by the propeptide domain, consisting of around 80 amino acids including a highly conserved cysteine switch sequence. The purpose of this sequence is to block the active site; the cysteine residue is positioned opposite the zinc atom in the active centre (Springman et al. 1990).

The catalytic domain of around 160 residues follows the propeptide and contains a highly conserved sequence in its C-terminal portion. This motif binds the catalytic zinc atom by three His residues.

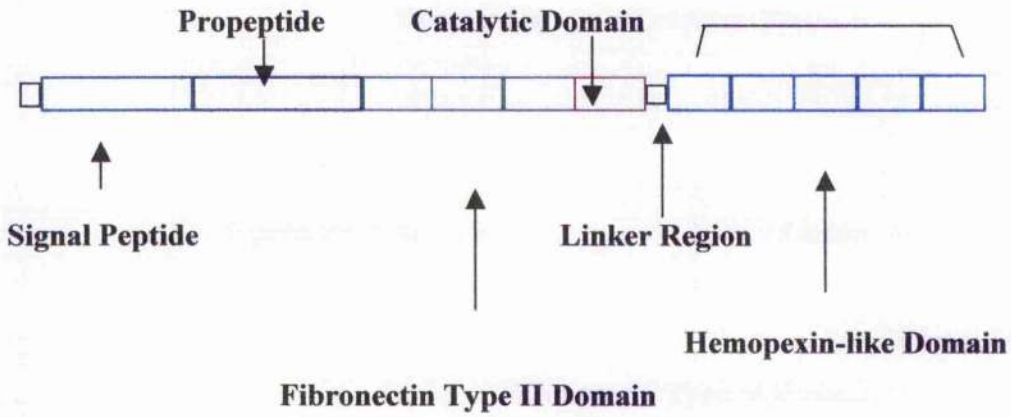
Following the catalytic domain there is a hinge region, of variable length of between 2-72 residues. Lastly there is a hemopexin-like domain (Hunt et al. 1987), thought to be involved in binding to the ECM (Clark et al. 1989).

Several of the MMPs have additional variations on this basic theme. In the membrane inserted MMPs such as MMP-14, a transmembrane domain follows the hemopexin domain (Takino et al. 1995) MMP-2 & 9 (figure 8) contain fibronectin

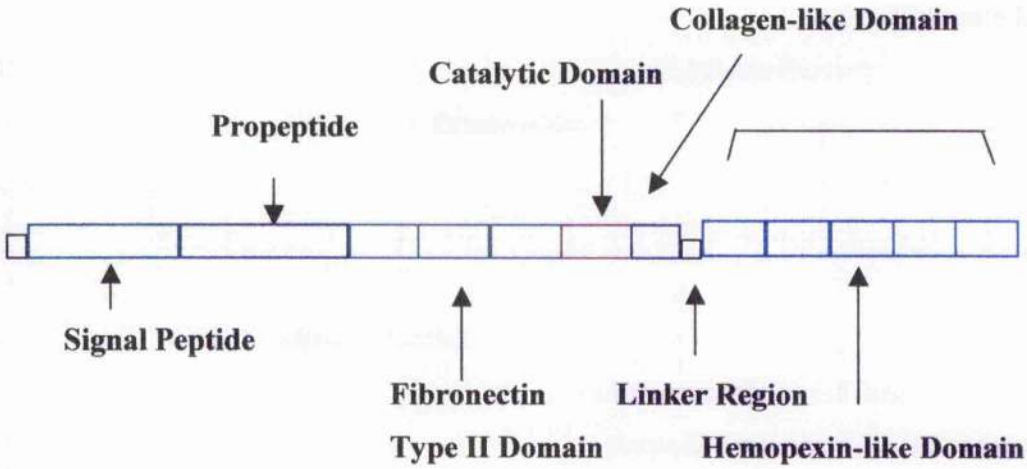
repeats - three tandem repeats of about 58 residues related to fibronectin type II domains. These repeats occur within the catalytic domain and assist binding to the substrate (Banyai and Patthy, 1991). Finally a Type V collagen insert is found only in MMP-9, between the active centre and the hemopexin domain (Wilhelm et al. 1989). It is 54 residues long, rich in proline and is an extension of the hinge region.

Figure 8 MMP-2 and MMP-9 structure

MMP-2



MMP-9



1.4.4 Activation of the zymogen and catalytic action

Since MMPs are secreted as inactive zymogens they must be activated by cleavage of the inhibitory propeptide before they can digest their substrates. One of the distinct features of the MMPs is their ability to be activated from the pro-form not only by proteinases but by a wide variety of non-proteolytic agents including mercurial compounds, other thiol reactive reagents, iodoactetate, N-ethyleimide, oxidized glutathione, SDS, NaSCN, NaI, reactive oxygens such as HOCl, NO_2, heat treatment and by exposure to acidic pH (Springman et al. 1990).

A hypothesis for activation of the zymogen was proposed based on the idea of a cysteine switch mechanism (Van Wart et al. 1990), which attempts to explain the activation by various reagents. In this model, the cysteine residue found in the conserved sequence of the pro-peptide interacts with the catalytic Zn^{2+}. This prevents the zinc ion associating with a molecule of water required for the hydrolysis of the propeptide. Thus the enzyme is maintained in the latent state. This Cys-Zn^{2+} bond must be disrupted for activation to occur. The term 'Velcro' mechanism is also used to describe this activation (Vallee and Auld, 1990). Proteolytic agents destabilise the zinc-cysteine bond by interacting with the proteinase at a 'bait' region. Non-proteolytic agents essentially achieve the same result by interacting with the transiently dissociated cysteine. The final stage in activation is the complete removal of the pro-peptide by the action of an active MMP or an MMP intermediate.

The mechanism by which MMPs catalyse hydrolysis of the peptide bond of their substrates has been modelled on the mechanism deduced for thermolysin due to similarities in 3-D structure (Spurlino et al. 1994, Becker et al. 1995). The glutamic residues in the catalytic motif play an active role in catalysis, along with the Zn^{2+} ion.

1.4.5 The substrates of MMPs: The ECM

As previously mentioned, MMPs are responsible *in vivo* for the breakdown of the ECM. The ECM surrounds and supports the cells within mammalian tissues. It is composed of 3 major classes of biomolecules: a) Structural proteins collagen and elastin; b) Specialized proteins such as fibrillin, fibronectin, and laminin; c) Proteoglycans: a protein core attached to long chains of repeating disaccharide units called glycosaminoglycans (GAGs). In order for cells to migrate though the ECM, as

happens in trophoblast invasion or in tumour cell metastasis, degradation must occur. The large number of MMPs and their specificity is accounted for by the different components of the ECM degraded.

MMPs have been named in accordance with the substrate they were originally observed to digest. In some cases this can be misleading. Despite being known as gelatinases, MMP-2 and -9 are also capable of degrading native Type I collagen, elastin and several other proteins.

1.4.6 Inhibitors of MMPs

1.4.6.1 Levels of control

It is obviously important that the activation of MMPs is tightly controlled to avoid tissue damage and this is achieved at several levels. The release of MMPs into the extracellular space occurs only in response to well defined signals at times when degradation is appropriate. Many MMPs remain near to the cell from which they were released, bound either to the cell surface or to matrix components. The latent pro-form must be cleaved to give the active form, providing another level of control. Lastly there are several inhibitors of MMPs, both general and specific, often produced by the same cell as secreted the MMP itself in an autocrine regulatory loop.

1.4.6.2 Tissue Inhibitors of Metalloproteinases

In vivo, once MMPs have been released into the extracellular space, their control is largely the responsibility of a family of inhibitors known as the tissue inhibitors of metalloproteinases (TIMPs). Four TIMPs have been described (table 2). They are important in the processes that MMPs are involved in and have been studied in a variety of pathologies including malignancy, regulation of cell morphology, inhibition of angiogenesis and tissue remodelling (Gomez et al. 1997).

1.4.6.3 TIMP function

Inhibition of the proteolytic activity of MMPs is the principal role of TIMPs *in vivo* (Woessner, 2000). The four members of the TIMP family each comprise 200-210

amino acid residues, arranged in three domains; a signal peptide, an N-terminal domain which is responsible for the inhibition of MMPs (Murphy, 1991; DeClerck et al. 1993) and a C-terminal domain which enhances binding to the MMP and helps to orient the TIMP to the MMP active centre (Willenbrock, 1993). The C-terminal domain of the TIMP interacts with the C-terminal domain of the MMP to orient the inhibitory end of the TIMP towards the active centre of the MMP. Due to this interaction, the active centre of the MMP is not free to interact with its substrates, and therefore ECM breakdown is controlled (Gomis-Ruth et al. 1997).

1.4.6.4 TIMP interaction with pro-MMPs

In addition to the general mode of inhibition, there are two known cases of TIMP interaction with pro-MMPs; the pro-form of MMP-9 with TIMP-1 (Murphy et al. 1989) and the pro-form of MMP-2 with TIMP-2 (Goldberg et al. 1989; Stetler-Stevenson et al. 1989). Interestingly, whilst these interactions reduce the activation of the pro-enzymes, the pro-MMP-2 / TIMP-2 complex interacts with the cell surface, anchoring the pro-enzyme in a position where it can be activated by the membrane type metalloproteinase MMP-14 (Emmert-Buck et al. 1995; Corcoran et al. 1996).

1.4.6.5 Other inhibitors

A variety of other compounds have also been found to inhibit MMP activity (Woessner, 2000). The principal means of inhibition is the chelation or replacement of zinc at the active centre. This is the method of inhibition by ethylenediamine tetraacetic acid (EDTA), cysteine (chelation) and DTT & gold compounds used to treat arthritis (replacement). Several natural antibiotics also act by chelation.

Table 2 Properties of Tissue Inhibitors of Matrix Metalloproteinases

	Mw (kDa)	MMPs Inhibited
TIMP-1	20.6	All
TIMP-2	21.5	All
TIMP-3	21.6	All
TIMP-4	22.3	MMP-1,2,3,7,9

1.4.7 Genetics

MMP-2 maps to chromosome 16q13. The gene is 17 kb long with 13 exons varying in size from 110 to 901 base pairs (bp) and 12 introns ranging from 175 to 4,350 bp. Alignment of introns showed that introns 1 to 4 and 8 to 12 of the type IV collagenase gene coincide with intron locations in the MMP-1 and MMP-3 genes, indicating a close structural relationship of these metalloproteinase genes. Analysis of the 0.4-kb 5'-flanking region of the MMP-2 gene showed that, in contrast to the genes of interstitial collagenase and stromelysin, there was no TATA box or 12-O-tetradecanoylphorbol-13-acetate-responsive element present in the promoter region, whereas there were two GC boxes. There is no CAAT box, but a potential binding site (CCCCAGGC) for the transcription factor AP-2 is located in the first exon (Huhtala et al. 1990; Tryggvason et al. 1990).

MMP-9 maps to 20q11.2-q13.1. Both MMP-2 and MMP–9 have 13 exons and similar intron locations (Huhtala et al. 1991). The number of exons is 3 more than has been found in other members of the gene family. The extra exons encode the amino acids of the fibronectin-like domain which has been found only in MMP-2 and MMP-9.

There have been many studies on the gene regulation of the metalloproteinases (Benbow and Brinckerhoff, 1997). The promoter region of MMP-9 more closely resembles those of the interstitial collagenase and stromelysin genes than MMP-2. It contains a TATA-like sequence and has TPA (Phorbol) response element like sequences. The regulatory processes for transcription of MMP-9 appear to be sensitive to oxygen through Hypoxia inducible factor 1α (HIF-1α) transcription factor which is degraded at normal oxygen levels, but not in hypoxic conditions (Caniggia et al. 2000b). MMP-2 on the other hand is not thought to be responsive to HIF 1α. This is discussed in section 1.5. Thus whilst MMP-2 and MMP–9 share many common features and have identical substrate specificity, the regulation of their genes appears to be quite distinct.

1.4.8 Methods of studying MMPs

Several methods have been used in the study of MMPs in physiology and pathology, each method having both advantages and disadvantages. A combination of various overlapping approaches is often the best strategy in deducing roles of MMPs in any system.

The inhibition of MMPs by sodium dodecyl sulphate (SDS) is a property that has been exploited in zymography, a popular method of studying MMP activity. After SDS-polyacrylamide gel electrophoresis (PAGE) on a gel containing a relevant protein substrate e.g. gelatin, the gel is washed to remove SDS, and incubated in a buffer containing Zn^{2-} and Ca^{2+}; the pro-enzyme is renatured and degrades the substrate e.g. gelatin in the gel. This method detects both pro and activated forms of MMPs.

Immunohistochemistry (IHC) of tissue sections can be used to precisely localize specific MMP expression to particular regions although it can be hard to ensure no cross reactivity between MMPs occurs and many antibodies do not distinguish between pro and active forms of MMPs. Semi quantitative analysis is possible however and this method has been widely used in studies of MMP expression in the placenta.

If fairly large quantities of MMPs are present in a sample, western blotting can be used as a semi-quantitative method for comparing amounts of MMPs between samples. As with IHC, the quality of the results will depend heavily on the choice of antibody, and often will not distinguish between pro and active forms of the enzyme.

The use of MMP assays has increased dramatically over the past few years with the growth in the number of commercially available kits. They have the advantage of being MMP specific, can be very sensitive and have a high throughput of samples. Unfortunately cross-reaction with other MMPs is fairly common. Moreover, in addition to detecting free MMPs, unquantifiable levels of MMP-inhibitor complexes may also be detected.

Quantitative PCR (polymerase chain reaction) has the advantage of being MMP specific and has the ability to measure changes in mRNA for multiple MMPs in small samples relatively quickly. However, because only mRNA levels are measured neither protein or activity levels can be accurately quantified. In conjunction with

other methods, however, it can provide useful insights into control at a transcriptional level.

1.4.9 MMPs and placental development

1.4.9.1 Trophoblast outgrowth models

The study of the secretion of proteolytic enzymes by invasive trophoblasts has its roots in studies performed during the 1970s and 80s. Experiments studying trophoblast outgrowth on radiolabelled extracellular matrices and cell monolayers found that outgrowing cells can invade and migrate through uterine stromal cells (Salomon and Sherman, 1975; Sherman and Salamon, 1975; Sherman, 1975a; Sherman, 1975b; Glass et al. 1979) and ECMs (Glass et al. 1983). It was proposed that the clear halo that formed slightly ahead of the outgrowth of the trophoblasts was the result of cytolytic enzymes.

1.4.9.2 The role of plasminogen activator in trophoblast invasion

Plasminogen activator (PA) is an enzyme that converts inactive plasminogen into the proteolytic enzyme plasmin. In addition to having a direct role in tissue digestion, plasmin may act as an upstream activator of MMPs. Trophoblast outgrowth models demonstrated that outgrowth in both humans and mice required urokinase like plasminogen activator (uPA) (Sherman et al. 1976; Strickland et al. 1976; Martin and Arias, 1982; Queenan et al. 1987). Studies suggesting that exogenous proteinase inhibitors can prevent blastocyst attachment *in vitro* (Blackwood et al. 1968) supported this interpretation. Blastocyst attachment in the mouse required a trypsin-like activity and the process of trophoblast outgrowth required both plasminogen activator and trypsin like activity (Kubo et al. 1981). Plasminogen activator inhibitor (PAI) type 1 is also associated with invading trophoblasts suggesting it may have a role as a regulator of uPA activity (Feinberg et al. 1989).

1.4.9.3 The role of gelatinases in trophoblast invasion

However, studies by Denker, (1977) and Denker and Fritz, (1979) found no inhibition of rabbit blastocyst attachment by epsilon amino caproic acid, an inhibitor of plasmin activity. The degradation observed by Glass et al. (1983) was not uPA dependent. The authors hypothesised that the degradative activity could be due to either secreted or cell bound proteinases or to endocytosis by the trophoblast cells. A later study (Behrendtsen et al. 1992) demonstrated that MMPs mediated the ECM degradation by cells from mouse blastocyst outgrowths.

It is generally accepted that CTB isolated from 1st, but not 2nd or 3rd trimester placenta, degrade and invade the ECM, corresponding with their invasive potential *in vivo*. The suggestion that trophoblasts mediate the developmentally regulated degradative properties of human chorionic villi was supported by evidence provided by Fisher et al. (1989) who used the method of Kliman et al. (1986) to isolate and compare the gelatin degrading proteinases produced by early gestation, invasive CTB with those produced by non invasive later gestation CTB and with placental fibroblasts. There were several proteinases uniquely produced by the invasive trophoblasts including a group of gelatinases ranging in Mwt from 68-200 kDa.

In support of the role of both MMPs and uPA in invasion, Yagel et al. (1988) showed that both inhibitors of plasminogen activator, and inhibitors of MMPs had the effect of blocking the invasion of a basement membrane by human trophoblast. It is possible that uPA acts upstream of MMPs by activating the zymogen.

The work of Librach et al. (1991) was pivotal in the study of metalloproteinases during trophoblast invasion. First trimester trophoblasts were shown to secrete the 92kDa type IV collagen-degrading metalloproteinase (MMP-9) and this was required for invasiveness *in vitro*. Its synthesis and activation peaked in the first trimester and correlated well with the invasive period *in vivo*. Inhibitors of the plasminogen activator system reduced but did not completely abolish invasion.

1.4.9.4 Temporal regulation of gelatinase expression – studies of cultured CTB

Several studies have cultured CTB from 1st/2nd/3rd trimester and analysed MMP secretion profiles in the culture medium. After implantation and before placental

development is complete in the 1st trimester, both MMP-9 and MMP–2 are secreted (Fisher et al. 1985; Shimonovitz et al. 1994; Librach et al. 1994). By the 3rd trimester it has been reported that MMP-9 is predominant, although levels are significantly less than in 1st trimester, and MMP-2 is secreted in only small amounts (Fisher et al. 1989; Shimonovitz et al. 1994).

Niu et al. (2000) found that 1st trimester villous tissue cultured alone secreted around 10x more pro-MMP-2 than pro-MMP-9, and that pro-MMP-2 levels decreased dramatically in the 2nd trimester. Conversely, 1st trimester CTB cultured in the absence of stromal cells had pro-MMP-9 levels 10x those of pro-MMP-2. Levels of TIMP-1 and TIMP–2 remained constant. Active MMP-2 was also secreted from villous tissue in the first trimester. This study illustrates the importance of the villous environment for influencing gelatinase production.

The first study to examine dynamics of MMP secretion during the first trimester was undertaken by Xu et al. (2000) who found a gradual increase in MMP-9 secretion by cultured CTB between week 7–11 and a corresponding decline in MMP-2 secretion. TIMP-1 secretion increased with gestation and no TIMP-2 was detected.

In contrast to previous studies Sawicki et al. (2000) found expression of MMP-2 and MMP–9 in 3rd trimester CTB culture.

1.4.9.5 MMP distribution patterns in the placenta

Several studies have been published examining the *in vivo* distribution patterns of MMPs and TIMPs in the placenta throughout gestation. Different protocols for tissue preparation and fixation were used in each of these and as such the data are difficult to compare. Several early studies on trophoblast invasion were carried out on primate placentae rather than human, which must also be taken into account when considering the data.

To determine the collagenase secreting cell type in first trimester human placenta. Moll and Lane (1990) cultured CTB from 8-12 weeks of gestation. Interstitial collagenase was localized to villous and EVT by light microscopy (paraffin embedded tissue) and immunogold labelling on cultures. The enzyme was present in the cytoplasm as well as on the cell surface of the trophoblast.

MMP-2 distribution at both protein and mRNA level has been studied in human placenta by Autio-Harmainen et al. (1992); Fernandez et al. (1992); Pollette et

al. (1994); Huppertz et al. (1998); Sawicki et al. (2000) and Isaka et al. (2003). A summary of these studies is given in table 3. The expression of MMP-2 in trophoblast cell columns of early pregnancy is a predominant finding of these studies. Huppertz et al. (1998) suggested that expression does not alter considerably in the later stages of pregnancy. In addition to a role in trophoblast invasion, Sawicki et al. (2000) suggested that basolateral polarised release of the gelatinases may be important in the villous tissue remodelling that occurs late in gestation.

MMP-9 localisation has also been studied by immunohistochemistry and in situ hybridization (Polette et al. 1994; Hurskainen et al. 1996; Huppertz et al. 1998; Sawicki et al. 2000 and Isaka et al. 2003). From these studies it has been reported that MMP-9 is expressed by EVT in early pregnancy but is downregulated towards term.

Most data published on the expression of TIMPs describe their expression as being restricted to the decidual cells at the maternal/fetal interface. (Graham and Lala, 1991; Damsky et al. 1993; Polette et al. 1994). Contradictory findings of EVT secretion of TIMPs were reported by Hurskainen et al. (1996) and Ruck et al. (1996). The data from these two studies supports the hypothesis that invasion may be controlled in an autocrine manner by the trophoblast cells themselves in addition to paracrine control by maternal decidua.

Table 3 Summary of published immunohistochemistry studies on MMP-9 and MMP-2 expression

Authors	Date Published	Type of Study	Number of samples studied	Gestation of samples studied	MMP-2 distribution	MMP-9 distribution	Notes
Autio-Harmainen et al.	1992	Immunohistochemistry (paraffin/cryostat sections) In situ hybridisation	7	8-11 weeks of gestation	mRNA and protein : EVT, villous stroma decidua Protein:vCTB/STB	Not studied	Blighted ovum/tubal pregnancy included
Fernandez et al.	1994	Immunohistochemistry (paraffin sections)	17	First trimester	Protein: EVT, vCTB decidua, villous stoma	Not studied	
Polette et al.	1994	Immunohistochemistry (paraffin sections) In situ hybridisation	14	First trimester and term	Protein and mRNA: EVT and decidua in 1st trimester, less at term Stroma at term	Protein and mRNA: EVT, vCTB and decidua in 1st trimester only	

					only		
Hurskainen et al.	1996	Immunohistochemistry (paraffin/frozen sections) In situ hybridisation	8	8-10 weeks of gestation	Not studied	mRNA and protein: EVT, stroma, endothelium mRNA only on vCTB	
Vettraino	1996	Immunohistochemistry (paraffin sections) In situ hybridisation	8	8 weeks of gestation (2 samples) and term (6 samples)	Not studied	Protein and mRNA: only in granulocytes of maternal sinusoidal spaces, fetal capillaries and EVT	
Huppertz et al.	1998	Immunohistochemistry (paraffin/frozen sections)	14	9, 10, 12, 16 and 38-41 weeks of	Protein: EVT throughout gestation	Protein: EVT in 1st trimester, unclear in later	Major differences between

				gestation		stages	paraffin/cryostat sections
Sawicki et al.	2000	Immunohistochemistry (paraffin)	Unknown	Term	Protein: vCTB, endothelium	Protein: vCTB, endothelium	
Isaka et al.	2003	Immunohistochemistry In situ hybridisation Film in situ zymography	30	First trimester/term	mRNA and protein: EVT throughout	mRNA and protein: EVT, vCTB, stroma throughout but neither on EVT at term	Gelatinase activity in 1st trimester but not at term

1.4.9.6 Regulation of MMP expression

Many studies have examined the influence of various hormones, cytokines and other chemicals on MMP expression in trophoblast invasion. Studies include the effect of progesterone (Shimonovitz et al. 1998), Interleukin-10 (Roth and Fisher, 1999), Interleukin-1β (Librach et al. 1994), hCG (Yagel et al. 1993), 8-Iso-Prostaglandin $F_2\alpha$ (Staff et al. 2000) on MMP production. However, much of the data published are confined to a single study and thus await further investigation. The studies by Caniggia et al on oxygen tension and regulation of MMP-9 are discussed in section 1.5.

1.4.9.7 MMPs and abnormal placentation

This subject will be discussed in Section 1.7. It suffices to state that MMP activity may be aberrant in several placental abnormalities including pre-eclampsia, IUGR, certain causes of miscarriage and choriocarcinoma, possibly being downregulated in the first three conditions and upregulated in the latter.

1.5 Placental development and oxygen tension

1.5.1 Oxygen tension in the placenta

Throughout pregnancy the placenta is exposed to changing levels of oxygen. Oxygen levels in the endometrial and trophoblast tissues can be measured using oxygen electrodes. Yedwab et al. (1976) showed that the oxygen tension (PO_2) in the lumen of the uterine body during the secretory phase is around 10 to 15 mmHg. In the first study to examine oxygen levels in 1st trimester pregnancies Rodesch et al. (1992) found that PO_2 values in the intervillous space increased from around 18mmHg at 8 weeks of gestation to about 60mmHg at 13 weeks of gestation. The oxygen tension in the endometrial stroma remained constant at around 40mmHg throughout pregnancy. In all cases between 8-10 weeks of gestation PO_2 values in the endometrium were higher than in the placenta, whereas between 10-12 weeks of gestation there was no difference between placental and endometrial values.

1.5.2 Intervillous blood flow and oxygen tension

It was thus established that early placentation occurs in a relatively hypoxic environment before the flow of blood into the intervillous space results in the CTB being exposed to increased oxygen tension. The presence of trophoblast plugs in the uterine spiral arteries (Hamilton and Boyd, 1960) is thought to prevent blood flow into the intervillous space in early pregnancy. The plugs are dispersed around the end of the first trimester at the same time that uteroplacental blood flow increases and oxygen tension in the placenta rises (Hustin and Schaaps, 1987). Injection of radiopaque dye into the aorta of pregnant women and the tracing of the dye in the intervillous space showed that at 6 weeks of gestation little dye entered the intervillous space, while at 12 weeks of gestation diffusion of the dye was observed throughout the intervillous space and continuous maternal blood flow was present (Burchell, 1967). Thus it has been speculated that the rise of oxygen tension in the intervillous space may be linked to the differentiation of CTB from the proliferative to the invasive state. In recent years *in vitro* experimentation has begun to unravel the effects of oxygen tension on CTB behaviour during early pregnancy. However interpretation of the data is not straightforward since proliferation and migration both

occur before and after the oxygen change and a simple theory is unlikely to be correct (Pijnenborg, 1980).

1.5.3 Oxygen tension and trophoblast invasion

Isolated CTB from the first trimester cultured on an ECM in 20% oxygen will invade the matrix on which they are cultured (Fisher et al. 1989). First trimester villi which have been explanted onto matrigel and cultured in 20% oxygen form new invasive sites at the tips of the explants (Vicovac and Aplin, 1996; Genbacev et al. 1997; Aplin et al. 1999) while those cultured in 2 or 3% oxygen are maintained in a proliferative, non-invasive immature state (Genbacev et al. 1997; Zhou et al. 1998; Caniggia et al. 2000a; Caniggia et al. 2000b). It has been accepted for some time that CTB proliferation is higher in hypoxia (Fox, 1964). CTB cultured in low oxygen do not undergo the switch in integrin receptors normally found as the invasive phenotype is acquired (Genbacev et al. 1997).

Several studies have examined other responses of trophoblasts to low oxygen including increased production of inflammatory cytokines, vascular endothelial growth factor (VEGF), PAI-1 and inhibition of differentiation to the syncytium (Alsat et al. 1996; Benyo et al. 1997; Taylor et al. 1997).

1.5.4 Oxygen concentration and regulation of transcription – the role of HIF-1α

The question of how oxygen tension regulates invasive behaviour centres on the transcription factor hypoxia inducible factor 1 (HIF-1). Low oxygen increases transcription of many genes in various cell types e.g. VEGF, erythropoietin and various glycolytic enzymes and transcription factors (Semenza and Wang, 1992; Forsythe et al. 1996; Semenza et al. 1994). In mammalian cells regulation of genes through oxygen tension is reliant on the transcription factor HIF-1 (Wang and Semenza, 1993a, 1993b). HIF-1 is a basic helix-loop-helix protein which binds to a short DNA motif in the 3'-flanking regions of hypoxia-inducible genes. HIF-1 is a heteromeric complex that is composed of two subunits, HIF-1β which is constitutively expressed and HIF-1α which is inducible. Although HIF-1α is present

at low oxygen tensions it is rapidly ubiquitinated and degraded by the proteasome under normoxic conditions (Salceda and Caro, 1997). The degradation occurs via an interaction with the tumour suppressor protein Von-Hippel-Lindau (Maxwell et al. 1999).

1.5.5 HIF-1α and trophoblast invasion

In a recent study, high expression of HIF-1α mRNA was shown in placental trophoblasts between 7-10 weeks of gestation. The levels then fell dramatically by 12-14 weeks (Cannigia et al. 2000b). The same study showed that incubation of villous explants from 7-10 weeks of gestation cultured in 3% oxygen with antisense HIF-1α inhibited proliferation and induced invasion.

Weiner et al. (1996) showed by northern blotting that both HIF-1α and HIF-1β are present in term placenta and others have shown the presence of HIF-1 DNA binding activity in isolated CTB cells at term (Seligman et al. 1997). mRNA activity of various transcription factors including HIF-1 in CTB was also recently reported (Janatpour et al. 1999).

In a comprehensive study, Rajakumar and Conrad, (2000) investigated the expression of HIF-1α and HIF-2α in normal human placenta from 1st trimester to term. They found that both proteins were localised to the STB, villous CTB and fetoplacental vasculature. Both proteins but not the corresponding mRNAs decreased significantly with gestation and both proteins could be induced by hypoxia in cultured placental villous explants. In addition the DNA binding activity of HIF-1α was increased by hypoxia. Caniggia et al. (2000b) reported that HIF-1α mRNA decreased with increasing gestation during the first trimester, but did not quantify HIF-1α protein expression.

1.5.6 Mechanisms of regulation of trophoblast invasion by HIF-1α

The evidence that MMPs are important in the degradation of the ECM during trophoblast invasion has led to speculation that oxygen tension may regulate MMP expression through HIF-1α. Caniggia et al. (1999a) reported that as oxygen tension increased, secretion of proteins that are induced in hypoxia such as TGF-β3 decreased

in villous explants. Caniggia et al. (2000a) also reported that TGF-β3 levels declined when HIF-1α mRNA was reduced by antisense treatment. In the same study, TGF-β was also reported to have an inhibitory effect on MMP-9 and integrin α1β1. Caniggia et al suggested that HIF-1α and TGF-β3 may act together to mediate the effects of oxygen on CTB invasion by modulating MMP-9 and integrin levels. Despite the findings of Caniggia et al, others have not been able to substantiate these results (section 1.2; Lyall et al. 2001a). However another study reported that CTB cultured in 20% O_2 dramatically up-regulated HIF-1α expression compared with those cultured in 2% O_2 (Jantapour et al. 1999), highlighting the need for caution in interpreting *in vitro* studies.

1.5.7 Defective trophoblast invasion and oxygen tension

Optimal perfusion of the placenta is achieved only if EVT invade deep into the decidua and remodel the spiral arteries. If exposure to increased oxygen does not occur at the critical stage, CTB differentiation into fully invasive cells could be impaired, a scenario which has been implicated in complications such as pre-eclampsia (section 1.7). Placental hypoxia has been implicated in abnormal CTB differentiation and placental development in pre-eclampsia (Conrad et al. 1997; Lim et al. 1997; Lindheimer et al. 1999; Caniggia et al. 1999b). This is discussed further in section 1.7.6. It has also been observed that CTB from pregnancies complicated by pre-eclampsia show defects in integrin switching, a process which may be regulated by oxygen (Zhou et al. 1993). If CTB invasion does not proceed normally then this would result in a lack of remodelling of the spiral arteries and impaired uteroplacental blood flow.

1.6 High altitude pregnancy

1.6.1 Physiological impact of living at high altitude

With increasing altitude the barometric pressure and hence partial oxygen pressure, falls linearly. Arterial PO2 at sea level is around 95mmHg while at 4000m arterial PO2 is around 50mmHg. Haemoglobin oxygen saturation decreases from 95-100% to around 80% with the same increase in altitude (Ward et al. 1995). High altitude hypoxia leads to several well documented cardiovascular, respiratory and electrolyte changes in the body (reviewed in Sarkar et al. 2003).

1.6.2 Increased incidence of pregnancy complications at high altitude

Pregnancy at high altitude has interested researchers for many years. Studies of pregnancy and fetal growth at altitudes of >2700m were undertaken as many as 45 years ago when Lichty et al. (1957) demonstrated that babies born to mothers living at 3100m were significantly smaller than those born at 1600m, irrespective of gestational age. Numerous studies since that time have confirmed that fetal growth is reduced at high altitude throughout the world (Moore et al. 1998). This lowered birth weight is independent of other characteristics such as gestational age, maternal weight gain, parity, smoking and hypertension (Jensen and Moore, 1997). Altitude related growth restriction frequency is negatively correlated with the length of time a population has resided at high altitude, suggesting an evolutionary shift towards sea level birth weight (Moore et al. 2000). Other than pre-term delivery, altitude reduces birth weight more than any other known factor; a decrease in birth weight of 100g/1000m has been reported (Yip, 1987; Jensen and Moore, 1997).

An increased rate of spontaneous pre-term birth has also been noted in some high altitude populations and there is also evidence that newborns at high altitudes have a higher mortality risk. Neonatal mortality is increased three-fold with increased altitude in urban and rural communities in Peru and Bolivia (PAH, 1994).

Perhaps most interestingly in relation to the current study, a dramatic increase in pre-eclampsia has been noted in populations living at >2800m (Palmer et al. 1999). This study reported a four-fold incidence in the prevalence of pre-eclampsia at an altitude of 3100m.

1.6.3 Types of placental hypoxia

Evidence of fetal hypoxia is suggested from the discovery that cord blood samples from high altitude newborns have a higher haemocrit and fetal haemoglobin than those at sea level (Ballew et al. 1986).

Women who are pregnant at high altitude experience hypobaric hypoxia (Espinoza et al. 2001; McAuliffe et al. 2001) and this results in pre-placental hypoxia (Kingdom and Kaufmann, 1997). Fetal hypoxia can be classified as one of three types (Kingdom and Kaufmann, 1997). In pre-placental hypoxia the maternal blood entering the intervillous space is hypoxic resulting in the mother, fetus and placenta being affected. Both high altitude pregnancies and pregnancies where the mother is anaemic fall into this category. Utero-placental hypoxia occurs when the maternal blood has normal oxygen levels but the intervillous space is compromised resulting in placental villi being exposed to lower oxygen, such as occurs in pre-eclampsia and some cases of IUGR. Finally, in post-placental hypoxia both the mother and the intervillous blood flow are normal but the fetal vascular perfusion is abnormal resulting in a reduced ability to obtain adequate oxygen. The various types of hypoxia affect placental villous development in different ways hence care must be taken in studies which attempt to relate one type of placental hypoxia to another.

1.6.4 Physiological parameters

Ultrasound studies have shown around a third reduction in uterine blood flow during the last trimester in high altitude, corresponding with a 30% reduction in oxygen delivery to the uterus (Zamudio et al. 1995). Impedance to uterine artery blood flow was lower at high altitude than at sea level suggesting a possible compensatory mechanism to this (Zamudio et al. 1995; Krampl et al. 2001). Recent studies have demonstrated a reduction in the invasion of the maternal spiral arteries by placental CTB at high altitude and have also shown a reduction in growth factors necessary for placental and fetal growth in these women (Zamudio et al. 2000; Tissot et al. 2000).

The normal pregnancy-associated fall in blood pressure during the second trimester is not seen in pregnant women at 3100m, even in those who remained normotensive throughout pregnancy (Palmer et al. 1999). This suggests failure of the normal cardiovascular adaptations to pregnancy since the fall in blood pressure seen

in normal pregnancy is possibly caused by primary peripheral vasodilation. The study also found a higher incidence of pre-eclampsia at 3100m (16% versus 3%), supporting an earlier study which had found that mean arterial blood pressures were increased late in the third trimester in women residing at the same altitude as was the prevalence of proteinuria, leading to an increase in diagnosis of pre-eclampsia (Moore et al. 1982).

1.6.5 Placental morphology and pathophysiology in high altitude pregnancy

The increases in complications of pregnancy found at high altitude are associated with increased markers of placental hypoxia such as formation of syncytial knots, persistence of vCTB at term and hypercapillarisation (Ali, 1997). The placenta is thought to adjust to some extent to the hypoxia by maintaining total oxygen diffusive conductance (Mayhew et al. 1990; Reshetnikova et al. 1994) even when growth in villous surface area is compromised (Jackson et al. 1987b). To compensate for this, it has been suggested that diffusion distances across the intravascular barrier might decrease, particularly across the trophoblast layer which may be sparser (Jackson et al. 1988). A decrease in perivascular cells around many of the villous capillaries has also been noted (Zhang et al. 2002) perhaps adding to the thinning of the maternal fetal interface.

Other compensatory mechanisms thought to occur include increased vasculosyncytial membrane formation and alterations in villous capillary diameter. Although the majority of studies have found an increase in diameter, others have found a decrease thus the situation is unclear (reviewed in Mayhew, 2003). At moderate altitudes (up to 2800m) growth of the villous tree and structure are maintained (Reshetnikova et al. 1994) but above 3600m surface area is reduced and to compensate, there is an enlargement of the intervillous space. Under hypoxic conditions, increased branching of fetal capillaries is thought to occur (Kaufmann et al. 1985; Kaufmann, Luckhardt and Leiser, 1988), possibly contributing to the altered configuration of the villous tree seen at altitude (Ali et al. 1996).

1.6.6 Maternal markers

Hypoxia is known to upregulate VEGF production and total VEGF has been shown to be increased in the maternal circulation at high altitude (Wheeler et al. 2002). Circulating levels of total VEGF are also increased in pre-eclampsia (Baker et al. 1995) again suggesting that maternal physiology at high altitude may be intermediate between normal pregnancy and pre-eclampsia. Women residing at high altitude also have elevated levels of pro-inflammatory cytokines and catecholamines during pregnancy suggesting that the maternal neural-immune axis may be altered, predisposing these women to complications (Coussons-Read et al. 2002).

1.6.7 Rationale for studying pregnancy at high altitude as a model for pre-eclampsia

The effects of altitude on pregnancy are relatively difficult to study due to the lack of communities residing at >2500m and the remote location of these in the US. In Europe no permanent communities reside at >2500m and studies on high altitude communities in South America and Asia are confounded by various factors relating to sub-standard maternal care.

It has been suggested that women living at high altitude may adapt to pregnancy in a way which is intermediate between that seen in normal versus pre-eclamptic pregnancy (Palmer et al. 1999). The fact that pre-eclampsia and other pregnancy complications occur more frequently at high altitude (Palmer et al. 1999; Mahfouz et al. 1994) supports this theory.

Pregnancy at high altitude may thus provide a natural experiment for studying the effects of hypoxia on maternal physiology and on placental and fetal development. It may thereby provide a model for the development of conditions such as pre-eclampsia.

1.7 Pre-eclampsia

1.7.1 Pre-eclampsia – epidemiology and clinical features

Pre-eclampsia is a disorder of late pregnancy, diagnosed by blood pressure >140/90mmHg and proteinuria (>300mg in 24 hours) after twenty weeks of gestation in women who were previously normotensive (Davey and MacGillivray, 1988). Widespread endothelial dysfunction is found in the maternal circulation (Roberts and Redman, 1993). The disease progresses from mild to severe in an unpredictable manner and is associated with maternal cerebral oedema, renal failure, liver capsule distension, pulmonary oedema, neurological manifestations, thromobocytopenia, coagulopathy and HELLP syndrome (hemolysis elevated liver enzymes low platelets) (reviewed in Lyall and Greer, 1994b). Pre-eclampsia when left untreated may progress to eclampsia, a life-threatening condition characterised by convulsions. By the stage that the clinical symptoms are evident, the pathology of the disease is advanced and no reliable predictors of pre-eclampsia have been found (Dekker and Sibai, 1991). Magnesium sulphate is currently the drug of choice for the management and treatment of the condition, to prevent development of eclampsia (Duley et al. 1995; Duley et al. 2002). Delivery of the fetus and placenta are the only way to relieve the symptoms reinforcing the role of the placenta in this condition.

Pre-eclampsia affects between 5-10% of pregnancies in the developed world (Zeeman et al. 1992) and is one of the leading causes of maternal mortality in the UK (Department of Health, 1999). It also results in a fivefold increase in perinatal mortality compared with normal pregnancy (Roberts, 1998). In the developing world pre-eclampsia accounts for 10-15% of all deaths connected with pregnancy (~50000 per year) (Duley, 1992). It is also associated with several complications of pregnancy including placental abruption, increased risk of premature delivery and an increase in the requirement of a caesarean section. Additionally, infants born to pre-eclamptic mothers have a higher incidence of IUGR (Witlin et al. 2000).

Various studies have implicated many different factors in the development of pre-eclampsia. These include maternal; nulliparity (Long et al. 1979), obesity (reviewed in O'Brien et al. 2003), age (Ziadeh et al. 2001) and new paternity (Trogstad et al. 2001), fetal e.g. multiple pregnancy (Long and Oats, 1987) and environmental factors (Neela and Raman, 1993). A correlation between several

maternal genes and pre-eclampsia has also been found (reviewed in Cooper et al. 1993).

1.7.2 Placental bed morphology in pre-eclampsia

There is much evidence that the placenta has a central role in the development of pre-eclampsia. The condition is relieved only after delivery of both fetus and placenta and pre-eclampsia has been observed in cases where there is no fetus present e.g. hydatidiform mole (Scott, 1958; Chun et al. 1964).

It has been known for many years that the placentae of pre-eclampsia women are poorly perfused (Page, 1939). In normal pregnancies, the lining of the maternal spiral arteries is replaced and remodelled by invading EVT from the placenta (section 1.1.7). This remodelling results in a four to six fold increase in the diameter of these arteries, transforming the vascular supply to a high-flow low-pressure system which can meet the demands of the developing fetus (section 1.2). It is widely recognised that invasion of trophoblasts into the maternal tissue is abnormal in pre-eclampsia. This observation was first made over thirty years ago in placental bed biopsies from pre-eclampsia patients and has been substantiated in several other studies (Brosens et al. 1972; Robertson et al. 1975; Sheppard and Bonnar, 1981; Gerretsen et al. 1981; Pijnenborg et al. 1991; Meekins et al. 1994). The morphological evidence to date suggests that the principal defect in invasion occurs in the endovascular invasive pathway and that interstitial invasion is relatively normal (Pijnenborg et al. 1996). In pre-eclamptic pregnancies the conversion of spiral arteries by invading EVT occurs only in the decidual segments (Brosens et al. 1972; Pijnenborg et al. 1991) and only a proportion of these are actually remodelled (Khong et al. 1986; Meekins et al. 1994).

As a result of impaired invasion the spiral arteries do not undergo full physiological change and hence blood flow to the placenta is reduced. Impaired invasion is also observed in some cases of IUGR (Gerretsen et al. 1981, Khong et al. 1986), miscarriage (Khong et al. 1987), premature labour (Salafia et al. 1995) and other complications of pregnancy. It is possible that the clinical syndrome of pre-eclampsia is the maternal response to the poorly perfused placenta (Friedman et al. 1991).

1.7.3 MMPs/TIMPs in pre-eclampsia

If trophoblast invasion is partially deficient in pre-eclampsia, it seems logical that one or more of the normal mechanisms governing invasion must be dysfunctional in the disease. MMPs and their regulators TIMPs are possible candidates.

Several studies have highlighted the importance of MMP-2 and MMP-9 in regulating normal CTB invasion (section 1.4.9). It was reported that MMP-9 was reduced in term placental extracts from women with pre-eclampsia (Kolben et al. 1996). Subsequent studies suggested that term trophoblast cell cultures from normal pregnancies secreted high levels of active MMP-9 while term trophoblast cultures from pre-eclamptic pregnancies secreted higher levels of inactive pro-MMP-9 (Graham and McCrae, 1996; Lim et al. 1997).

One study found that decidual microvascular endothelial cells obtained from cases complicated by pre-eclampsia expressed lower levels of MMP-1 than control cases but no difference in either MMP-9 or TIMP-1 was found (Gallery et al. 1999), suggesting that MMP-1 may have a role in regulating invasion into the maternal blood vessels.

There is a lack of *in vivo* information regarding MMP expression in pre-eclampsia. Since pre-eclampsia does not develop until mid-late gestation, levels of MMP-9 and MMP-2 in placentae from early gestations cannot be easily correlated with future disease. It is thus difficult to establish any connection between MMP expression early in gestation and the later development of pre-eclampsia. A very recent study (Huisman et al. 2004) found no relation between MMP expression in early chorion villous samples and the eventual occurrence of the disease.

1.7.4 Cell adhesion molecules in pre-eclampsia

Cell adhesion molecules and their ligands regulate the adhesion of cells to each other, to other cell types and to the ECM (section 1.2). Invasion of the maternal uterus and arteries by trophoblast cells requires modification of the adhesion molecule repertoire, a process which is thought to be deficient in several disorders including pre-eclampsia and IUGR (reviewed in Lyall, 1998).

Some immunohistochemical studies on placentae from cases complicated by pre-eclampsia have shown abnormal CAM expression by invasive CTB particularly

with regard to integrin switching. Cell adhesion molecule expression in normal pregnancy is discussed in section 1.2.3. Zhou et al. (1993) reported that cultured CTB from placentae from pre-eclamptic pregnancies failed to downregulate $\alpha6\beta4$ and to upregulate $\alpha1\beta1$ in distal cells columns and in the uterine wall. The expression patterns of $\alpha3\beta1$ and $\alpha5\beta1$ were not different in pre-eclampsia compared with normal pregnancy. The same study showed an increase in laminins A and B2 in pre-eclampsia pregnancies compared to uncomplicated pregnancies. Zhou et al later reported changes in CTB expression of endothelial CAMs in pre-eclampsia compared with normal pregnancy with differences in all three αv family members. Pre-eclampsia was also associated with differences in cadherin expression and in CAMs associated with leukocyte trafficking. (Zhou et al. 1997a; Zhou et al. 1997b). However these findings are hard to reconcile with the fact that in the third trimester spiral arteries are re-endothelialised and therefore not lined with CTB. In contrast, Divers et al. (1995) reported no differences in integrin expression in either the amniochorion or the basal plate in pre-eclampsia compared to normal pregnancy. Pijnenborg et al. (1998) and Lyall et al. (2001b) did not identify any PECAM-1 expression on CTB in placental bed biopsies in either pre-eclampsia, IUGR or in normal pregnancy suggesting they may not adopt endothelial cell properties. Thus aberrant cell adhesion in pre-eclampsia has not been firmly linked with abnormal CAM expression and further studies are required.

1.7.5 Cell adhesion molecules and the maternal inflammatory response in pre-eclampsia

Cell adhesion molecules are associated with leukocyte trafficking, a process which has significance in the inflammatory response seen in pre-eclampsia. Platelets and neutrophils are thought to be mediators of maternal endothelial dysfunction in the condition and both are activated in pre-eclamptic pregnancies (Greer et al. 1989). Platelets cause vascular damage and obstruction, resulting in tissue ischaemia and damage (Greer, 1992). Neutrophils, once activated, release a variety of vasoconstrictive agents and other mediators of vascular damage (Harlan, 1987).

The recruitment and activation of platelets and neutrophils is partly controlled by the expression of cell adhesion molecules on the surface of the

endothelium of the maternal blood vessels and on the surface of circulating cells (Harlan and Liu, 1992). The major CAMs expressed on the endothelial surface which are involved in platelet and neutrophil activation are platelet endothelial cell adhesion molecule (PECAM), E-Selectin, intracellular adhesion molecule-1 and 2 (ICAM-1 and 2) and vascular cell adhesion molecule-1 (VCAM-1). Increased concentrations of soluble VCAM-1 and E-Selectin have been described in the maternal circulation in pre-eclampsia (Lyall, 1994a; Lyall et al. 1995).

1.7.6 The role of O_2 in pre-eclampsia

Oxygen tension is reported to be vital in regulating CTB proliferation and invasion in the early stages of pregnancy (section 1.5). Both pre-eclampsia and IUGR have been associated with placental hypoxia although this is controversial (Kingdom and Kaufmann, 1997). Several recent studies have focussed on the role of oxygen in modulating CTB proliferation and invasion. One study reported that CTB cells cultured under hypoxic conditions (2% O_2) failed to upregulate the integrin $\alpha 1\beta 1$ in a similar manner to CTB from pre-eclamptic pregnancies (Genbacev et al. 1996). Hypoxic Inducible Factor (HIF-1α) may be a key mediator of this response to hypoxia. Placentae from pre-eclamptic pregnancies overexpress both HIF-1 α and HIF-2 α proteins (Rajakumar et al. 2001). Additionally, Rajakumar et al showed by western blotting that cells in placentae from normal pregnancies upregulate HIF-1α and HIF-2 α, in response to low oxygen and that the proteins are degraded when oxygen levels are increased. In contrast, explanted villous tissue from pre-eclamptic pregnancies failed to degrade HIF-1 α and HIF- 2α when transferred from a low to a high oxygen environment (Rajakumar et al. 2003).

Caniggia et al also found abnormal elevation of HIF-1 mRNA and protein expression in placental tissue from pre-eclamptic pregnancies compared to normal placental tissue (Caniggia and Winter, 2001). The same group had previously shown an association between the expression of TGF-β3 and HIF-1 α and demonstrated that inhibition of the later reduced TGF- β3 mRNA levels, reduced trophoblast proliferation and triggered both MMP-9 and α1 integrin expression (Cannigia et al. 1999). Moreover, inhibition of TGF-β has been reported to restore the invasive capability of CTB from explants from patients with pre-eclampsia and it was

suggested this may act through HIF (Caniggia et al. 2000b). The implications of these studies are that if oxygen levels fail to increase in pre-eclampsia, or if any increase is not detected, then levels of both HIF-1α and TGF-β3 remain high, impairing trophoblast invasion. The authors reported that TGF-β3 was increased in pre-eclampsia placentae. However, other studies do not support an involvement of TGF-β in the development of pre-eclampsia (Lyall et al. 1998; Lyall et al. 2001a).

1.7.7 Two-stage model of pre-eclampsia

It has been suggested that pre-eclampsia could be explained by a two-stage model in which a poorly perfused placenta releases factor(s) into the maternal circulation which result in generalised endothelial dysfunction (Redman, 1991). Poor perfusion of the placenta, resulting from insufficient trophoblast invasion and placental bed vascular remodelling, may cause the placenta to secrete cytokines or growth factors in response to decreased oxygen (Benyo et al. 1997). Alternatively the reduced perfusion may cause an increase in trophoblast apoptosis/necrosis, leading to an increase of particulate material in the maternal circulation and subsequent endothelial activation (DiFederico et al. 1999; Redman and Sargent, 2000). Another possibility is that oxidative stress caused by the decreased oxygen flow to the placenta creates free radicals, which then cause an inflammatory response in the maternal circulation (Hubel et al. 1989; Hubel, 1999). This pathway could involve the activated leukocytes present in the maternal circulation in pre-eclampsia (Sacks et al. 1998; Redman et al. 1999). This is discussed further in section 1.7.8. Indeed since a maternal inflammatory response occurs in normal pregnancy, the extent of endothelial activation seen in pre-eclampsia may represent an over-exaggeration of the normal maternal response to pregnancy.

1.7.8 The role of oxidative stress in the development of pre-eclampsia

As discussed previously, in pre-eclampsia a heightened maternal inflammatory response to pregnancy occurs as evidenced by an increase in several maternal markers (section 1.7.5). Accumulating evidence suggests that the link between the maternal syndrome and the compromised placenta may be found in placental synthesis of

reactive oxygen species (ROS) and the consequent free-radical associated dysfunction of the maternal vasculature. There is considerable evidence of oxidative stress in women with pre-eclampsia (reviewed in Hubel, 1999).

Hypoxia can affect cells through increased generation of free radicals (Halliwell and Gutteridge, 1999). While metabolism is aerobic electrons passed along the mitochondrial respiratory chain are combined with oxygen to form water. At times electrons are leaked and combine with molecular oxygen to form the superoxide anion O_2^- or other ROS. In hypoxia the formation of ROS is increased. ROS attack all types of biological molecules resulting in damage to DNA strand breakage, alteration of DNA bases, lipid peroxidation and protein misfolding. Furthermore ROS also increase cellular calcium concentrations leading increased apoptosis and necrosis (Halliwell, 1996). Ischaemia-reperfusion where tissue is subjected to a period of hypoxia followed by perfusion with blood and hence oxygen is an even more potent inducer of free-radical formation than hypoxia alone. Interestingly, generation of oxygen free radicals is also increased under hyperoxic conditions (Freeman and Crapo, 1981).

It was reported many years ago that mitochondria in the placenta of women with pre-eclampsia are both more abundant and of abnormal appearance (Jones and Fox, 1980) and show signs of oxidative damage (Wang and Walsh, 1998). This could lead to increased electron leakage and so increase the formation of free radicals. ROS are likely to be generated through the xanthine oxidase/dehydrogenase pathway and this may also be stimulated by ischaemia/reperfusion injury as described above. Failure to detoxify ROS because of a lack of antioxidant defences may also contribute to oxidative stress in pre-eclampsia. Alternatively or additionally, ROS may be generated by leucocytes, which are activated in pre-eclampsia or by the endothelium (reviewed in Poston, 2003).

In uncomplicated pregnancies it is likely that blood flow to the placenta is generally adequate for fetal and placental development, although some intermittent perfusion may occur, resulting in low levels of ischaemia/reperfusion injury. This may explain the low levels of oxidative stress seen in normal placentae (Hung, Skepper and Burton, 2001). In cases of pre-eclampsia with IUGR, chronic hypoxia in the placenta is likely to occur, as a result of incomplete transformation of the spiral arteries due to reduced trophoblast invasion (section 1.7.6). Furthermore, the lack of transformation of the spiral arteries may lead to increased intermittent

vasoconstriction of the proximal segments of these arteries as a result of maternal vasomotor control being maintained. Narrowing of the spiral arteries through acute atherosis is also observed. Both of these situations could result in ischaemia-reperfusion type injury and initiate oxidative stress within the placenta at an early stage. In late onset pre-eclampsia with normal birthweight, there is evidence of increased oxidative stress (Hubel, 1999) in the placenta but little evidence to support chronic intraplacental hypoxia. In this scenario there may be an exaggeration of the situation in normal pregnancy, perhaps with greater fluctuations in oxygen concentrations due to incomplete conversion of the spiral arteries or deficiencies in antioxidant defences in the placental tissue.

In normal pregnancy it has been reported that the production of protective antioxidant molecules is upregulated at the same gestational age as maternal blood begins to perfuse the placenta (Jauniaux et al. 2000). This may protect against the potential increase in oxidative stress that would accompany the rise in oxygen tension in the placenta. Thus antioxidant therapy using vitamins C and E may offer protection against pre-eclampsia by reducing oxidative stress (Chappell et al. 1999) and clinical trials are currently underway.

1.8 Aims of the current study

As outlined in this chapter, successful trophoblast invasion is critical to the outcome of pregnancy. Failure of this process has been implicated in several complications of pregnancy including pre-eclampsia.

MMPs and their regulators may be of critical importance in regulating trophoblast invasion. In order to determine the role of these proteins in pre-eclampsia it is first important to understand their expression and activity in normal pregnancy. As previously discussed, there is no consensus of opinion at which stage in gestation and where in the placenta these molecules are expressed. The aims of the current study were initially to investigate placental MMP expression and activity throughout gestation and thereafter to study various regulators of MMP activity, specifically TIMP-1, TIMP-2 and HIF-1α. Although several studies have investigated these molecules, it was hoped to carry out a more comprehensive analysis than had been previously undertaken. Integral to achieving this aim would be determining which methods of studying MMP expression were most suited to placental tissue.

In addition to obtaining a comprehensive understanding of placentation in normal pregnancy and relating this to placentation in pre-eclampsia, *in vivo* and *in vitro* models of pre-eclampsia may be useful in elucidating the underlying pathology in the disease. Since oxygen has been implicated as a critical regulator of trophoblast invasion high altitude pregnancy where the mother is subjected to chronic hypoxia has been suggested as one such model.

MMP expression is decreased in pre-eclampsia and in low oxygen tension, hence it was hypothesised that placenta at high altitude may show alterations in MMP expression. Thus an aim of the study was to quantify MMP–9 and MMP–2 expression in term placentae from various altitudes in order to determine whether MMP expression was altered in placentae at high altitude compared to low altitude.

Maternal circulating concentrations of CAMs are markers of endothelial activation and are elevated in pre-eclampsia. Hence the subsequent objective of the study was to measure concentrations of circulating CAMs in the maternal serum from placentae at various altitudes.

Placental villous explant culture in low oxygen tension has also been used in many previously published studies as a model for pre-eclampsia. The current study

aimed to set up this model in a laboratory where it had not previously been used and to initially examine explant behaviour at normal and low oxygen tensions before studying MMP expression in both conditions. The model would then be extended to utilise villous tissue from chorionic villous samples (CVS), as this had not previously been attempted.

In summary the aims of the thesis were to determine the expression and activity of MMP-9 and -2 and their regulators in normal pregnancy and to investigate two different models of pre-eclampsia.

Chapter 2: Materials and Methods

This chapter describes the methodology used in this thesis and the source of reagents and relevant equipment.

2.1 Buffers

Water (dH_20) used in buffer preparation was Milli-Q RG Ultrapure.

2.1.1 General buffers

PBS Phosphate buffered saline: A solution of one PBS tablet per 100ml dH_2O, stored at 4°C.

PBST PBS with 0.4% polyoxyethylene sorbiton monolaurate (tween-20), stored at 4°C.

TBS Tris buffered saline: 20mM tris, 0.5M NaCl, pH 7.5, stored at 4°C.

TBST TBS with 0.4% tween-20, stored at 4°C.

TBSTB TBST with 0.25% Bovine serum albumin (BSA), stored at 4°C.

2.1.2 Cell culture reagents

Culture medium for explant culture

1: 1 ratio of Dulbecco's Modified Eagle's Medium (DMEM): Hams F12 culture medium, supplemented with 10% fetal bovine serum (FBS), 1% 100x L-glutamine, 1% 100x fungizone and 1% 100x penicillin/streptomycin.

2.1.3 Tissue homogenisation buffer

25mM tris, pH 7.6, 250mM sucrose. The buffer was sterilized by autoclaving and stored at 4°C. 12.5µl/ml protease inhibitor cocktail designed for use with mammalian tissue was added on the day of use.

2.1.4 Western blotting and zymography buffers

Resolving gel buffer

1.5M tris, 0.4% SDS, pH 8.8.

Stacking buffer

1M tris, 0.4% SDS, pH 6.8.

5x running buffer

25mM tris, 190mM glycine, diluted 1:5 in dH_2O on the day of use.

2x loading buffer

1.2ml of 1M tris, pH 6.8, 2ml glycerol, 4ml of 10% SDS, 0.8ml dH_2O and a few bromophenol blue crystals. 1M dithiolthreitol (DTT) was frozen at –20°C in 1ml aliquots and on the day of use was added to loading buffer at a ratio of 1:4. For non-reducing gels DTT was omitted.

Transfer buffer

2mM tris, 190mM glycine, 20% methanol.

Zymography wash buffer

50mM tris, pH 7.4, 150mM NaCl, 2.5% triton X-100.

Zymography reaction buffer

50mM tris, pH 7.4, 5mM $CaCl_2$, 0.02% NaN_3.

MMP activation buffer

50mM tris, pH 7.5, 0.15M NaCl, 5mM $CaCl_2$

2.2 Antibodies used in western blotting and immunohistochemistry

MMP-9

Anti-MMP-9 (gelatinase B), sheep polyclonal Immunoglobulin G (IgG) (Biogenesis, catalogue no. 5980-0911).
Anti-MMP-9 mouse monoclonal, IgG1, directed against the carboxy-terminal domain (residues 626-644) of human MMP-9 (Chemicon, catalogue no. MAB 3309).

MMP-2 (Biogenesis)

Anti-MMP-2 (gelatinase A), sheep polyclonal IgG (Biogenesis, catalogue no. 5980-0211).

LP34 (Novocastra)

Anti-Cytokeratin 5/6 and 18 intermediate filament proteins, mouse monoclonal IgG1, clone LP34 (Novacastra, catalogue no. NCL-L-LP34).

TIMP-1 (Biogenesis)

Anti-TIMP-1, rabbit polyclonal purified Ig (Biogenesis, catalogue no. 9013-1108).

TIMP-2 (Biogenesis)

Anti-TIMP-2, sheep polyclonal, purified Ig, raised against loop 6 of synthetic human TIMP-2 (Biogenesis, catalogue no. 9013-2609).

HIF-1α (Serotec)

Anti-HIF-1α, goat polyclonal, purified IgG, Raised against peptide mapping near the carboxy-terminus of HIF-1α of human origin (Serotec catalogue no. Sc-8711).

Ki67 (Novacastra)

Anti-Ki67 Liquid mouse monoclonal antibody IgG1. Raised against prokaryotic recombinant fusion protein corresponding to a 1086bp Ki67 motif-containing cDNA fragment (Novacastra catalogue no. NCL-L-Ki67-MM1).

Table 4 Antibody dilutions

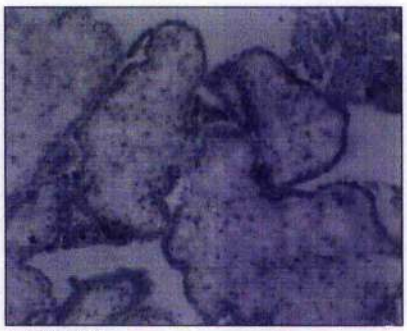
Antibody	Immunohistochemistry dilution	ABC kit used for ICC	Western blotting dilution
MMP-9 (Biogenesis)	1/500	Sheep	1/1000
MMP-9 (Chemicon)	N/A*	N/A*	1/1000
MMP-2 (Biogenesis)	1/250	Sheep	1/1000
TIMP-1	1/250	Universal	N/A
TIMP-2	1/300	Sheep	N/A
HIF-1α	1/250	Goat	N/A
LP34	1/200	Universal	N/A
Ki67	1/250	Universal	N/A

*N/A = Not applicable

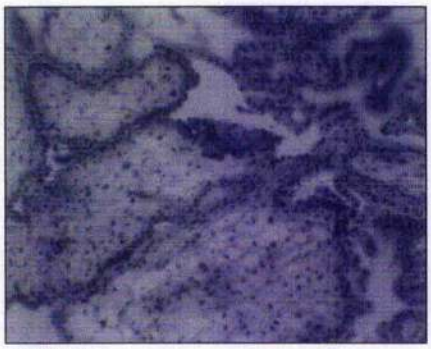
The primary antibody was omitted and substituted with either PBS or the relevant Ig fraction of non-immune serum from sheep, goat, rabbit or mouse at the same concentration as the primary antibody as a negative control in each experiment performed. No staining was present on any of the controls. A representative control for each antibody is shown in figure 9.

Figure 9 **Representative negative control for primary antibodies used in immunohistochemistry (Relevant Ig fraction substituted for primary antibody)**

MMP-9

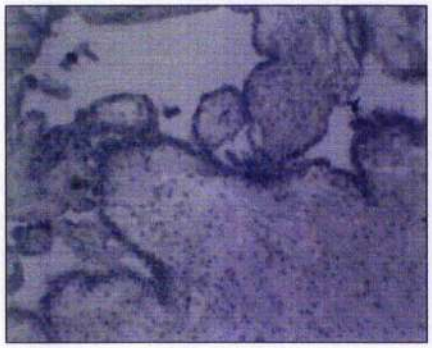


MMP-2



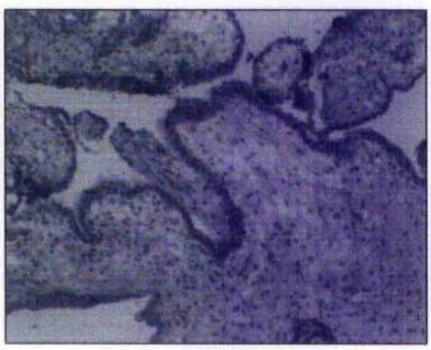
TIMP-1

TIMP-2



HIF-1α

LP34



Ki67

2.3 Purified MMP proteins used as controls in western blotting and zymography

2.3.1 Purified MMPs

MMP-9 (Biogenesis)

Purified human pro-MMP-9 (gelatinase B), 0.4mg/ml, >95% purity, 92kDa single band which upon activation exhibits a relatively stable band at 84kDa (Biogenesis Catalogue no. 5980-0957).

MMP-2 (Biogenesis)

Purified human pro-MMP-2 (gelatinase A), 0.202mg/ml, >98% purity, 72kDa single band which upon activation produces a band at 68kDa. However this 68kDa form is readily degraded to smaller species (Biogenesis Catalogue no. 5980-0257).

2.3.2 Activation of purified MMPs

Method 1: APMA activation of MMP-9

0.021g 4-aminophenylmercuric acetate (APMA) was dissolved in 210µl dimethylsulphoxide (DMSO) by vortexing. 211µl of dH_2O was added to the solution resulting in a 142mM solution of APMA and 50% DMSO. A working concentration was prepared by adding 10µl of the 142mM APMA solution to 345µl of H_2O to give a final concentration of 4mM APMA. 20µg MMP-9 was added in a 1:1 ratio (2µl + 2µl) to 4mM APMA, the eppendorf was vortexed and incubated at 37°C for 18 hrs. The activated MMP-9 was then diluted with dH_2O to the required concentration for loading onto the gel.

Method 2: Trypsin activation of MMP-9

500µg TPCK-trypsin was diluted in 1ml activation buffer (section 2.1.4) and stored in aliquots at -20°C. 20µl of this solution was added to a 10µl aliquot of 0.4mg/ml MMP-9, and the total volume made up to 100µl with activation buffer. The reaction

mixture was incubated at 37°C for 20 min. Following this, 10µl of a 1mg/ml solution of aprotinin in activation buffer was added to stop the reaction. For varying concentrations of MMP-9, the trypsin and aprotinin concentrations were scaled accordingly.

2.4 Placental sample collection

2.4.1 Gestational study samples

First and second trimester placentae (20 of each) were obtained from women undergoing termination of apparently normal pregnancies at the Royal Victoria Infirmary, Newcastle-upon-Tyne. Informed consent was obtained from each woman. The samples were collected and stored for a previous study and full details of the samples are given in previous publications (Lyall et al. 1998; Lyall et al. 1999a; Lyall et al. 2000a; Lyall et al. 2001a; Robson et al. 2002; Simpson et al. 2002). An initial ultrasound scan was performed to confirm fetal viability and determine gestational age. After evacuation of the uterine contents, placental villous samples were collected from all cases. 30 term placenta were obtained from women undergoing elective caesarean sections (ELCS).

All samples were collected directly into liquid nitrogen-cooled isopentane and stored sealed at –70°C. These samples were transported to Glasgow on dry ice and stored immediately upon arrival at –70°C.

2.4.2 High altitude study samples

2.4.2.1 Placental collection

The study comprised 6 women residing at 1600m (Denver, Colorado), 10 women residing at 3100m (Leadville, Colorado) and 10 women residing at sea level (Glasgow, Scotland). Inclusion criteria were that subjects were healthy non-smokers and without chronic conditions predisposing to pre-eclampsia such as renal disease, diabetes or obesity. Subjects resided at their respective altitudes of residence from conception to birth. All Denver and Leadville specimens were collected and supplied

by Dr Stacy Zamudio, Dept of Obstetrics, Gynecology and Women's Health, Newark, USA. Informed consent was obtained from each patient, according to the procedures approved by the Institutional Review Board of the University of Colorado or by the Ethics Committee of Yorkhill Hospital, Glasgow as appropriate. Placentae were collected immediately following birth by ELCS. Gross examination was performed to exclude any major abnormalities and the placenta was weighed. After removing 2cm around the cord insertion and 2cm of the perimeter, the placenta was cut longitudinally into 5 strips. The placental strips were dissected to produce $1cm^2$ blocks and a random 5 of these were chosen to process further.

2.4.2.2 OCT/liquid nitrogen freezing

One full thickness block from each placenta to be included in the high altitude study was snap frozen directly in liquid nitrogen. The blocks received from Leadville and Denver had been processed according to the OCT method described below, thus it was necessary to treat the blocks from Glasgow (low altitude) placentae in the same way to exclude any differences due to experimental method.

For OCT processing full thickness placental blocks were rinsed three times in PBS until the blood was washed away. The blocks were then dehydrated in a stepwise manner through a series of sucrose solutions in PBS (2, 4, 6, 8, 10, 12%) for one minute in each, before being placed into a universal container on ice containing 1ml of a 1:1 ratio of OCT: 15% sucrose in PBS. After 30 min the blocks were removed from the universal container, and placed in a bed of OCT in plastic holders. The holder was wrapped securely in tinfoil and placed in liquid nitrogen. After snap freezing the samples were placed onto dry ice and stored at $-70^{\circ}C$.

2.4.2.3 Maternal serum collection

Fifteen women from Denver, Colorado (moderate altitude group, 1600m) and 19 women from Leadville, Colorado (high altitude group, 3100m) gave informed consent to the study procedures, approved by the University of Colorado Health Science Institutional Review Board. Healthy primipara were identified by referral or by response to approved flyers placed in waiting rooms. Interested women were recruited to participate as early in pregnancy as possible. Approximately 10ml of blood was

withdrawn from the antecubital vein once per month throughout pregnancy and at 3 months postpartum. Blood was drawn into a serum separator vacutainer. Blood was allowed to clot for 1 hour, serum was separated by centrifugation for 5 min at 10,000rpm and placed in liquid nitrogen or a minus 70°C freezer. After the birth of the baby, gestational ages at the time of the blood draws were back-calculated from the clinically assessed gestational age and these dates are reported here as the gestational age at which the blood samples were obtained.

2.4.3 Termination of pregnancy (TOP) collection for explant culture

TOPs (6 to 12 weeks of gestation) were carried out at Gartnaval General Hospital, Glasgow. Ethical approval to use the placentae obtained from TOPs was granted by Yorkhill ethics committee. Terminations were performed by vacuum suction and the evacuation products were transferred immediately, on ice, to the laboratory.

2.4.4 Chorionic villous sample (CVS) collection for explant culture

CVS samples were obtained from women undergoing cytogenetic investigation in the Institute of Medical Genetics, Yorkhill Hospital. Each sample was transported in transport medium to the cytogenetics diagnostic laboratory. Once tissue had been removed for diagnosis, any remaining tissue was available for research purposes. CVS tissue was always processed on the same day the sample was taken. Ethical approval to use the placental tissue obtained from CVS was granted by Yorkhill ethics committee.

2.5 Explant culture methods

2.5.1 Tissue dissection

The method used was based on the method described by Genbacev et al. (1992). Villous tissue was obtained from CVS performed in the Queen Mother's Hospital or from TOPs obtained from Gartnaval General Hospital (section 2.4.3/2.4.4).

CVS contained only very small pieces of villi and were rinsed in PBS and not dissected further. TOP tissue was rinsed with PBS and any fetal tissue removed.

Villous tissue, identified under a dissecting microscope by its attachment to the chorionic membrane and by its white fingerlike fluffy projections, was transferred to a separate 35mm dish. Following a further rinse, the tissue was dissected to give small (5-20mg wet weight) pieces of villi. Each piece of villi, from CVS and TOPs to be used was weighed in a sterile petri dish.

2.5.2 Culture of explants on matrigel

All explant culture was carried out in a Class II laminar flow cabinet. Frozen aliquots of matrigel were thawed on ice for 1hr, and 150µl of matrigel was added to each insert (Millicell 12mm culture plate inserts (pore size 4µm)) using pre cooled pipette tips. Each insert was placed in a well of a 24-well plate. The incubators used were Forma Scientific water-jacketed incubators. The low oxygen incubator was set at 2% O_2/5%CO_2/93%N_2 and the normal oxygen incubator was set at 18%O_2/5%CO_2/77%N_2. The oxygen and carbon dioxide tension in the incubators was checked regularly using a Fyrite calibrator. Dissolved oxygen levels 125ml of test culture medium in a T75 vented lid flask was measured using a Jenway Model 970 dissolved oxygen meter and electrode (Jencons, UK) (discussed in chapter 8).

The 24-well plate with inserts was incubated for 30 min at 37°C in 18% O_2 until the matrigel had polymerised. Thereafter one piece of villous tissue was carefully placed centrally on each insert using a pipette tip and teased until it was lying as uncurled as possible. Any excess liquid medium was removed and the plate was incubated for 60 min at 37°C in 18% O_2. 400µl of culture medium was added to the lower chamber of the culture plate and the plate was incubated overnight at 37°C in 18% O_2. The following morning, 200µl of culture medium was added to the upper surface of the insert to submerge the explant. The explant was incubated for 96/144 hrs at 37°C in either the normal or low oxygen incubator and the culture medium was collected and changed every 24/48 hrs. All culture medium was stored at –20°C until assayed.

Photographs were taken of the explant at each culture medium change using an Olympus S2H microscope with an Olympus C35DA2 camera attachment. The number of sites of outgrowth of CTB cells in each explant were recorded and migration of cells into or through the matrigel was scored on a scale of 0 – 5 (0 – no

migration, 1 – one or two sites of localised migration, 2 – several sites of localised migration, 3 – moderate migration, 4 - moderate to extensive migration, 5 - extensive migration from several sites around the explant). Cells migrating into the matrigel were easier to identify and score when viewed directly under the microscope than in the final photographic images.

2.5.3 Explant freezing

The matrigel was carefully dissected out of the insert using a surgical blade and gently blown onto a cork disk. Subsequently the disk was placed upside down in liquid nitrogen cooled-isopentane for 30 sec to freeze the tissue. The corks were transferred to pre-cooled pots for storage at -70 °C.

2.6 Immunohistochemistry

2.6.1 Preparation of frozen tissue sections

2.6.1.1 Silane coating of slides

Double frosted microscope slides were silane coated (all procedures were carried out in a fume hood). Slides were arranged in racks so that both sides of the slide were treated. The rack was dipped in acetone for 5 min, followed by 2% dimethylchlorosilane solution in acetone for 5 min. The slides were rinsed twice in acetone followed by a 30 min rinse in running distilled water. The slides were then air-dried in the racks overnight in a Hybaid hybridisation oven at 37°C.

2.6.1.2 Cryosectioning

6µm sections of all samples were cut on a cryostat. The internal temperature of the cryostat was –25°C. Placental blocks or corks containing explants on matrigel were mounted onto a chuck using OCT and successive sections were cut using a disposable microtome blade. Any section that appeared to fold on cutting was rejected. One

section of a tissue block or six sections of an explant were mounted onto each slide. The slides were air-dried overnight before being placed in a –70°C freezer for storage.

2.6.2 Avidin/Biotin immunoperoxidase staining of frozen tissue sections

The slides were brought to room temperature in a pre-cooled container, and a ring was marked round the placental section using a DAKO pen. The sections were fixed in acetone for 5 min followed by ethanol for 5 min and rinsed several times in dH_2O. Sections were blocked with the appropriate serum from a Vectastain ABC kit (100µl serum in 5ml PBS) for 30 min at 37°C, then washed in PBS for 5 min.

Primary antibody was diluted appropriately (table 4) in the blocker, 50µl was pipetted onto each section, followed by incubation for 90 min at 37°C, and 2 x 5 min washes in PBS.

The secondary antibody was diluted 1/50 in the blocker. 50µl was placed on the sections followed by incubation for 30 min at 37°C, and 2 x 5 min washes in PBS. The slides were soaked in 1% hydrogen peroxide in methanol for 15 min at room temperature in order to quench any endogenous peroxide activity present, then washed for 2 x 5 min in PBS.

Avidin Biotin complex (ABC) was prepared by adding 100µl reagent A and 100µl reagent B from the kit to 5ml PBS. This was incubated at room temperature for 30 min. 100µl was placed on each section and left for 30 min at room temperature, followed by 2 x 5 min washes in PBS. 3,3 diaminobenzidine (DAB) was prepared by adding one DAB tablet and one urea tablet to 5ml dH_2O. The resultant solution was filtered and 100µl placed on each section for 10 min.

The slides were washed in running water for 10 min, counterstained with Harris' haemotoxylin, progressively dehydrated in ethanol (30%, 50%, 70%, 100% each for 1 min, then 100% for 5 min) and soaked in histoclear for 5 min. Coverslips were mounted using DePeX mounting medium and the slides were air dried overnight. Slides were viewed using an Olympus BH2 microscope and images were captured using an Olympus DP10 camera and Flashpath software. The staining was scored by an observer blinded to the tissue identity on a semi-quantitative scale where 0 = no staining, 1 = very mild staining, 2 = mild staining, 3 = moderate staining and 4 = strong staining.

2.7 Placenta homogenisation

Tissue homogenisation buffer (4ml) (section 2.1.3) was aliquoted into a universal container and kept on ice. The frozen placental block to be homogenised was weighed, then ground in liquid nitrogen with a mortar and pestle to a fine powder. The liquid nitrogen was allowed to evaporate before the powder was placed in the buffer. Homogenisation buffer was added to the tissue at a final ratio of 4:1 (v:w).

The tissue was homogenized in 6 bursts of 10 sec at full speed, with cooling on ice between bursts using a Polytron homogeniser. The homogenate was then aliquoted into eppendorfs and spun at 5000g for 10 min at 4°C. The supernatant was aliquoted into fresh tubes and spun again at 50,000g for 20 min at 4°C. The supernatant was removed and kept as the cytosol fraction and the pellet was resuspended in 400ul of 25mM tris, pH 7.6. The amount of protein in the sample was quantified by the method of Bradford (section 2.10.1).

2.8 Western Blotting

2.8.1 Denaturing polyacrylamide gel electrophoresis (PAGE) and protein transfer

A Mini Protean II western blotting apparatus was used in all experiments except for the experiment in which a large gel was required when a Protean II xi cell tank was used. A 7.5% SDS polyacrylamide resolving gel was poured (4.5ml of 30% acrylamide, 1.2ml of 50% glycerol, 4.5ml resolving gel buffer (section 2.1.4), 7.7ml dH_2O, 90µl of 10% ammonium persulfate (APS) and 9µl N,N,N',N' tetramethylethylenediamine (TEMED)). The gel was overlayered with isopropanol and left to set for 45 min at room temperature, after which the isopropanol was washed out with distilled water.

The stacking gel was poured (2.7ml of 30% acrylamide, 5ml stacking gel buffer (section 2.1.4), 12.2ml dH_2O, 100µl 10% APS and 20µl TEMED) and a 10 well comb with 0.75mm spacers was inserted. The gel was left to set for 30 min, the comb was removed and the wells were washed out with dH_2O. Samples were mixed 1:1 with 2x loading buffer (section 2.1.4). The samples were boiled for 5 min at 95°C

before loading. The total amount of sample loaded per well was variable depending on the protein concentration required but did not exceed 20µl or 100µg protein. Pre-stained SDS-PAGE low range molecular weight (Mwt) markers range 20,000-113,000 kDa were loaded on each gel.

The gel tank was assembled, running buffer (section 2.1.4) was added to the tank and the gels were run at a constant current of 15mA and at maximum voltage (400V) until the dye front had reached the bottom of the gel (Electrophoresis power supply 400L). Protein was transferred overnight in transfer buffer (section 2.1.4) at a constant voltage of 30V and at maximum current (250mV), to nitrocellulose membranes. The transfer was carried out at 4°C. Immunoblotting was then carried out as described in section 2.8.3

2.8.2 Spotting of purified MMP proteins on nitrocellulose membrane for antibody comparison

Purified MMP-9 and MMP-2 purified proteins (2.3) were spotted at varying concentrations using a pipette onto nitrocellulose membrane. The membrane was left to dry until the spots could no longer be detected by eye (about 90 sec). Immunoblotting was then carried out as described in section 2.8.3.

2.8.3 Immunoblotting

Filters were blocked for 1 hr at room temperature in 5% non-fat dried milk (Marvel) in PBST (section 2.1.1) or in 5% donkey serum in PBST.

The filters were incubated with the primary antibody at an appropriate dilution which was predetermined for 1 hr at room temperature (table 2.2) after which they were washed 3 x 5 min in PBST. The secondary antibody (horseradish peroxide conjugated donkey anti sheep/goat IgG) was diluted 1/1000 in 1% marvel in PBST, and incubated with the filters for 1 hr at room temperature. The filters were then washed 3 x 5 min in PBST.

Proteins were detected using the Amersham Enhanced Chemiluminescence (ECL) detection system and the filters were exposed to hyperfilm ECL for various times under safety illumination. The film was developed in an X-ray film processor.

The positions of the molecular weight markers were recorded, a graph was plotted on semi log scale and the molecular weights of any visible bands were calculated.

2.9 Zymography

2.9.1 Denaturing polyacrylamide gel electrophoresis (PAGE)

The method used was based on the method described by Herron et al. 1986. The same apparatus was used as for western blotting (section 2.8.1). Samples were separated on a 7.5% SDS polyacrylamide resolving gel containing varying concentrations of gelatin. A 3% solution of gelatin in dH_2O was prepared and stored in aliquots at –20°C. Table 5 shows the composition of gels with varying final gelatin concentrations. Buffer A and B were the same as used in western blotting.

Table 5 Composition of zymography gels

Final % gelatin	30% acrylamide	Buffer A	dH_2O	3% gelatin	10% APS	TEMED
0.1%	4.5ml	4.5ml	8.3ml	0.6ml	90µl	9µl
0.05%	4.5ml	4.5ml	8.6ml	0.3ml	90µl	9µl
0.025%	4.5ml	4.5ml	8.75ml	0.15ml	90µl	9µl

The gel was poured and layered with isopropanol and left to set for 45 min at room temperature, after which the isopropanol was washed out with dH_2O. The stacking gel was then poured (2.7ml of 30% acrylamide, 5ml stacking gel buffer, 12.2ml dH_2O, 100µl 10% APS and 20µl TEMED) and a 10 well comb with 0.75mm spacers was inserted.

The gel was left to set for 30 min, the comb was removed and the wells were washed out with dH_2O. Molecular weight markers were the same as for western blotting (section 2.8.1). Samples were loaded and then the gels were run at a constant current of 15mA and maximum voltage (400V) until the dye front had reached the bottom of the gel.

2.9.2 Zymographic detection of gelatinases

The gel was subsequently washed in zymography wash buffer (section 2.1.4) for 3x 30 min to wash out SDS and incubated in reaction buffer (section 2.1.4) for 18 hrs at 4°C. The gel was then stained in 0.3% coomassie brilliant blue stain (BDH) in dH_2O for 30 min and destained for 1 x 5 min, 2 x 30 min, 1 x 5 min in destain buffer (20% methanol, 10% acetic acid). The bands on the gel were analysed by scanning densitometry using a GS-700 imaging densitometer in conjunction with Multianalyst software and optical density (OD) was determined for each band.

2.10 Assays

2.10.1 Bradford protein assay

The method is that described by Bradford, (1976). BSA stock standard solution was stored at –20°C in 100µl aliquots of 1.4mg/ml. On the day of use 180µl H_2O was added to give a total volume of 280µl and a concentration of 500µg/ml. A series of standard solutions (0-500µg/ml) using the dilutions below was prepared.

Concentration of standards (µg/ml)	Volume of standards (µl)	Volume dH_2O (µl)
0	-	50
100	10	40
200	20	30
300	30	20
400	40	10
500	50	-

Procedure

10µl of standard solutions and samples were added to duplicate wells on microtitre plates followed by 200µl of bradford's reagent and the plate was placed on a plate

shaker for 10 sec to ensure thorough mixing. After a period of 5 min to 1 hr, the absorbance (Abs) at 595nm was read using a biochromatic plate reader and analysed using Genesis software. A standard curve was automatically plotted and total protein values calculated from the standard curve.

2.10.2 MMP-9 activity assay (Amersham Pharmacia)

The reagents were prepared as described in the reagent preparation protocol, as were the working standards (0, 0.5, 1, 2, 4, 8, 16 ng) for the normal standard curve. For the lower range standard curve the values were 0, 0.125, 0.25, 0.5, 1, 2 and 4ng.

Assay buffer (110µl) was added to the zero wells, and 100µl of each standard to the appropriate wells. 100µl of the samples was then added to the appropriate wells. The plate was covered with the plate lid and incubated at 4°C overnight.

The following morning each of the wells was aspirated, washed 4 times with wash buffer, and blotted on tissue paper. 50µl of APMA (1mM in DMSO) was added to each well containing the standards and those wells where total (i.e. activated and pro) MMP-9 was to be measured and 50µl of assay buffer was added to each well where active MMP-9 was to be measured. The plate was shaken for 20 sec, covered with the plate lid and incubated at 37°C for 90 min.

The detection reagent was prepared just before the end of this incubation period as described in the reagent preparation section. 50µl of the detection reagent was added to each well and the plate was shaken for 20 sec. The plate was read at 405nm to obtain a zero time reference value. The plate was then covered and incubated at 37°C. At 1hr intervals thereafter the plate was shaken for 20 sec and optical density (OD) at 405nm determined and absorbance calculated. This was continued until saturation point was reached. A standard curve was plotted for each of the time-points, and the values of MMP-9 in the samples calculated from the best standard curve.

2.10.3 Vascular Cell Adhesion Molecule-1 (VCAM-1), Intracellular Cell Adhesion Molecule-1 (ICAM-1) and Endothelial Selectin (E-Selectin) assays (Parameter Assays, R&D Systems)

Samples and reagents were prepared as described on page 5 of the instruction manuals. The serum samples to be assayed were diluted 1:50 in sample diluent for the VCAM-1 assay and 1:20 for the ICAM-1 and E-Selectin assays. Standards were assayed in duplicate.

100µl diluted conjugate was added to each well followed by 100µl of standards, samples, and control, with thorough mixing. The plate was covered and incubated at 37°C for 90 min. The wells were then emptied by aspiration and washed 6 times with 300µl wash buffer. On the final wash, the plate was tapped on paper towels to ensure all liquid was removed.

100µl substrate was then added to each well followed by incubation at room temperature for 20 min. Finally 100µl of stop solution was added to each well.

The OD was determined after 5 min at 405nm with correction at 620nm. The standards, from which a standard curve was plotted, were in the range 0–75.78ng/ml, 0–49.55 ng/ml and 0–10.52 ng/ml for each of the assays respectively and the control samples were well within the required range. Levels of VCAM-1, ICAM-1 or E-Selectin in the serum samples were computed automatically from the standard curve. The inter and intra-assay precision coefficient of variance (% CV) values are shown for each assay.

VCAM-1: Inter-assay precision CV = 8.5–10.2%, Intra-assay precision CV = 4.3–5.9%

ICAM-1: Inter-assay precision CV = 6.0–10.1%, Intra-assay precision CV = 3.3–4.8%

E-Selectin: Inter-assay precision CV = 5.7–8.8%, Intra-assay precision CV = 4.7–5%

2.11 Statistical methods

All data analysis was performed on the statistical analysis package SPSS for Windows. Below is an outline of the tests used in this thesis.

2.11.1 Descriptive statistics

2.11.1.1 Mean

The mean of a group of data was calculated by dividing the sum of all the cases by the number of cases. This gives a central value of the data and can only be used reliably when the data is not skewed i.e. there is an even or normal distribution.

2.11.1.2 Median

The median of a group of data refers to the middle value when the information is sorted in ascending order. This can also be the average of the two middle cases if there is an even number of data. As it measures central tendency the median is a useful descriptive parameter to use when data is skewed since it is not disrupted by outlying values.

2.11.1.3 Standard deviation

Standard deviation (SD) refers to the dispersion of the data around the mean and was calculated using the equation

$$SD = \sqrt{\frac{Ey^2 - (E\,y)^2 / N}{(N-1)}}$$

y = individual case values

N = number of cases

2.11.1.4 Standard error of the mean

This figure is a measure of the variance of the mean from sample to sample from the same distribution. The standard error (SE) of the mean was calculated by dividing the standard deviation by the square root of the number of cases.

2.11.2 Analysis of distribution of data

Before statistical comparison of different groups could be carried out, it was necessary to determine whether the distribution of the data was parametric. For this purpose Shapiro-Wilk testing was carried out. The Shapiro-Wilk test detects departures from normality, without requiring that the mean or variance of the hypothesized normal distribution be specified in advance. If the probability (p) value for the test >0.05 the data has a normal distribution and parametric testing may be carried out. If the p-value <0.05 then the data has a non-normal distribution and non-parametic testing should be used. The test result does not indicate the type of non-normality. Examination of the calculated skewness and kurtosis, and of the histogram, boxplot, and normal probability plot for the data may provide clues as to why the data failed the Shapiro-Wilk test.

2.11.3 Correlation analysis

2.11.3.1 Pearson's correlation coefficient

The correlation coefficient provides a measure of the degree of linear association between two variables. The Pearson's correlation analysis is used on data which has a normal distribution. A correlation coefficient (R) is obtained in addition to a p-value. R-values range from −1 to +1 with −1 indicating a high negative correlation and +1 indicating a high positive correlation. A p-value is also given – if this <0.05, a significant association between the variable is assumed.

2.11.3.2 Spearman's correlation coefficient

The Spearman's correlation analysis is the non-parametric equivalent of the Pearson's correlation.

2.11.4 Analysis of variation between groups

2.11.4.1 Independent samples t-test

The parametric t test is used for the comparison of means in two samples. The test gives a p-value which indicates the probability that the samples come from different populations. A p-value < 0.05 is taken as significant and indicates that there is a less than 5% chance that the samples are from the same population.

2.11.4.2 Mann-Whitney U test

This non-parametric test is equivalent to the parametric t-test. It measures whether two independent samples are likely to have come from the same population. Both groups are combined and placed in ascending order and a calculation is made of how many times a value in one group precedes the value in the other group (U). If one of the groups has a larger proportion of higher rankings the groups are likely to have different distributions. A value of $p<0.05$ is taken as indicating significance.

2.11.4.3 Wilcoxon-signed ranks test

This test is used to determine whether the median difference in paired data is zero. It consists of sorting the absolute values of the differences from smallest to largest, assigning ranks to the absolute values and then finding the sum of the ranks of the positive differences. A value of $p<0.05$ is taken as indicating significance.

2.11.4.4 Two-way ANOVA

Analysis of Variance (ANOVA) is used to test the hypothesis that samples from more than two independent groups have equal means. A two way ANOVA produces a two-

way analysis of variance of the effect of a dependant variable (eg altitude) on an independent variable (e.g. antibody staining). This test is basically an elaborated version of the t-test. A value of $p<0.05$ is taken as indicating significance.

2.11.4.5 Fisher's PLSD

Once a two-way ANOVA has been carried out, post hoc analysis shows where the differences are amongst several means.

2.11.4.6 Kruskal-Wallis

The Kruskal-Wallis test is a non-parametric method of testing the hypothesis that samples from more than two independent groups have the same continuous distribution versus the alternative that measurements tend to be higher in one or more of the populations and has some similarity to an ANOVA. A value of $p<0.05$ is taken as indicating significance.

2.12 List of suppliers of chemicals and apparatus

Amersham Pharmacia, Biotechnologies, UK

ECL western blotting detection reagent, hybond ECL, hyperfilm ECL, MMP-9 activity assay

Bacharach Instruments, Denmark

Fyrite gas analyser

Bayer (Germany)

Bayer 288 blood gas analyser

BD Biosciences, Bedford, UK

Matrigel

BDH laboratory Supplies, Poole, UK

Acetone, APS, coomassie brilliant blue, dimethyldichlorosilate, DPX mounting reagent, DTT, ethanol, harris' haematoxylin, isopentane, methanol, NaCl, NaOH, OCT compound, SDS, sucrose, trichloroacetic acid, triton-X100

Biochem Immunosystems, UK

MAIA Clone HCG assay

Biogenesis

MMP-2 protein, MMP-9 protein, anti-MMP-2 antibody, anti-MMP-9 antibody, anti-TIMP-1 antibody, anti-TIMP-2 antibody

Biorad Laboratories Ltd, UK

Protean II xi western blotting apparatus, and mini protean II xi western blotting apparatus, low range molecular weight markers, GS-700 imaging densitometer and multianalyst software

Bioquell

Microflow biological safety cabinet BSC1/LG01

Boehringer Mannheim, Germany

Tris base

Chemicon

Anti-MMP-9 antibody

Cryoservice, UK

Nitrogen gas

Diagnostics Scotland, Carluke, UK

Normal donkey, horse and human serum, horseradish peroxide conjugated donkey anti sheep/goat IgG

Fisher Scientific UK Loughborough, UK

Acetic acid, isopropanol

Forma Scientific Inc, UK

CO_2 water jacketed incubator model no. 3141

Gibco-BRL, UK

FBS, 100x L-glutamine, 100x fungizone, 100x penicillin/streptomycin, DMEM, Hams F12 cell culture medium

Glasgow University Central Stores

Ethanol

Greineir Labortechnik

Microtitre plates

Heidolph

Plate mixer

Heraeus, UK

Biofuge centrifuge

Hybaid, UK

Hybridisation oven

Jencon Scientific Ltd, UK

Jenway 970 portable dissolved O_2/°C meter and electrode

Kinematica AG, Switzerland

Polytron homogeniser

Labsystems, UK

Multiskan biochromatic plate reader and Genesis software

Life Technology Ltd, Paisley, UK

Electrophoresis power supply 400L

Millipore, UK

Millicell culture plate inserts (12mm diameter, pore size 4□m)

Multi RO Plus water purification system

National Diagnostics, Hull, UK

Histoclear

Novocastra

Anti-Cytokeratin LP34 antibody, Ki67 antibody

Nunc, UK

Sterelin 24-well plates

Olympus, UK

S2H microscope and C35DA2 camera attachment

Oxoid Ltd, Basingstoke, UK

PBS (Dulbecco A)

Premier Beverages, Stafford, UK

Marvel powdered milk

Raymond Lamb Ltd, UK

1cm cork discs

Rectangular $2cm^2$ plastic holders

R&D

Parameter assays – VCAM-1, ICAM-1 and E-Selectin

Santacruz Biotechnology

Goat control Ig, catalogue number: sc-2028

Mouse control Ig, catalogue number: sc-2025

Rabbit control Ig, catalogue number: sc-2027

Sheep control Ig, catalogue number: sc-2717

Sigma-Aldrich Company, Poole, UK

30% acrylamide/bis-acrylamide (ratio 37:5:1), APMA, aprotinin, bradford reagent, bromophenol blue, BSA, $CaCl_2$, Fast 3,3' diaminobenzide tablets, DMSO, EDTA, gelatin, glycerol, glycine, hydrogen peroxide 30% solution, NaN_3, protease inhibitor cocktail (P8340 for use with mammalian cell and tissue extracts), TEMED, TPCK-trypsin, tween-20

Shandon,UK

Cryostat microtome

Swann Norton, UK

Surgical blades

Thermashandon, UK

Double frosted microscope slides

Vector Laboratories

DAKO pen

Vectastain ABC kits (universal, sheep and goat)

Chapter 3: Evaluation of various methods for detecting and quantifying MMP-9 and MMP-2 in placental homogenates

One key aim of the current study was to quantify MMP-2 and MMP-9 expression and activity in normal placenta throughout gestation. These MMPs have been implicated in CTB invasion in many studies yet widely differing conclusions on when and where they are expressed have been reported (section 1.4). One likely source of these differences lies in the methodology used to examine MMP expression.

Therefore it was necessary to evaluate potential methods that could be used to study MMP expression. Initially it was decided to attempt to quantify MMP-9 and MMP-2 expression and activity in placental homogenates. The overall aim of this section was to determine which methods would be most appropriate for detecting and quantifying MMP-9 and MMP-2 in placental homogenates. Initially western blotting was tested as a potential method of MMP quantification as it was a technique already in common use in our laboratory. Secondly zymography, a method widely used in determining MMP activity in cell culture medium was investigated. Finally an MMP-9 activity assay was evaluated.

3.1 Western blotting

3.1.1 Validation of commercially available MMP antibodies

The purpose of this experiment was to determine which MMP antibodies were most sensitive and specific for MMP-2 and MMP-9 detection in studies herein.

The properties and availability of MMP-9 and MMP-2 antibodies on the market were investigated. Two MMP-9 antibodies were purchased, one from Biogenesis and one from Chemicon (section 2.2). One MMP-2 antibody was purchased from Biogenesis (section 2.2) for initial studies.

The antibodies selected were then tested on western blots (section 2.8). Three gels were run, each with 50μg total protein homogenates (section 2.7) from a term and from a 10 weeks of gestation normal placenta.

For MMP-9 (Chemicon Antibody) after initial washing and detection (30 sec exposure) there appeared to be some bands present (figure 10a). However after an overnight wash these bands were not present (10 min exposure), suggesting they were non-specific (figure 10b).

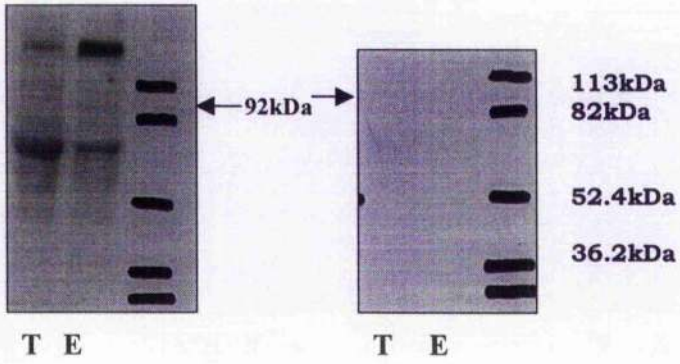
For MMP-9 (Biogenesis Antibody), high background initially obscured any bands (30 sec exposure) (figure 10c). After overnight washing, clean bands were evident but this was not at the correct molecular weight of 92kDa (10 min exposure) (figure 10d).

For MMP-2 (Biogenesis Antibody), high background initially obscured any bands (30 sec exposure) (figure 11a). After overnight washing, clean bands were evident (10 min exposure) (figure 11b) but these seemed to be in a very similar position to the bands detected with the MMP-9 Biogenesis antibody. This heightened the suspicion that these bands may be nonspecific.

Figure 10 Detection of MMP-9 in placental homogenates under reducing conditions using two different antibodies

Chemicon MMP-9 Ab

a) Initial detection b) Detection after overnight wash

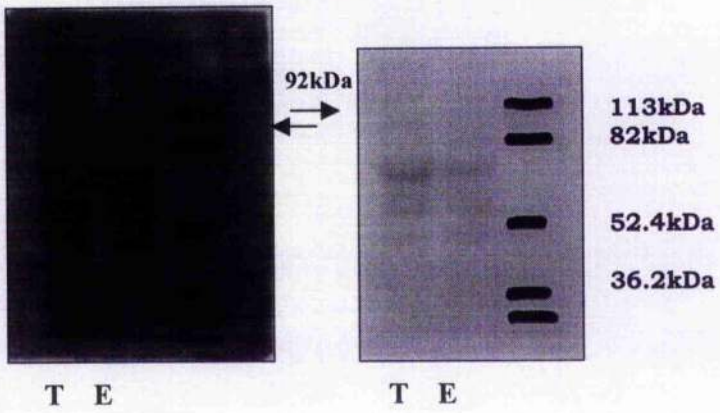


T = Term placental homogenate

E = 10 weeks of gestation placental homogenate

Biogenesis MMP-9 Ab

c) Initial detection **d) Detection after overnight wash**

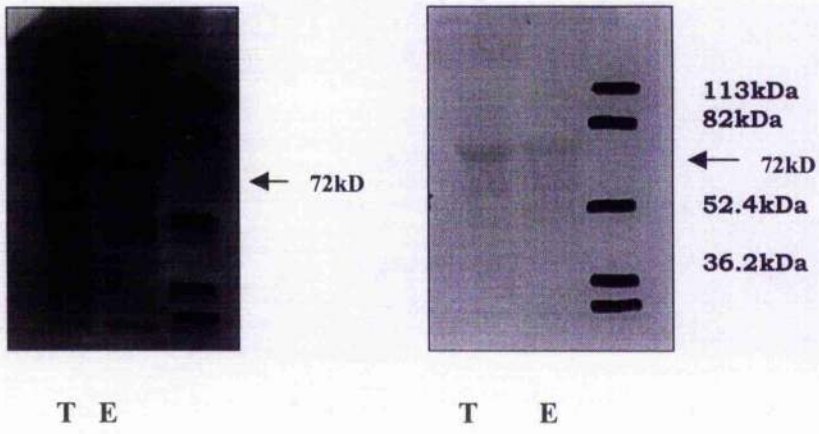


T = Term placental homogenate

E = 10 weeks of gestation placental homogenate

Figure 11 **Detection of MMP-2 in placental homogenates using Biogenesis MMP-2 antibody under reducing conditions**

a) Initial Detection **b) Detection After Overnight Wash**



T = Term placental homogenate

E = 10 weeks of gestation placental homogenate

3.1.2 Detection of MMP-9 and MMP-2 purified proteins with commercially available antibodies using a 'spot' method.

Since the western blotting in section 3.1.1 did not confirm that the antibodies were successfully detecting MMP-9 and MMP-2 in placental homogenates, it was decided to test their ability to detect purified MMPs which were commercially available. The 'spotting' method (section 2.8.2) was chosen as it is less time consuming than western blotting.

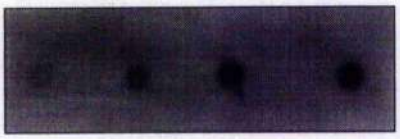
MMP-2 and MMP-9 purified protein (Biogenesis, section 2.3.1) were spotted at various concentrations onto nitrocellulose and the ability of the antibodies used in 3.1.1 to detect the MMPs was tested. A comparison was also made between using 5% Marvel in PBST (section 2.1.1) compared to 5% donkey serum in PBST as the blocking reagent.

Detection of the MMP-9 purified protein was successful using both the Chemicon and Biogenesis MMP-9 antibodies, however the Biogenesis antibody produced slightly better detection sensitivity - 6.5ng of protein was easily detectable after a 5 min exposure (figure 12a) while the Chemicon antibody only just detected this amount (figure 12b). Since the Biogenesis antibody was four times cheaper than the Chemicon antibody, and detection of purified MMP-9 seemed to be slightly better with Biogenesis, it was decided to use the Biogenesis antibody in further studies.

For MMP-9, blocking with 5% serum (figure 12c and 12d) gave a clearer background particularly with the Biogenesis antibody; therefore this was used in all subsequent blots.

Figure 12 **Detection of purified MMP-9 protein spotted onto nitrocellulose using Biogenesis and Chemicon antibodies (5 min exposure)**

a)



6.5ng 12.5 ng 25ng 50ng

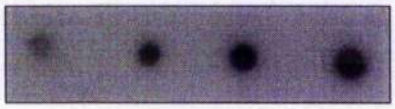
MMP-9 (Biogenesis) Marvel blocker

b)

6.5ng 12.5 ng 25ng 50ng

MMP-9 (Chemicon) Marvel blocker

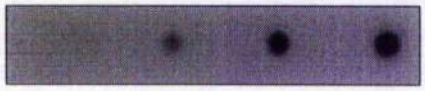
c)



6.5ng 12.5 ng 25ng 50ng

MMP-9 (Biogenesis) Serum blocker

d)



6.5ng 12.5ng 25ng 50ng

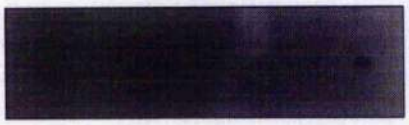
MMP-9 (Chemicon) Serum blocker

MMP-2 detection was initially weak with the Biogenesis antibody after 5 min (figure 13a), however the 50ng spot of MMP-2 was clearly visible after 30 min exposure to film (figure 13b). Since the antibody was able to detect MMP-2, it was decided to use it in further studies.

For MMP-2, blocking with 5% serum also gave a much clearer background; therefore this was used in all subsequent blots.

Figure 13 Detection of purified MMP-2 protein spotted onto nitrocellulose using Biogenesis antibody

a) 5 min exposure



MMP-2 (Biogenesis) Marvel blocker

6.5ng 12.5 ng 25ng 50ng

MMP-2 (Biogenesis) Serum blocker

6.5ng 12.5 ng 25ng 50ng

b) 30 min exposure

MMP-2 (Biogenesis) Marvel Blocker

6.5ng 12.5 ng 25ng 50ng

MMP-2 (Biogenesis) Serum blocker

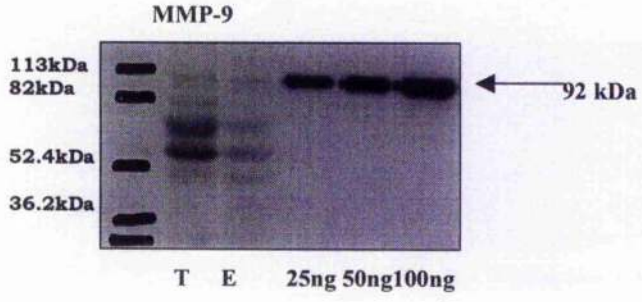
6.5ng 12.5 ng 25ng 50ng

3.1.3 Detection of purified MMP-9 and MMP-2 by western blotting

In experiments in 3.1.1 it had proved difficult to detect MMPs in placental homogenate samples using western blotting. However the subsequent spotting experiments in 3.1.2 showed that the antibodies were capable of detecting purified MMP-2 and MMP-9. Therefore the next set of experiments was designed to test whether the purified MMPs could also be detected using western blotting. For MMP-9 this was tested using control proteins loaded at a concentration of 25 to 100ng. In addition, early and term placental homogenates were loaded for comparison on the same gel.

Control protein (25ng) was readily detectable as one clear band at an estimated molecular weight of 92kDa (the known molecular weight of pro-MMP-9) after an exposure of 5 min. The lanes in which the placental homogenates were loaded also had bands at the same molecular weight as the control protein, although these were not particularly strong (figure 14).

Figure 14 Detection of purified MMP-9 protein by western blotting (reducing conditions)



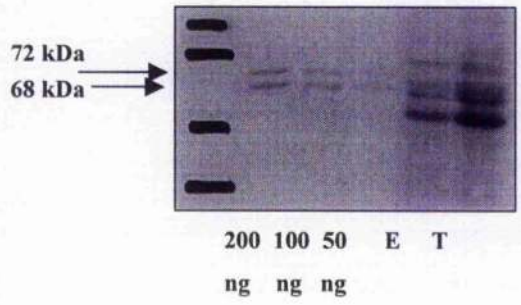
T = Term placental homogenate

E = 10 weeks of gestation placental homogenate

For MMP-2, detection of the purified protein was tested using protein loaded at a concentration of 50 to 200ng, since the antibody had been shown in section 3.1.2 to be less sensitive than the MMP-9 antibody. Early and term placental homogenates were also loaded for comparison on the same gel. 50ng of control protein was just detectable after an exposure of 5 min and 100 and 200ng were readily detectable. The lanes in which the placental homogenates were loaded also had bands at the same molecular weight as the purified protein, although other bands were also present (figure 15).

Interestingly in the control protein lanes two bands were observed – one band at 72kDa and one at 68kDa. Since the activated form of MMP-2 has a molecular weight of 68kDa this additional band probably represents the activated from. Since the MMP-2 protein had not been purposely activated, only one band (72kDa) was expected to be present. The protein had been aliquoted on arrival and frozen at –70°C until used as advised by the manufacturers. The manufacturers (Biogenesis) were consulted and advised that the lower band was probably the activated form of MMP-2. They also advised that the activation had probably occurred as a result of unavoidable autocatalytic cleavage of the enzyme during transit and storage.

Figure 15 **Detection of purified MMP-2 protein by western blotting (reducing conditions)**



T = Term placental homogenate

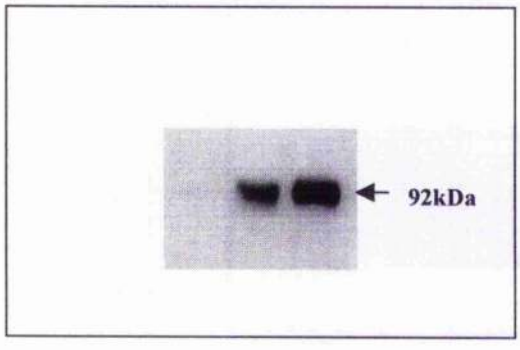
E = 10 weeks of gestation placental homogenate

3.1.4 Does the MMP-9 antibody detect both activated and pro forms of MMP-9?

The previous experiment in section 3.1.3 showed that the MMP-9 antibody successfully detected non-activated forms of MMP-9. However in tissues both non-activated and activated forms of MMP-9 are present. It was therefore important to establish whether this antibody could also detect the activated form. The following experiment was performed.

A Western blot (section 2.8) was run to examine detection of activated and pro-MMP-9. MMP-9 can be activated *in vitro* by incubation with 2mM Amino Phenyl Mercuric Acetate (AMPA) for 18 hrs (section 2.3.2). The APMA cleaves the pro sequence from the MMP-9 in a similar way to activation *in vivo*. MMP-9, which had been subjected to the activation procedure minus the addition of APMA, was also run as a control. 100ng of protein was loaded in each lane. The results are shown in figure 16.

Figure 16 Detection of pro and APMA activated purified MMP-9 protein by the Biogenesis antibody under reducing conditions



No band was present in the lane containing MMP-9 which was treated with APMA. In the lanes containing the control and untreated MMP-9, a large band corresponding to a molecular weight of 92kDa was seen.

It was clear that this antibody did not detect the activated form of MMP-9. However the gels were run under reducing conditions. It is known that some antibodies are sensitive to whether the conditions are reducing or non-reducing. Alternatively it was possible that the activation had failed and the protein had been completely degraded and was not detected.

Therefore it was decided to test whether the activated form could be detected under non-reducing conditions.

3.1.5 Detection of activated and pro-MMP-9 under non-reducing conditions

Since Western Blots are time consuming, it was decided to do a preliminary investigation of non-reducing conditions using spotting (section 2.8.2) before going on to carry out a full western blot. 200ng spots of pro, activated and control MMP-9 were spotted onto nitrocellulose and incubated with the antibody (figure 17).

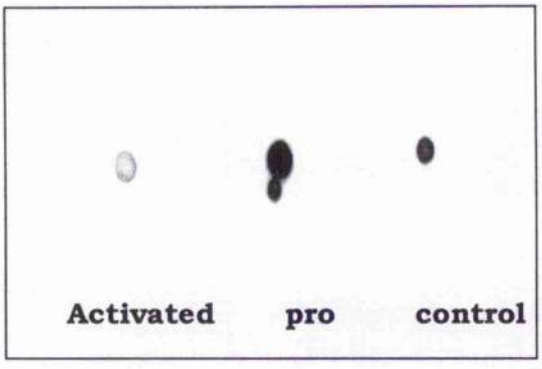
A large spot was detected where non-activated MMP-9 was spotted. The control spot was smaller and lighter and the activated spot was even lighter. The antibody thus seemed to detect the pro form more readily than the activated form. Alternatively it was possible that the small spot in the activated sample represented a small amount of pro form which had not been activated, if the antibody was not capable of detecting the active form. Since MMP-9 can be cleaved to smaller fragments than the predominant 84kDa from, it was also possible that incubation with AMPA had resulted in smaller fragments which were not detected by the antibody. Interestingly, the control seemed to show some spontaneous activation even in the absence of APMA.

In summary, it was still unclear whether the antibody could detect the activated form of MMP-9. Although APMA was the recommended activating agent for the purified MMP-9 protein, the manufacturers advised that in certain circumstances this activation protocol may be too harsh leading to over activation.

Trypsin is an *in vivo* activator of MMP-9 (section 1.4.4) and is without the biological hazards associated with APMA. The protocol to activate MMP-9 with

trypsin is also less time consuming (section 2.3.2). Therefore it was decided to activate MMP-9 protein with trypsin to determine whether or not the antibody could detect MMP-9 activated in this way.

Figure 17 Detection of pro and APMA activated purified MMP-9 protein under non-reducing conditions



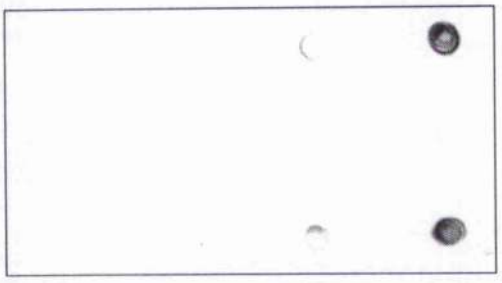
3.1.6 Detection of MMP-9 activated by trypsin

Since trypsin activation of MMP-9 is both less hazardous and requires a shorter activation time than AMPA, this was attempted (section 2.3.2). The spotting procedure was carried out as before (section 2.8.2). The results are shown for a 90 sec (a) and 5 min (b) exposure to film (figure 18).

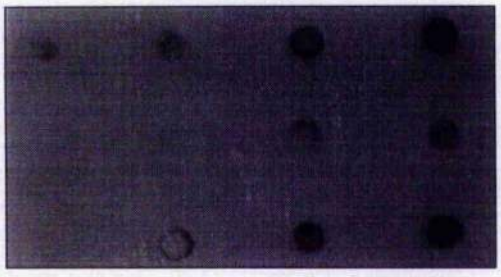
Almost exactly the same results were obtained as with activation by APMA. The pro-form and control were detectable at 100ng after a 90 sec exposure and the activated form at 200ng after a 90 sec exposure. After a 5 min exposure the pro-form was detectable at 25ng, the control at 50ng and the activated at 100ng.

Figure 18 Detection of pro and trypsin activated purified MMP-9 protein

a) 90 sec exposure



b) 5 min exposure



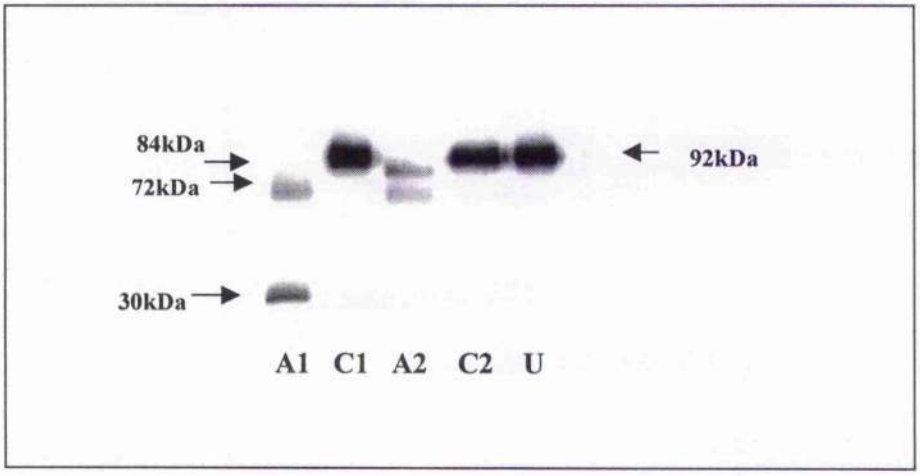
Since the molecular weight of the MMP-9 protein could not be determined from these blots it was impossible to determine whether the antibody detected activated MMP-9 in the middle spots or whether it simply detected the remaining unactivated form. It was clearly necessary to carry out a full western blot to determine the molecular weight of each of the activated, non-activated and control forms of MMP-9.

3.1.7 Western blot of AMPA and trypsin activated MMP-9 and pro-MMP-9 under non-reducing conditions

Western blotting was performed in order to determine the molecular weights of the MMP-9 activated with trypsin and its control, and the MMP-9 activated with APMA, and its control. It was hoped this would determine which forms of MMP-9 the antibody detected under non-reducing conditions.

Since under reducing conditions no activated MMP-9 was detected, a western blot was run under non-reducing conditions with APMA activated MMP-9 (A1) and control MMP-9 (C1) and trypsin activated MMP-9 (A2) and control MMP-9 (C2) (figure 19). 200ng of each was loaded. This western blot was carried out exactly as described previously with the exception that the loading buffer was non-reducing (section 2.1.4).

Figure 19 Western blot of AMPA and trypsin activated purified MMP-9 and pro-MMP-9 protein under non-reducing conditions



A1: APMA activated MMP-9

C1: Control for APMA activated MMP-9

A2: Trypsin activated MMP-9

C2: Control for trypsin activated MMP-9

U: Pro-MMP-9

The trypsin activation produced an 84kDa activated form of MMP-9 and a 72kDa form whereas APMA activation released 72 and 30kDa forms. Neither of the controls were activated under these conditions. Since the 84kDa form is the predominant active species *in vivo* trypsin activation was used from this stage onwards. These results have clearly shown that the Biogenesis antibody detects both active and pro forms of MMP-9 under non-reducing conditions. This is important not only for Western Blotting but also for immunohistochemistry, which is carried out under non-reducing conditions.

The next step was to revisit the experiments performed in section 3.1.1 but this time using non-reducing conditions.

3.1.8 Detection of MMP-9 in term placental homogenates by non-reducing western blotting

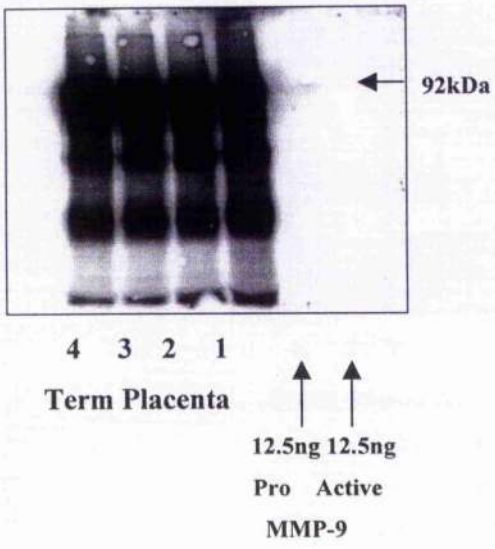
As the western blots carried out on placental homogenates prior to this stage had all been carried out under reducing conditions, it was decided to investigate if detection of MMP-9 would be improved if the gel was run under non-reducing conditions.
A block of frozen term placenta was homogenised (section 2.7) using various conditions. The conditions used were:

1) Homogenisation buffer containing 5mM PMSF and 12.5 µl/ml Sigma protease cocktail inhibitor.
2) Homogenisation buffer containing 12.5 µl/ml Sigma protease cocktail inhibitor.
3) Homogenisation buffer containing 5mM PMSF.
4) Homogenisation buffer containing neither inhibitor.

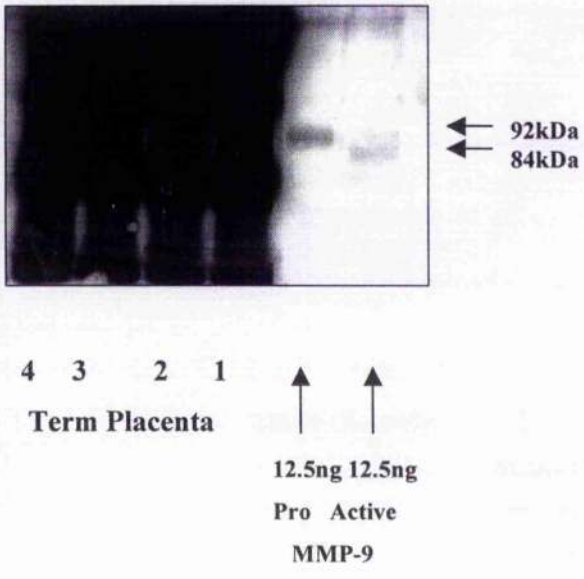
The cytosolic fraction (75µg) was loaded from each of these different experimental conditions and a western blot carried out with the MMP-9 Ab (figure 20).

Figure 20 **Detection of MMP-9 in term placental homogenates by non-reducing western blotting**

a) **5 min exposure**



b) **30 min exposure**



When the blot was exposed to film for 5 min, a band was present in the placental homogenates (4,3,2,1) at 92kDa. However there were also several other bands present making it difficult to identify pro and active MMP-9 separately. It was not possible to detect the control pro and activated MMP-9 until the blot had been exposed to the film for 30 min. By this stage the bands present in the homogenate were totally obscured by dark background.

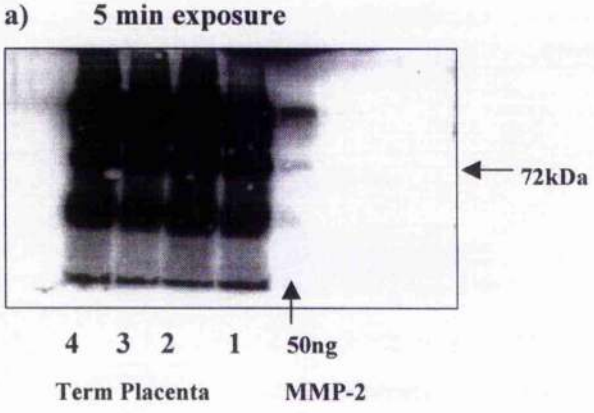
It was decided to attempt non-reducing western blotting on placental homogenates with the MMP-2 antibody to determine if the same difficulties would be encountered.

3.1.9 Detection of MMP-2 in term placental homogenates by non-reducing western blotting

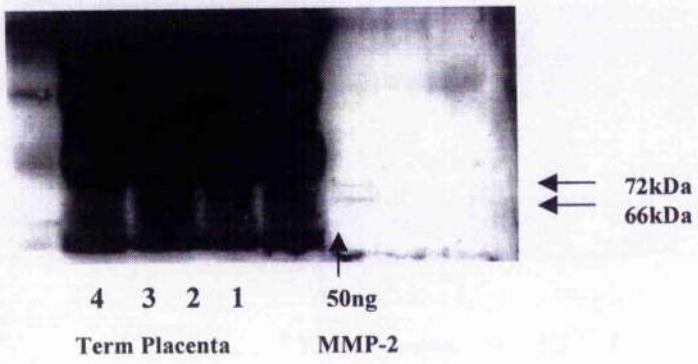
A block of frozen term placenta was homogenised using various conditions. The conditions used were as described above (section 3.1.8) The cytosolic fraction (75μg) was loaded from each of these different experimental conditions and a western blot carried out with the MMP-2 Ab (figure 21).

Figure 21 Detection of MMP-2 in term placental homogenates by non-reducing western blotting

a) 5 min exposure



b) 30 min exposure



Similar difficulties were encountered with western blotting of placental homogenates using MMP-2 antibody as had been found with MMP-9. While there appeared to be a band present at the correct molecular weight (72kDa) after a 5 min exposure, other bands were also present. Further inspection revealed that the bands detected by the MMP-2 antibody were very similar to the bands detected by the MMP-9 antibody, suggesting they may be non-specific. Moreover, a 30 min exposure was required to detect 50ng of control pro and active MMP-2. This resulted in extremely dark bands in the homogenates.

Thus it seemed that western blotting of homogenates would not be straightforward and it was decided to determine whether zymography would provide a more accurate method of detecting MMP-9 and MMP-2 in placental samples.

3.2 Zymography

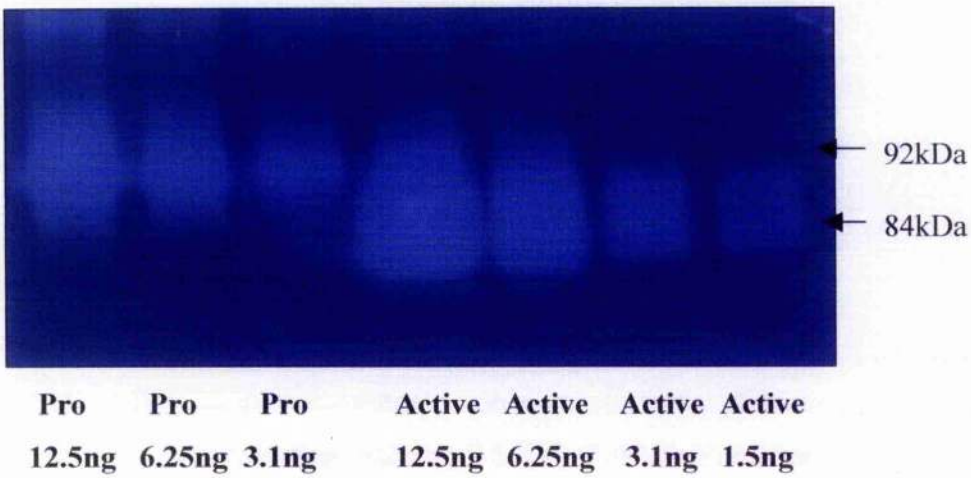
Western blotting did detect both pro and active forms of purified MMP-9 however the presence of several other bands detected in placental homogenates raised doubt as to whether the bands at 92 and 84kDa were definitely MMP-9. Zymography offers an alternative method to detect MMP-9 (section 1.4.8). Not only does it detect both active and pro forms but it also reflects activity. Thus it was decided that zymography may provide an alternative to western blotting for detecting MMP-9 activity. It also has the advantage of detecting MMP-2 active and pro forms in the same gel as MMP-9 since both degrade the substrate gelatin (section 1.4.5).

3.2.1 Detection of pro and active MMP-9 by zymography

Previous published studies have all been performed using non-reducing conditions. Thus all zymography experiments herein were performed under non-reducing conditions. To first of all establish the technique was working adequately the zymography method (section 2.9) was tested using commercially available MMP-9 protein (section 2.3).

MMP-9 protein was mixed with non-reducing loading buffer and separated on an SDS-PAGE gel impregnated with 0.05% gelatin. Pro-MMP-9 was loaded at concentrations of 12.5, 6.25 and 3.125ng. Activated MMP-9 (section 2.3) was loaded at concentrations of 12.5, 6.25, 3.125 and 1.5ng (figure 22).

Figure 22 Detection of purified pro and active MMP-9 protein by zymography

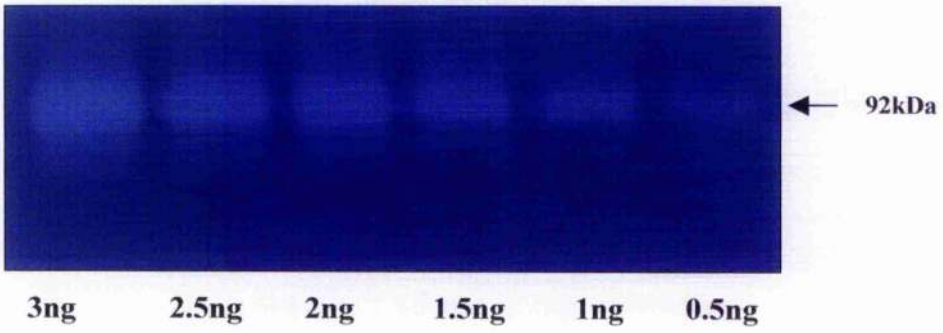


Pro MMP-9 was still clearly detectable at 3.1ng and active MMP-9 at 1.5ng. This was much more sensitive than western blotting and it was concluded that this would provide a more sensitive method for detecting MMP-9 in placental homogenates.

3.2.2 Detection of 0.5ng–3ng purified MMP-9 protein by zymography

Since 3.1ng of purified MMP-9 was easily detectable by zymography, a 0.05% gelatin impregnated gel was loaded with 3, 2.5, 2, 1.5, 1 and 0.5ng of pro-MMP-9 to determine whether smaller amounts of MMP activity were detectable and if so, was the method sensitive enough to detect small changes in activity (figure 23).

Figure 23 **Detection of 0.5ng–3ng purified pro-MMP-9 protein by zymography**



0.5ng of pro-MMP-9 was still clearly detectable as a single band at 92kDa and there was a definite change in band intensity as concentration decreased at least at double dilutions.

3.2.3 Detection of pro and active MMP-2 by zymography

Since the attempt at detecting MMP-9 by zymography had been successful, it was decided to attempt to detect purified MMP-2 protein by the same method. Since, unlike the MMP-9, the purified commercial MMP-2 contained both pro and active forms (section 3.1), there was no need to activate MMP-2 further. 12.5ng, 6.25ng and 3.1ng of purified MMP-2 protein were mixed with non-reducing loading buffer and separated on an SDS-PAGE gel impregnated with 0.05% gelatin (figure 24).

Figure 24 **Detection of purified pro and active MMP-2 protein by zymography**

As with MMP-9, zymography was also much more sensitive in detecting MMP-2 than western blotting. The technique clearly differentiated between the activated and pro forms.

3.2.4 Effect of protease inhibitors used in placental homogenisation on detection of MMPs by zymography

Since purified MMP-2 and MMP-9 were clearly detected by zymography, the next stage was to determine whether MMP activity could be as easily detected in placental homogenates.

It is known that certain protease inhibitors used in homogenisation affect MMP activity, therefore it was decided to homogenise a term placenta with different combinations of protease inhibitors.

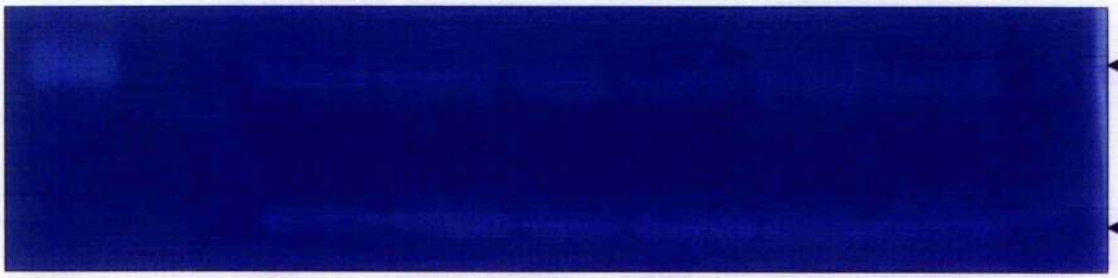
A block of frozen term placenta was homogenized under 4 different conditions:

1) Homogenisation buffer containing 5mM PMSF and 12.5 µl/ml Sigma protease cocktail inhibitor.
2) Homogenisation buffer containing 12.5 µl/ml Sigma protease cocktail inhibitor.
3) Homogenisation buffer containing 5mM PMSF.
4) Homogenisation buffer containing neither inhibitor.

The homogenisation was carried out as described (section 2.7). EDTA was omitted from the homogenisation buffer due to its inhibitory effect on MMPs.

75µg total and cytosolic protein from conditions 1-4 were separated by SDS-PAGE on a gel impregnated with 0.05% gelatin. Both the cytosolic and total protein fractions were separated to determine if all the MMP activity was present in the cytosolic fraction as expected. 1.5ng of purified pro-MMP-9 protein was loaded as a control (figure 25).

Figure 25 Effect of protease inhibitors used in placental homogenisation on detection of MMP-9 and MMP-2 by zymography



T = Total Protein (Membrane and Cytosolic)

C = Cytosolic Fraction

Both MMP-9 and MMP-2 were detectable in all of the homogenates. With both MMPs only the higher molecular weight pro forms were detectable. There did not appear to be much difference between the various combinations of protease inhibitors added, although the homogenate that had no protease inhibitors added did seem to have less MMP activity and the homogenate with both inhibitors appeared to have slightly stronger bands. Thus homogenisation buffer containing 5mM PMSF and 12.5 μl/ml protease cocktail inhibitor was used in all homogenisations thereafter. As expected, since the protein is found in the cytosolic fraction, the amounts detected in the total and cytosolic fractions appeared to be the same.

3.2.5 Is detection of MMPs by zymography as sensitive on large SDS-PAGE gels as it is on mini-gels?

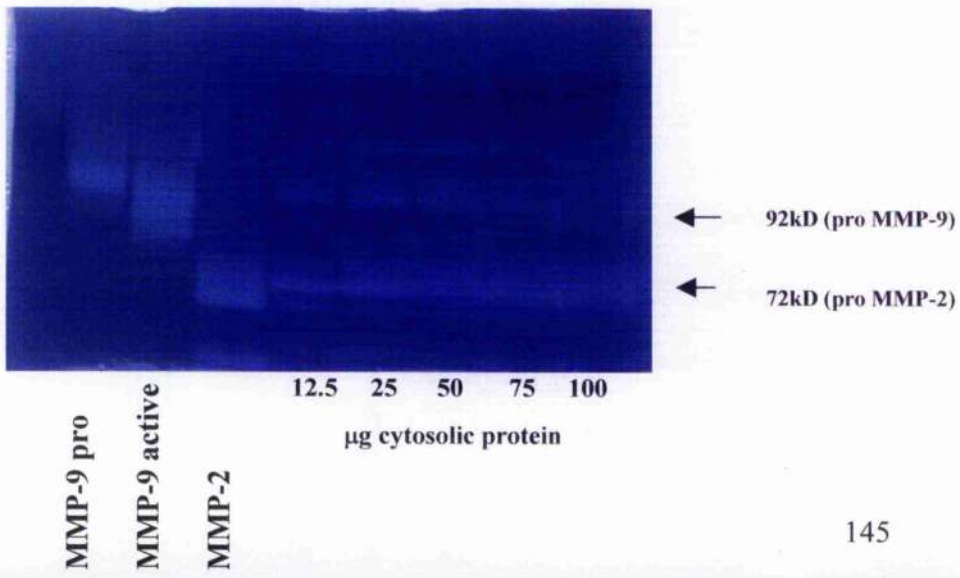
All experiments to date had been performed on mini gels. Due to the large number of samples to be analyzed zymography was next attempted on large gels. However several problems were encountered. Firstly the bands were not as clear, this is probably because of the large well size. The concentration of the sample in each well could be increased but as the samples were small there may not be enough of each to

allow this. Although the band separation is clearer and it would therefore be easier to differentiate between pro and active forms, the sharpness of the bands on the mini-gel seemed more suitable for analysis by densitometry. The large gels were therefore not further investigated.

3.2.6 Does reducing the gelatin concentration increase detection of MMP-9 and MMP-2 in placental homogenates?

While the zymography was clearly effective at detecting pro- MMP-9 and –2 in homogenates it was not clear whether there was little or no active forms in the homogenates, or whether the method was not sensitive enough to detect them. Since published studies do not show gels containing less than 0.05% gelatin, it was decided to determine if a 0.025% gelatin impregnated gel would be more sensitive for detection of active MMP-9 and MMP-2 in placental homogenates. An 11 weeks of gestation frozen placental block was homogenised, the cytosolic protein concentration was determined by Bradford's assay (section 2.10) and increasing concentrations were run on a 0.025% gelatin zymography gel. Each was loaded with 2ng pro and active MMP-9, 10ng MMP-2 and 12.5, 25, 50, 75, and 100 µg cytosolic protein of the 11 weeks of gestation placental homogenate (figure 26).

Figure 26 **A reduced gelatin concentration (0.025%) for detection of MMP-9 and MMP-2 in placental homogenates is not as effective as 0.05% gelatin**



It was clear that an increase in protein loaded resulted in increased gelatinase activity until around 50 μg when it appeared to plateau. MMP-9 seemed to be present almost exclusively in the pro form. In contrast, while the pro form of MMP-2 predominated, there appeared to be some evidence of active MMP-2. Densitometrical analysis of the bands showed that they were too diffuse to be quantified accurately by densitometry. To improve this there were two possible options. The concentration of gelatin could be increased or the amount of protein loaded could be decreased to give sharper bands. This was attempted in the next section.

3.2.7 Homogenate concentration optimisation

This experiment was designed to expand the range of concentrations of homogenates loaded onto both 0.025% and 0.05% gelatin gels. Two gels were prepared: a 0.05% gelatin gel loaded with 6.25, 12.5, 25, 50, 75, and 90μg cytosolic protein, and a 0.025% gelatin gel loaded with 2.5, 7.5, 10, 15, and 20μg cytosolic protein.

The bands on the 0.025% gel still looked diffuse. It was difficult to distinguish a reduction in band intensity with reduced protein loading (figure 27a). However on the 0.05% gelatin gel, the lower bands were much sharper and were able to be quantified by densitometry (figure 27b). Quantification of bands by densitometry on the 0.05% gelatin gel revealed that the bands increased semi-linearly with increased protein concentration (figure 28).

Figure 27 Sample concentration optimisation for zymography

a) 0.025% Gelatin

20 15 10 7.5 5 2.5

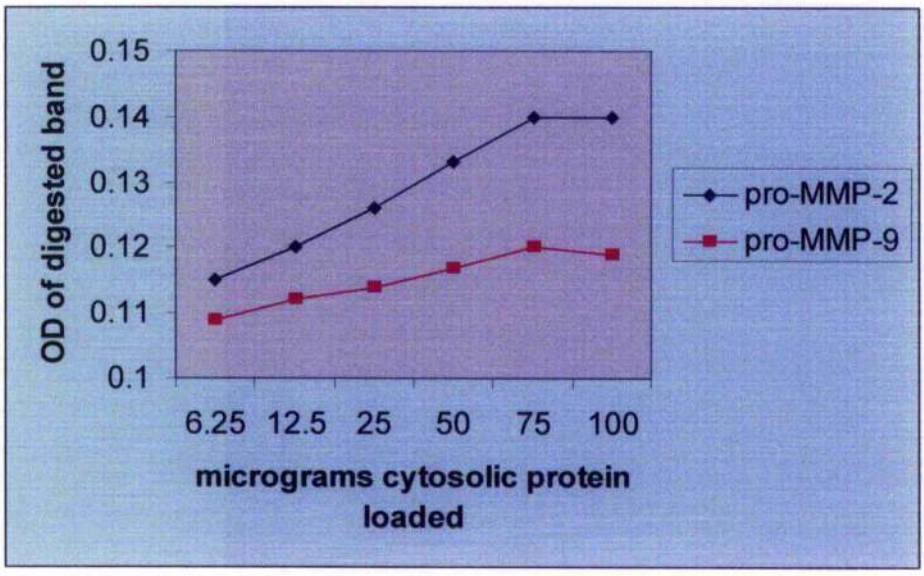
µg cytosolic protein

b) 0.05% Gelatin

100 75 50 25 12.5 6.25

µg cytosolic protein

Figure 28 Sample concentration optimisation - densitometry



3.2.8 Trial zymography of placental homogenates

Before homogenising the whole collection of placentae to be investigated (> 40 cases), a pilot study was carried out on one placenta from each week of gestation (7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 weeks and term) to check that the gel would be suitable for placentae from all gestations to be investigated. A 0.05% gelatin gel was loaded with 6.5µg of protein from the cytosolic fraction of each placental homogenate.

In addition it was decided to test a concentration of gelatin higher than 0.05% to be absolutely sure that 0.05% was the optimum concentration.

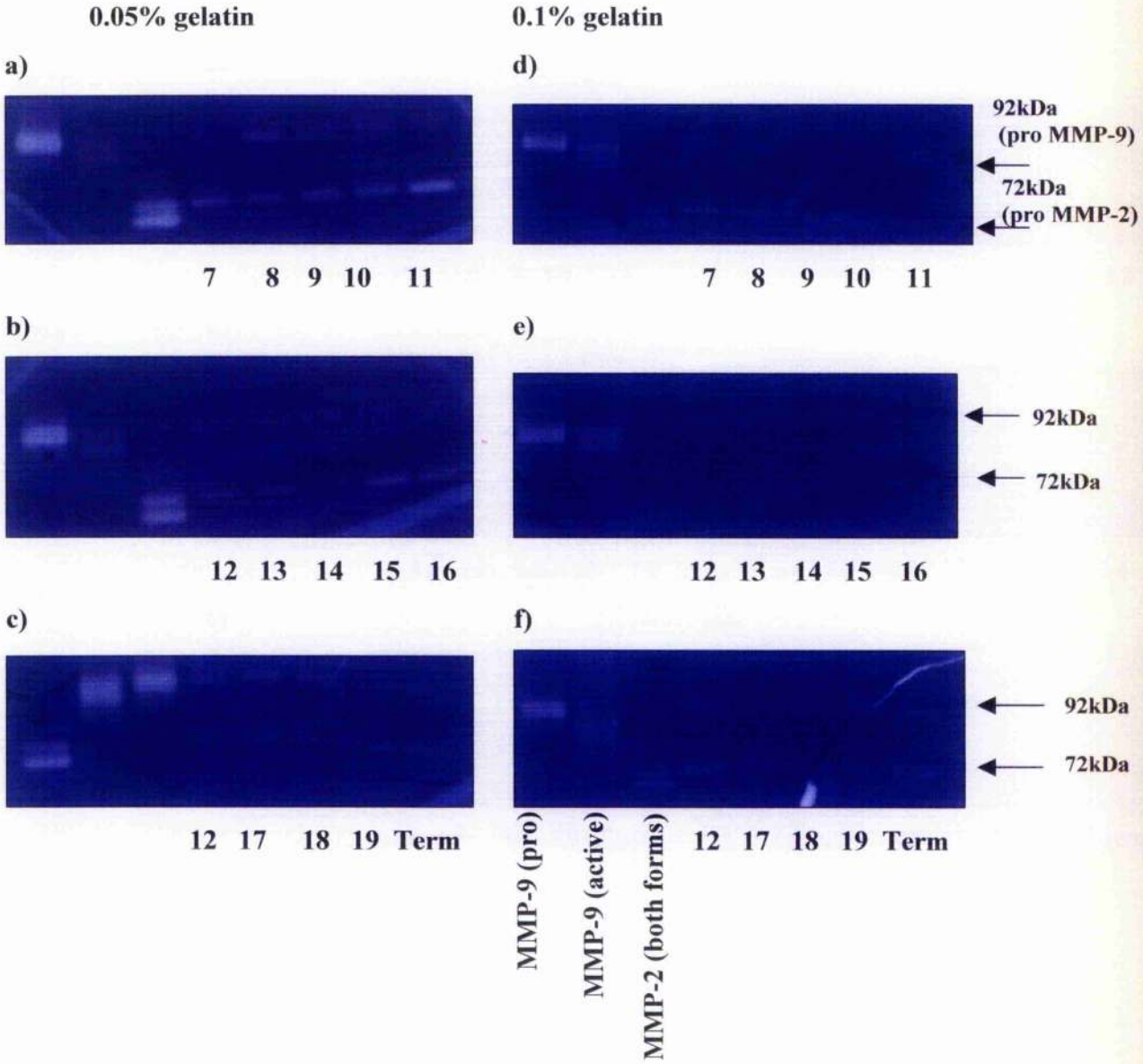
1) **0.05% gelatin / 6.5µg protein loaded**

7,8,9,10,11 weeks of gestation	(Fig 29a)
12,13,14,15,16 weeks of gestation	(Fig 29b)
112,17,18,19 weeks of gestation and term placenta	(Fig 29c)

2) **0.1% gelatin / 6.5µg protein loaded**

7,8,9,10,11 weeks of gestation	(Fig 29d)
12,13,14,15,16 weeks of gestation	(Fig 29e)
112,17,18,19 weeks of gestation and term placenta	(Fig 29f)

Figure 29 **Trial zymography of placental homogenates: 0.05% vs 0.1% gelatin**



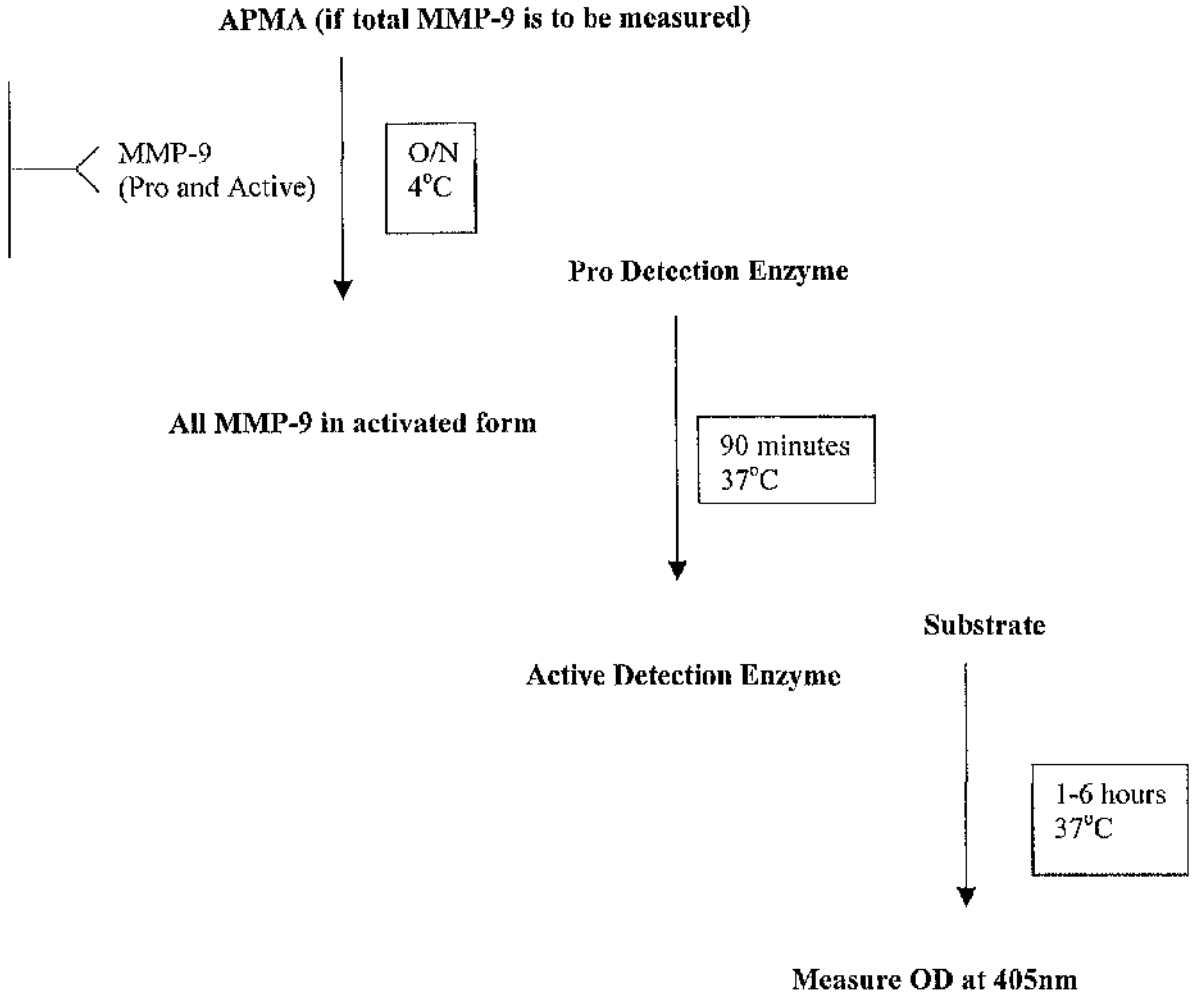
While both pro-MMP-9 and pro-MMP–2 were detectable at all gestations on both 0.05% and 0.1% gelatin gel, the 0.1% gel gave much poorer results. It was concluded that using 6.5μg cytosolic protein on a 0.05% gelatin zymography gel would give good quantifiable results and this combination was subsequently used in analysing placental samples throughout gestation (chapter 4).

3.3 Trial of a commercially available MMP-9 assay for detecting pro and active MMP-9 in placental homogenates

Zymography was successful in detecting pro-MMP-2 and pro-MMP-9 in placental homogenates. However it was not sensitive enough to be used to detect and quantify active MMP-2 or MMP-9. While zymography is a very cost effective method it has the disadvantage of being only semi-quantitative for measuring MMP activity. Thus it was decided to investigate if the commercially available MMP-9 assay from Amersham would be a viable method for more accurately quantifying MMP-9 activity in placental homogenates, and for detecting both pro and active forms of MMP-9. The zymography and assays of placental homogenates could be used in parallel to give a more comprehensive analysis of MMP-9 levels in placentae throughout gestation. An MMP-2 assay is also available but due to cost considerations, only the MMP-9 assay was investigated at this stage.

In the MMP-9 activity assay, both pro and active MMP-9 are captured by a specific antibody. Active MMP-9 is measured directly after addition of modified urokinase (UKCOL) and peptide substrate. Pro-MMP-9 is activated by APMA in an overnight incubation before activity can be measured. Each sample is assayed twice, activated and unactivated. Thus both total and active MMP-9 levels are obtained in the one assay. Specificity for pro-MMP-9 is reported to be 100% and specificity for active MMP-9 is reported to be around 65%. Complexes of pro and active MMP-9 with both TIMP-1 and TIMP-2 are known to have a degree of cross-reactivity in the assay (between 20 and 38%). Figure 30 shows a flow diagram of the assay protocol.

Figure 30 MMP-9 assay flow chart



The assay had been commercially optimised for serum and plasma samples and in spiked samples gave recovery rates of above 80%. The assay had not, however been optimised for tissue homogenates, although it was stated these could be successfully assayed. No guidance on dilutions of homogenates or on expected accuracy was given so it was decided that it was necessary to investigate these aspects. Thus placental homogenates prepared prior to this stage (section 2.7) were used to test the efficiency of the assay at detecting MMP-9 in homogenates themselves.

3.3.1 Results for measuring MMP-9 in placental homogenates using the commercial MMP-9 assay

To determine if the assay would detect MMP-9 in placental homogenates from throughout gestation, a 1st (10 weeks of gestation), 2nd (19 weeks of gestation) and 3rd (38 weeks of gestation) trimester placental homogenates were assayed. The cytosolic fraction from each of the samples was diluted in assay buffer to give a final protein concentration of 2.5mg/ml. Each sample was assayed in the activated and non-activated form to measure active and total MMP-9 and each of these was assayed in duplicate. In addition each activated homogenate was duplicated with the addition of a 5ng/ml active MMP-9 spike to determine how much of the spike would be detected. The standard curve was prepared using concentrations of 0.5, 1, 2, 4, 8 and 16ng/ml MMP-9 as described in the manufacturer's instructions.

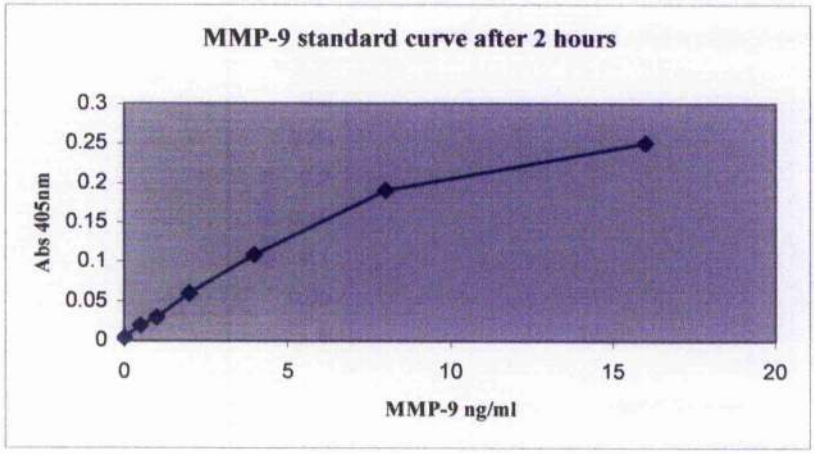
The assay was carried out as described in section 2.10.2. The manufacturers recommended reading the plate at 2 hours. The results shown in figure 31a show that the standard curve (0-16ng/ml) obtained at this time shows linearity for 0-8ng/ml. All the homogenate dilutions fell within this range. From the standard curve shown in figure 31a the concentration of MMP-9 in each of the homogenates was calculated. Standard curves after this 2-hour time-point did not show the same degree of linearity (figure 31b). The results for the homogenates are shown in table 6.

When active and total MMP-9 levels in the samples were compared, this initial experiment suggested that there was more total than active in all three gestations studied which is as would be expected. While these results were based on

only one sample from each gestation, it seemed that at 10 weeks of gestation total MMP-9 levels were 3x higher than active, at 19 weeks of gestation total levels of MMP-9 were only slightly higher than active and at 38 weeks of gestation total MMP-9 levels were 12x higher than active. Active MMP-9 levels were fairly constant throughout. Only between 47% and 77% of the spiked MMP-9 was detected in the wells. It was possible that MMP-9 was bound by proteins in the homogenate, possibly TIMPs, preventing some of the MMP-9 from being captured in the assay, since TIMP/MMP-9 complexes only show partial cross-reactivity with the assay. When the homogenate values were corrected for the spike the active MMP-9 levels were lowest at ten weeks of gestation, and the levels at 19 weeks of gestation were almost double that found at ten weeks of gestation. The levels of active MMP-9 at term were intermediate. Total MMP-9 levels at 10 and 19 weeks of gestation were comparable to each other while total levels at term were around 10x higher than during the first and second trimesters.

Figure 31 Standard curves for measuring MMP-9 in placental homogenates

a)



b)

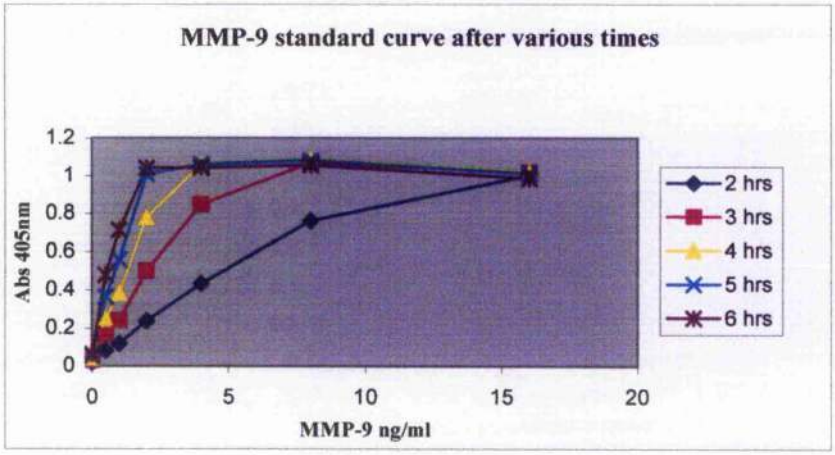


Table 6 **MMP-9 assay of placental homogenates from 1st, 2nd and 3rd trimester**

Homogenate	Active MMP-9 in homogenate ng/ml	Total MMP-9 in homogenate ng/ml	% Spike detected in duplicate well	Active MMP-9 in homogenate ng/ml (corrected for spike)	Total MMP-9 in homogenate ng/ml (corrected for spike)
10wks	2.03	6.08	77	2.63	7.90
19wks	2.17	3.16	47	4.62	9.83
38wks	2.04	24.96	59	3.46	42.31

Since the active MMP-9 levels lay at the lower end of the standard curve, it was necessary to perform the assay again, this time using the lower range standard curve protocol described in the manufacturer's instructions to ensure the accuracy of the detected active MMP-9. No further dilutions of the homogenates were performed at this stage since the active MMP-9 levels were fairly low already.

3.3.2 MMP-9 assay of placental homogenates from 1st, 2nd and 3rd trimester: lower range standard curve

The assay was repeated with 3 different homogenates from 1st (9 weeks of gestation), 2nd (17 weeks of gestation) and 3rd (36 weeks of gestation) trimester placental homogenates. The procedure was carried out exactly as before with the exception that the wells used to construct the standard curve contained 0, 0.125, 0.25, 0.5, 1, 2 and 4ng of MMP-9 as opposed to the higher amounts used in the previous assay. This was to ensure that the range of the standard curve was appropriate to the amount of active MMP-9 likely to be present in the samples. Each of the samples was diluted in assay buffer to give a final protein concentration of 2.5mg/ml. The standard curve obtained after 5 hours was the best fitting curve (figure 32) so this was used in calculating values of MMP-9 from the unknown samples (table 7).

As in the assay carried out in 3.3.1, there wasn't a large difference in the amount of active MMP-9 present in the samples of different trimesters, however there was a clear difference in the total MMP-9 detected in these homogenates. Much higher levels of pro-MMP-9 than active MMP-9 were detected, which agreed with the results from the preliminary zymographical analysis. Total MMP-9 levels in the 9 and 36 weeks of gestation homogenate were above the standard curve. The 17 weeks of gestation homogenate had the lowest levels of total MMP-9 as in the previous assay before correction with the spike.

To rectify the difficulty of detecting both the low levels of active MMP-9 and the high levels of pro-MMP-9 it would be necessary to construct a larger standard curve than the ones recommended in the assay protocol.

Figure 32 **Standard curve: lower range assay for measuring MMP-9 in placental homogenates**

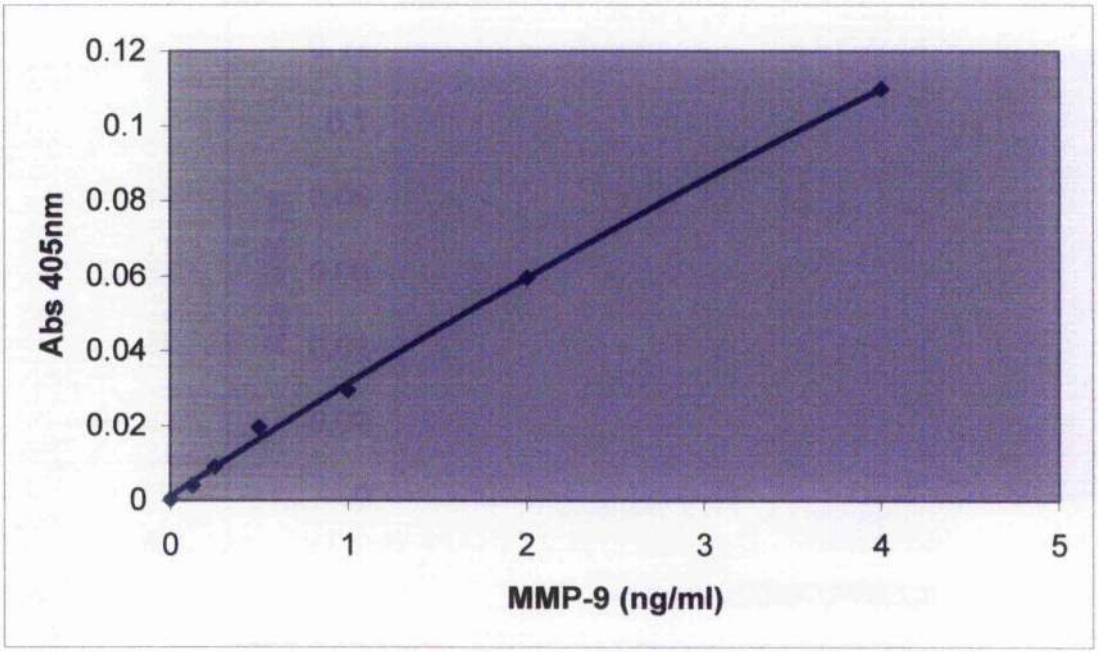


Table 7 **MMP-9 assay of placental homogenates from 1st, 2nd and 3rd trimester: lower range standard curve**

Sample	Active (ng/ml)	Total (ng/ml)
9wks	0.52	Above Std Curve
17wks	0.44	4.4
36wks	0.4	Above Std Curve

3.3.3 Recovery of a 5ng/ml MMP-9 spike at various homogenate concentrations and at various BSA concentrations

Since the experiments in 3.3.1 suggested that detection of an MMP-9 spike was not satisfactory, the effect of increasing protein concentration on MMP-9 detection in the assay was investigated. Various concentrations (0 mg/ml, 1mg/ml, 2mg/ml and 4 mg/ml) of BSA were added to wells containing 5ng/ml MMP-9 in PBS to determine the effect of increasing non-specific protein concentration on MMP-9 detection. Various concentrations of a term placental homogenate (1.2mg/ml, 2.5mg/ml and 5mg/ml) were also assayed with a 5ng/ml spike to determine if altering the dilution of the homogenate would affect the detection of the MMP-9 spike. A larger standard curve than had been used before was constructed over the range 0.1-16ng/ml from the standards provided in the kit as this would be necessary if both active and total MMP-9 levels are to be measured in the same assay. From the standard curve plotted after 2 hours of incubation (figure 33) the recovery of a 5ng/ml spike at various BSA concentrations (table 8) and at various homogenate concentrations (table 9) was calculated.

Increasing the concentration of BSA in the sample from 0-4mg/ml protein had virtually no effect on the recovery of the 5ng/ml spike (table 8) which was constantly over 100%.

Detection of the MMP-9 spike in placental homogenates (table 9) was greater at lower protein concentrations. However no more than 56% of the spike was recovered in any sample in this assay and detection varied between 31 and 56 %. The main concern therefore was that similar percentages of MMP-9 in the sample itself may be detected. The manufacturers of the assay (Amersham) were queried about this situation. Their reply was that this was probably as accurate as could be expected but that comparisons could still reliably be made between sample groups. It would not be desirable to dilute the homogenates much more than was done in this assay due to the small quantities of active MMP-9 which are present.

Figure 33 **Standard curve for determining recovery of a 5ng/ml MMP-9 spike at various homogenate concentrations and at various BSA concentrations**

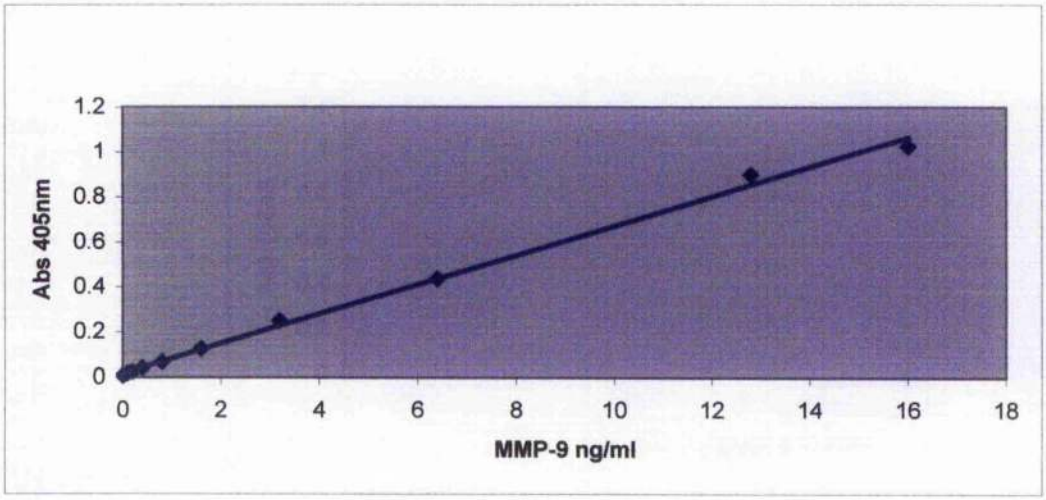


Table 8 **Recovery of a 5ng/ml Activated MMP-9 spike at various BSA concentrations**

BSA mg/ml	% Spike Recovery
0	116.1466
1	115.3092
2	115.5484
4	112.9163

Table 9 **Recovery of a 5ng/ml MMP-9 spike at various homogenate concentrations**

Homogenate Concentration	% Spike Recovery
5mg/ml	31.29786
2.5mg/ml	41.73047
1.2mg/ml	56.27871

From these experiments it was concluded that while the assay does detect both active and pro- MMP-9 in placental homogenates from 1st 2nd and 3rd trimester, detection of spiked MMP-9 protein was not entirely satisfactory, nor consistent. This may be due to binding of MMP-9 by TIMPs present at varying amounts in different homogenates. Due to time constraints it was therefore decided not to undertake further investigation of the assay at the current time.

3.4 Summary and discussion

Evaluation of various methods for detecting and quantifying MMP-9 and MMP-2 in placental homogenates

The aim of this section of the study was to determine which methods would be most appropriate for determining MMP expression levels in placental homogenates. Western blotting was initially evaluated as it was a technique already in common use in the laboratory. In addition it was also hoped to establish the detection potential of the MMP-9 and MMP-2 antibodies that were later to be used in immunohistochemistry using western blotting. Several studies have attempted semi-quantitative analysis of MMP expression in medium from cultured trophoblast cells using zymography or activity assays. However no studies have examined MMP expression/activity in placental homogenates using these techniques and hence each of these were investigated to determine their suitablity. Important considerations in evaluating the methods were cost, time, ease of method, repeatability, and the potential for quantitative analysis.

3.4.1 Western Blotting

Initially the experiments aimed to validate two commercially available antibodies for detecting MMP-9 and one for detecting MMP-2. There are numerous other MMP-9 and MMP-2 antibodies on the market the majority of which detect both pro and active MMP-2 and MMP-9. The datasheets from the particular antibodies evaluated gave no indication which forms would be detected. Although western blotting is often carried out under reducing conditions the detection properties of some antibodies can be affected by whether or not the conditions are reducing. Initially the gels were run

under standard reducing conditions since no indication was given to the contrary on the antibody datasheets.

3.4.1.1 Detection of MMPs in placental homogenates under reducing conditions

The first attempt to detect either MMP in 50µg of total protein placental homogenates from a term and an early placenta using the Biogenesis MMP-9 and MMP-2 antibodies or the Chemicon MMP-9 antibody was unsuccessful and only non-specific bands were present. An attempt was then made to detect pro-MMP-9 and pro-MMP-2 purified proteins 'spotted' on nitrocellulose. This experiment showed that the Biogenesis MMP-9 antibody, which clearly detected 6.5ng of MMP-9 protein, was more sensitive than the Chemicon MMP-9 antibody. MMP-2 detection was not as sensitive and 50ng of purified protein was the smallest quantity that could readily be detected. It was decided to concentrate on the Biogenesis MMP-9 and MMP-2 antibodies from this stage.

3.4.1.2 Detection of purified MMPs under reducing conditions

When the purified proteins were then run alongside homogenates on full western blots the MMP-9 antibody detected one clear band at 92kDa in the lanes where the purified protein had been loaded. There were some bands present in the homogenate lanes at around 92kDa although there were also numerous unspecific bands at other molecular weights. The MMP-2 antibody detected two bands in the purified protein lanes at 72kDa and 68kDa, which was surprising as the protein was described as being pro-MMP-2 (72kDa). The manufacturers proposed that this extra band was probably the result of autocatalytic cleavage of the pro form of MMP-2. The protein was stored at -70C in accordance with the manufacturers instructions however it was possible that autocatalysis may still occur at a slow rate. It was clear from these experiments however that the MMP-2 antibody detected both pro and active MMP-2 under non-reducing conditions.

3.4.1.3 Activation of purified MMP-9 and determination of antibody specificity

Although the MMP-2 antibody detected both the pro and active forms of MMP-2, it was uncertain however whether the MMP-9 antibody detected both pro and active forms of MMP-9. Since both forms are likely to be present in placental tissue, it was important to determine which forms the antibody detected. An initial attempt to activate MMP-9 was made using APMA as described on the manufacturers data sheet and western blotting was carried out under reducing conditions. No protein was detected in the lane where the activated MMP-9 was run. Since APMA activation is fairly harsh there was the possibility that all of the protein had been degraded. Alternatively it was possible that the antibody did not detect active MMP-9 under reducing conditions. Activated, non-activated and control MMP-9 were then spotted onto nitrocellulose and detected under non-reducing conditions. The pro-form spot was considerably more intense than either the control or activated spot. Although this would seem to suggest that the active form is not as readily detected as the pro-form even under non-reducing conditions, it could also have been the case that the spot remaining in the activated lane was some remaining pro-form.

Trypsin is an *in vivo* activator of MMP-9 and is without the biological hazards associated with APMA. Therefore it was decided to activate MMP-9 protein with trypsin to determine whether or not the antibody could detect MMP-9 activated in this way. With spotting almost exactly the same results were obtained as with activation by APMA.

A full western blot was then carried out to determine the molecular weight of each of the activated, non-activated and control MMP-9 to determine which forms of MMP-9 the antibody was actually detecting in each. A western blot was run under non-reducing conditions with APMA activated MMP-9 and control MMP-9 and trypsin activated MMP-9 and control MMP-9. The trypsin activation produced an active form of MMP-9 at 84kDa and a 72kDa form whereas APMA activation released 72kDa and 30kDa forms, consistent with the concept that it is a harsh activator of MMPs. Neither of the controls were activated. Since the 84kDa form is the predominant active species *in vivo* trypsin activation was used from this stage onwards. The results clearly showed that the Biogenesis antibody detected both active and pro forms of MMP-9 under non-reducing conditions although it more readily

detected the pro-form. This is important not only for Western Blotting but also for immunohistochemistry, the conditions in which are non-reducing.

3.4.1.4 Detection of MMPs in placental homogenates under non-reducing conditions

Cytosolic fraction (75μg) from placenta homogenised using various different protease inhibitors were then run on a gel under non-reducing conditions and western blotting was carried out using firstly the MMP-9 antibody and secondly the MMP-2 antibody. The different protease inhibitors were tested to determine if either of the inhibitors regularly used in our laboratory had adverse effects on MMP detection. This is discussed further in the section on zymography. When the blot used to detect MMP-9 was exposed to film for 5 min, a band was present in the homogenates at 92kDa. However there were also several other bands present. It was not possible to detect the 12.5ng control pro and activated MMP-9 until the blot had been exposed to the film for 30 min. This is longer exposure time than had been previously required for this amount of protein. By this stage the bands present in the homogenate were totally obscured by dark background. It is possible that the non-reducing conditions increase the non-specific protein detection. If western blotting was to be used in detecting MMP-9 in homogenate samples, it seems likely two exposure times would be necessary even if the amount of control was increased. Similar difficulties were encountered with western blotting of placental homogenates using MMP-2 antibody. While there appeared to be a band present at 72kDa after a 5 min exposure, other bands were also present. Further inspection revealed that the bands detected by the MMP-2 antibody were very similar to the bands detected by the MMP-9 antibody, suggesting they may be non-specific. Moreover, a 30 min exposure was required to detect 50ng of control pro and activated MMP-2. This resulted in extremely dark bands in the homogenates.

3.4.1.5 Conclusions of western blotting trial

Since the detection of any MMP-9 and MMP-2 in placental homogenates was difficult it is likely that any quantitative analysis of the bands would be almost impossible, although the technique is very cost and time effective. Thus western blotting of

placental homogenates may not be straightforward and it was decided to determine whether zymography would provide a more accurate method of detecting MMP-9 and MMP-2 in placental samples. It is possible that other antibodies from other manufacturers may give better results. However, due to time considerations these were not evaluated. The validation of these antibodies for use in immunohistochemical studies was also satisfactorily achieved.

3.4.2 Gelatin Zymography

Gelatin zymography is an alternative method for detecting MMP-9 and MMP-2. Not only does it detect both active and non-active species but it also reflects activity and is more sensitive than western blotting. It also has the advantage of detecting MMP-2 in the same gel as MMP-9 since both degrade the same substrate gelatin.

3.4.2.1 Detection of purified MMP-9 and MMP-2 protein by zymography

Previous published studies have all been performed using non-reducing conditions. Thus all zymography experiments herein were performed under non-reducing conditions. To establish the technique was working adequately the zymography method was tested using commercially available MMP-9 protein on a gel containing 0.05% gelatin. This concentration of gelatin is lower than that used in many of the previously published studies which have used 0.3 or 0.1% gelatin and was chosen as a base point to determine sensitivity. Pro-MMP-9 was clearly detectable at 3.1ng and active MMP-9 at 1.5ng. A second gel showed that 0.5ng of MMP-9 was clearly detectable as a single band at 92kDa and there was a definite change in band intensity as concentration increased at least at double dilutions. The third experiment showed that zymography was also much more sensitive in detecting MMP-2 than western blotting. The technique detected 3.1ng of purified MMP-2 protein and clearly differentiated between the pro and activated forms.

3.4.2.2 Detection of MMP-9 and MMP-2 in placental homogenates by zymography

Since purified active and pro-MMP-2 and MMP-9 were clearly detected by zymography, the next stage was to determine whether MMP activity could be as

easily detected in placental homogenates. Placentas were homogenised using various combinations of protease inhibitors and 75μg of both the total protein and cytosolic fractions were run. Both MMP-9 and MMP-2 were detectable in all of the homogenates on a 0.05% gelatin gel. With both MMPs only the higher molecular weight pro-forms were detectable. There did not appear to be much difference between the various combinations of protease inhibitors added, although the homogenate that had no protease inhibitors added did seem to have less MMP activity and the homogenate with two inhibitors appeared to have slightly stronger bands and so both inhibitors were used in all the subsequent homogenisations. As expected, since MMPs are cytosolic proteins, the amounts detected in the total and cytosolic fractions were comparable. No EDTA was used in any homogenisation as it has an inhibitory effect on MMPs, an important fact to take into consideration when considering the results of some previously published studies.

Until this stage all experiments had been carried out on mini-gels. It was suggested that using large zymography gels as opposed to mini-gels would allow more protein to be added to each well and give clearer bands. However, the opposite occurred and while the bands obtained were larger they were also more diffuse and harder to quantify. This may have occurred for several reasons; e.g. diffusion of the protein over the longer running time required or an over abundance of MMP activity for the amount of gelatin present in the confined space hence mini gels were used in all experiments hereafter.

3.4.2.3 Attempt to detect active MMP-9 and MMP-2 in placental homogenates

While the zymography was clearly effective at detecting pro-MMP-9 and –2 in homogenates it was not clear whether there were little or no active forms in the homogenates, or whether the method was not sensitive enough to detect them. Most previous studies have used 0.1% or 0.3% gelatin (Fisher et al. 1989; Librach et al. 1991; Xu et al. 2000) and although lower than this concentration had been used for the initial study, it was decided to determine if a 0.025% gelatin impregnated gel would be more sensitive for detection of active MMP-9 and MMP-2 in placental homogenates. On a 0.025% gelatin impregnated gel an increase in protein loaded resulted in increased gelatinase activity until around 50μg. MMP-9 seemed to be

present almost exclusively in the pro-form. In contrast, while the pro-form of MMP-2 predominated, there was some active MMP-2 at the correct molecular weight. Densitometrical analysis of the bands showed that they were too diffuse to be quantified accurately by densitometry. When the amount of protein loaded was decreased the active bands on the 0.025% gel still looked diffuse and it was difficult to distinguish between different concentrations of protein. When the concentration of gelatin was increased to 0.05%, the lower concentration pro-bands were much sharper and were able to be quantified by densitometry. They also increased semi-linearly. However the active bands were undetectable. Hence it was decided to measure only the pro-form of the enzymes in the study.

3.4.2.4 Pilot study and conclusions from zymography trial

A pilot study was carried out on one placenta from each week of gestation to be investigated, to check that a 0.05% gelatin gel would be suitable. In addition, a concentration of gelatin higher than 0.05% was tested to be absolutely sure that 0.05% was the optimum concentration for detection of MMPs in placental homogenates. The gels were loaded with 6.5μg of protein from the cytosolic fraction of each placental homogenate. While both pro-MMP-9 and –2 were detectable at all gestations on both 0.05% and 0.1% gelatin gel, the 0.1% gel gave much poorer results. It was concluded that loading 6.5μg cytosolic protein on a 0.05% gelatin impregnated zymography gel would give quantifiable results using a time and cost effective method.

3.4.3 MMP-9 Activity Assay

An MMP-9 activity assay (Amersham) was also evaluated for detecting MMP-9 in placental homogenates. It had the advantage of being able to measure both active and pro-MMP-9 in the same assay and was very time effective. An MMP-2 assay was also available but due to cost considerations, only the MMP-9 assay was investigated. In the instruction manual specificity for pro-MMP-9 was reported to be 100% and specificity for active MMP-9 around 65%. Complexes of pro and active MMP-9 with both TIMP-1 and TIMP-2 were reported to have a degree of cross-reactivity in the assay (between 20 and 38%).

The assay had been commercially optimized for serum and plasma samples and in spiked samples gave recovery rates of above 80%. It had not, however, been optimized for tissue homogenates, although it was stated in the manual that these could be successfully assayed. No guidance on dilutions of homogenates or on expected accuracy was given.

3.4.3.1 Initial assay trial

To determine if the assay would detect MMP-9 in placental homogenates throughout gestation, 1st (10 weeks of gestation), 2nd (19 weeks of gestation) and 3rd (38 weeks of gestation) trimester placental homogenates were assayed. The homogenates were assayed at a concentration of 2.5mg/ml as this was the concentration of the most dilute sample after homogenisation. Each activated homogenate was duplicated with the addition of a 5ng/ml active MMP-9 spike to determine how much of the spike would be detected. Only between 47% and 77% of the spiked MMP-9 was detected. It is possible that MMP-9 was bound by proteins in the homogenate, possibly TIMPs, preventing some of the MMP-9 from being captured in the assay since TIMP/MMP-9 complexes only show partial cross-reactivity with the assay. These results of the initial experiment showed that there was more total than active MMP-9 at all three gestations studied. After each sample had been corrected for the spike, active MMP-9 levels were fairly constant in comparison to total MMP-9. At both 10 and 19 weeks of gestation total MMP-9 levels were around 2x higher than active, and at 38 weeks of gestation total MMP-9 levels were 10x higher than active. This was fairly surprising since while many studies have reported the importance of MMP-9 in the first trimester, few have implicated it at term (section 1.4.9).

3.4.3.2 Lower range standard curve

A second assay was carried out using a lower range standard curve, as the active MMP-9 in the homogenates had been near the lower end of the standard curve. Different homogenates (9 and 17 weeks of gestation and term) were used from those in the first assay. Pro-MMP-9 levels were above the standard curve in two out of three homogenates. As in the first assay there was not a large difference in the amount of active MMP-9 present in the samples of different trimesters. The total MMP-9 levels

in the 9 and 36 weeks of gestation homogenate were above the standard curve. The 17 weeks of gestation homogenate had the lowest levels of total MMP-9 as in the previous assay before (but not after) correction with the spike. No spike was included in this assay due to space constraints. To rectify the difficulty of detecting both the low levels of active MMP-9 and the high levels of pro-MMP-9 it would be necessary to construct a larger standard curve than the ones recommended in the assay protocol.

3.4.3.3 Determination of effect of increasing protein concentration on assay detection of MMP-9

To determine the effect of increasing protein concentration on MMP-9 detection in the assay, various concentrations (1mg/ml, 2mg/ml and 4 mg/ml) of BSA were added to wells containing a 5ng/ml MMP-9 in PBS. Various concentrations of a term placental homogenate (1.2mg/ml, 2.4mg/ml and 5mg/ml) were also assayed to determine if altering the dilution of the homogenate would affect the detection of the MMP-9 spike. Increasing the concentration of BSA from 0-4mg/ml only slightly decreased the recovery of the 5ng/ml spike implying that non-specific protein concentration at these dilutions does not affect MMP-9 detection in the assay. Detection of the MMP-9 spike in placental homogenates was better at lower protein concentrations. However no more than 56% of the spike was recovered in any sample and detection varied between 31 and 56 %. The main concern was that this may imply that similar percentages of MMP-9 in the sample itself are detected. The manufacturers were queried about this situation. Their reply was that this was probably as accurate as could be expected but that comparisons could still reliably be made between sample groups.

3.4.3.4 Conclusions from MMP-9 assay trial

The conclusion from these experiments were that while the assay did detect both active and pro-MMP-9 in placental homogenates from 1st 2nd and 3rd trimester, detection of spiked MMP-9 protein was not entirely satisfactory, nor consistent. This may be due to binding of MMP-9 by TIMPs present at varying amounts in different homogenates. It is possible that this could be controlled for by spiking the samples with purified MMP-9 protein, although this would be both time and resource consuming and not necessarily accurate. The assay is also very expensive compared

to other methods of MMP detection. It was therefore decided not to undertake further investigation of the assay at the current time.

3.4.4 Summary of conclusions on methodology for MMP detection and quantification

Zymography was the method that gave the most reliable semi-quantitative measurements of MMP-9 and MMP-2 activity in placental homogenates. Since the MMP-9 and MMP-2 antibodies had been validated using western blotting and since immunohistochemistry was already a well-established technique in our laboratory it was hoped that immunohistochemistry in conjunction with zymography would provide the most viable means of examining MMP-2 and MMP-9 distribution and activity in placentae throughout gestation.

Chapter 4: Detection and quantification of MMP-9 and MMP-2 throughout pregnancy using zymography and immunohistochemistry

Further to the trial of various methods (chapter 3) it was concluded that zymography was the optimal method for semi-quantitative analysis of MMP activity in placental homogenates. Immunohistochemistry was chosen as the preferred method for determining cellular location of MMP-2 and MMP-9 since the technique had been extensively used in the laboratory where the study was undertaken and to enable comparison with previous placental studies.

Hence zymography and immunohistochemistry were carried out to study the expression and activity of MMP-2 and MMP-9 in placental homogenates and placental sections from 7-19 weeks of gestation and from term placentae. The aims of this were several-fold:

- To perform semi-quantitative analysis of MMP-2 and MMP-9 activity in placentae throughout gestation using zymography
- To perform semi-quantitative analysis of MMP-2 and MMP-9 expression in placentae throughout gestation using immunohistochemistry
- To determine in which cells MMP-2 and MMP-9 are expressed in the placenta
- To compare the results of the semi-quantitative immunohistochemical analysis of MMP-2 and MMP-9 with the zymography results

There is currently no consensus on precisely when and where MMP-2 and MMP-9 are expressed in the placenta during pregnancy. Thus a further aim was to compare the data obtained with those studies previously published.

4.1 Zymography of placental homogenates from 7-19 weeks of gestation and from term placentae

Thirty-one placental blocks which had been collected immediately after delivery by ELCS in the 3rd trimester or TOP, and frozen at –70C, were homogenized as described in section 2.7. The gestations were as follows: 2x 7 weeks, 3x 8 weeks, 3x 9 weeks, 2x 10 weeks, 2x 11 weeks, 2x 12 weeks, 2x 13, weeks 2x 14, weeks 2x 15 weeks, 3x 16 weeks, 2x 17 weeks, 1x 18 weeks, 1x 19 weeks and 6x 40 weeks of gestation.

Cytosolic protein (6.5μg) from each homogenate was then separated on 0.05% gelatin zymography gels. Low range molecular weight markers were also run on each

gel. The gels were washed in zymography wash buffer (section 2.1.2) to remove SDS and incubated in zymography reaction buffer (section 2.1.2) overnight, then stained in Coomassie blue and destained as described in section 2.9.2

Each band of digested gelatin representing pro-MMP-9 (92kDa) or pro-MMP-2 (72kDa) was quantified by negative densitometry and the background of the gel was subtracted from each reading. Thereafter each reading was divided by an internal control sample which was run on each gel to account for any day-to–day variations which may occur. No MMP-9 or MMP-2 activated bands were intense enough to be quantified. The final data was analysed to determine if there were any variations in either pro-MMP-2 or pro-MMP-9 with gestation.

Figures 34 and 35 show the zymography gels for pro-MMP-2 (figure 34) and pro-MMP-9 (figure 35). For pro-MMP-2 a band at 72kDa was detectable in all the samples. For pro-MMP-9 a band at 92kDa was detectable in all the samples. Table 10 shows the raw data and the values for these zymograms once they had been divided by the internal control sample value. The columns on the left show the values for pro-MMP-2 and the columns on the right show the values for pro-MMP-9.

Next the data was grouped into trimesters and represented as scattergrams. Figure 36 shows the scattergram for pro-MMP-2 and figure 37 shows the scattergram for pro-MMP-9. The bars show the median values. Following this, statistical analysis was carried out to determine if there were any statistical differences in pro-MMP-2 or pro-MMP-9 activity between trimesters.

Table 11a shows the descriptive statistics for pro-MMP-2 and table 11b shows the results of the statistical analysis for pro-MMP-2. No assumptions were made as to the normality of the data and non-parametric testing was used throughout. A Kruskal-Wallis analysis showed a highly significant difference between the trimesters. There was significantly less pro-MMP-2 activity in the 2nd trimester compared with the 1st trimester ($p<0.01$). There was also significantly less pro-MMP-2 activity in the 3rd trimester compared with the 1st trimester ($p<0.01$). There was no significant difference in pro-MMP-2 activity between the 2nd and 3rd trimesters.

Table 12a shows the descriptive statistics for pro-MMP-9 and table 12b shows the results of the statistical analysis for pro-MMP-9. A Kruskal-Wallis analysis

showed no significant differences between the three trimesters ($p>0.05$). Mann-Whitney analysis also showed no significant differences ($p>0.05$) between any of the trimesters.

Figure 34 Pro-MMP-2 activity in placental homogenates - zymography

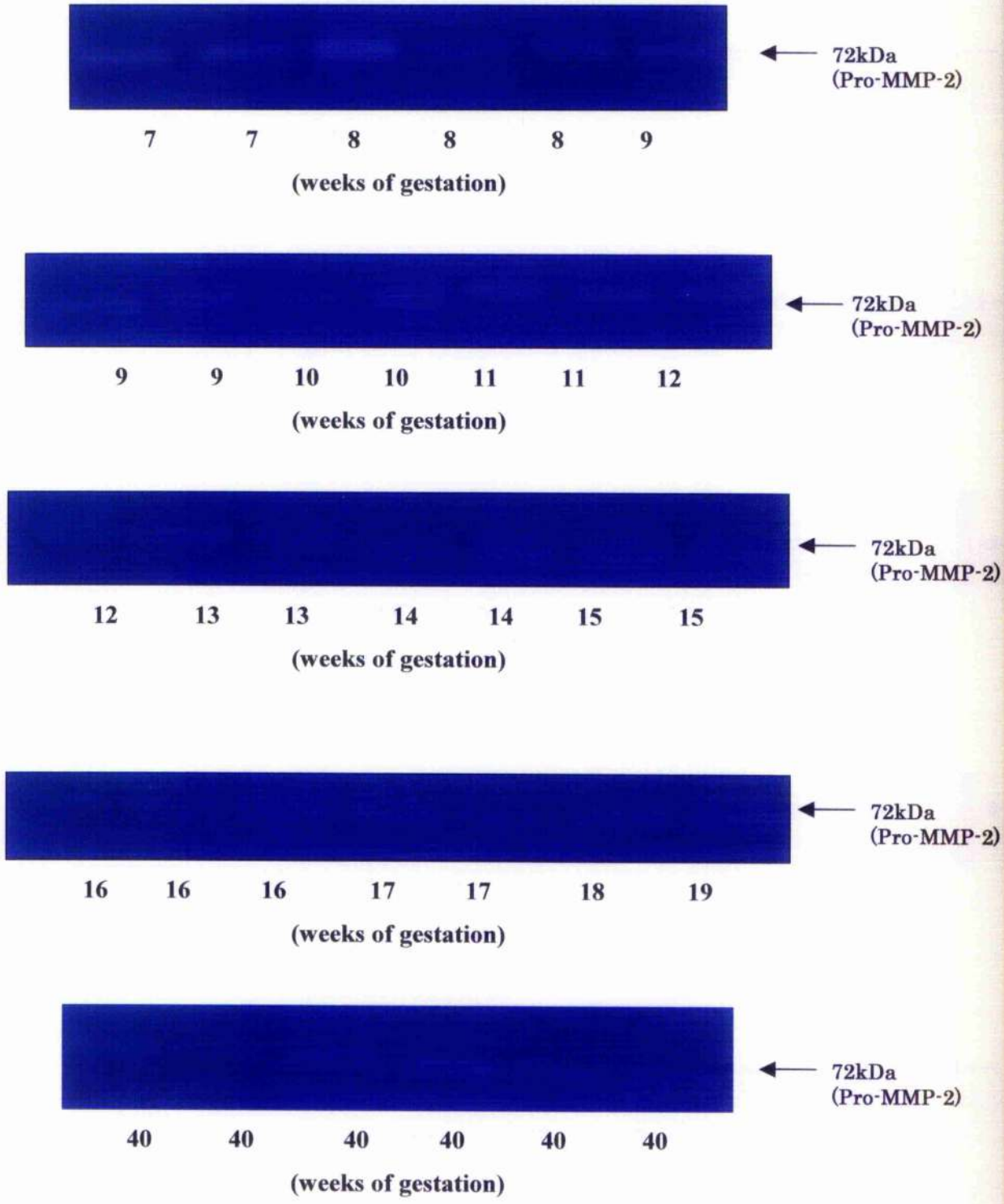


Figure 35 Pro-MMP-9 activity in placental homogenates- zymography

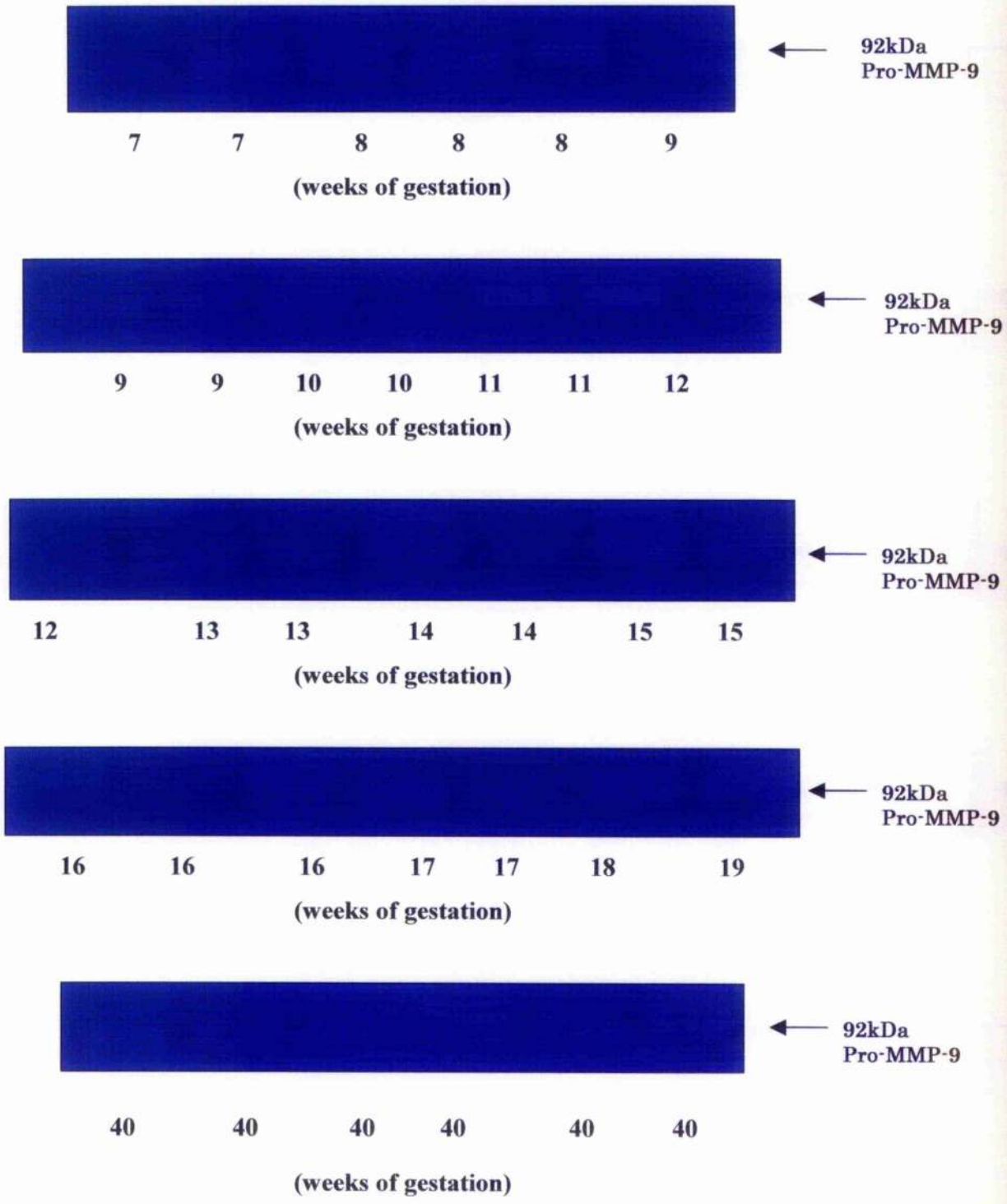


Table 10 Densitometrical analysis of zymography of placental homogenates – raw and corrected data

	pro-MMP-2			pro-MMP-9		
Gestation	Pro-MMP-2 OD	MMP-2 Control OD	Final Pro-MMP-2 (OD/con)	Pro-MMP-9 OD	MMP-9 Control OD	Final Pro-MMP-9 (OD/con)
7	0.23	0.14	1.64	0.05	0.02	2.5
7	0.31	0.14	2.21	0.15	0.02	7.5
8	0.41	0.14	2.92	0.15	0.02	7.5
8	0.21	0.14	1.50	0.19	0.02	9.5
8	0.17	0.14	1.21	0.14	0.02	7.0
9	0.19	0.14	1.36	0.08	0.02	4.0
9	0.23	0.19	1.21	0.08	0.03	2.6
9	0.23	0.19	1.21	0.09	0.03	3.0
10	0.12	0.19	0.63	0.09	0.03	3.0
10	0.19	0.19	1.00	0.03	0.03	1.0
11	0.22	0.19	1.15	0.12	0.03	4.0
11	0.23	0.19	1.21	0.15	0.03	5.0
12	0.19	0.19	1.00	0.06	0.03	2.0
12	0.08	0.15	0.53	0.08	0.04	2.0
13	0.17	0.15	1.13	0.13	0.04	3.25
13	0.11	0.15	0.73	0.06	0.04	1.5
14	0.10	0.15	0.67	0.19	0.04	4.75
14	0.18	0.15	1.20	0.06	0.04	1.5
15	0.16	0.15	1.07	0.22	0.04	5.5
15	0.17	0.15	1.13	0.10	0.04	2.5
16	0.02	0.13	0.15	0.07	0.03	2.3
16	0.06	0.13	0.46	0.04	0.03	1.3
16	0.10	0.13	0.77	0.09	0.03	3.0
17	0.12	0.13	0.92	0.11	0.03	3.7
17	0.05	0.13	0.38	0.04	0.03	1.3
18	0.12	0.13	0.92	0.12	0.03	4.0
19	0.15	0.13	1.15	0.12	0.03	4.0
40	0.04	0.14	0.29	0.14	0.03	4.7
40	0.04	0.14	0.29	0.07	0.03	2.3
40	0.12	0.14	0.86	0.06	0.03	2.0
40	0.14	0.14	0.77	0.05	0.03	1.7
40	0.11	0.14	0.46	0.08	0.03	2.7
40	0.11	0.14	0.46	0.10	0.03	3.3

Figure 36 **Scattergraph of pro-MMP-2 activity in 1st, 2nd and 3rd trimesters**

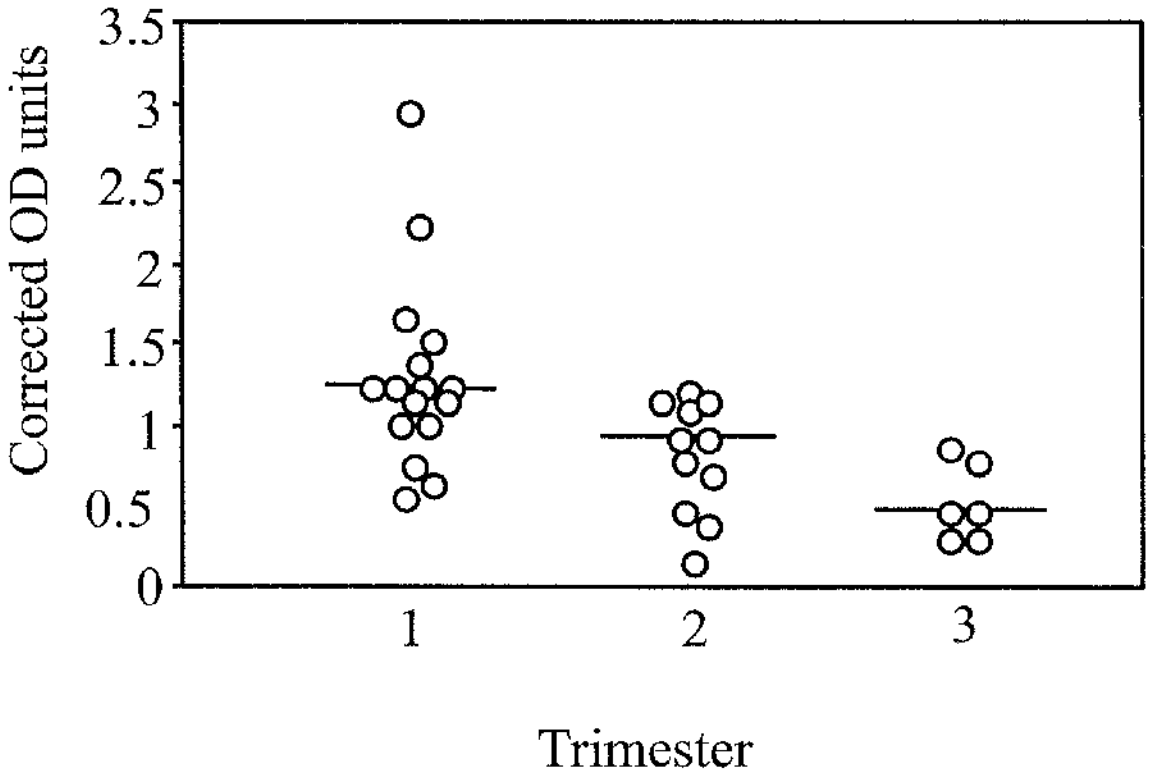


Figure 37 **Scattergraph of pro-MMP-9 activity in 1st, 2nd and 3rd trimesters**

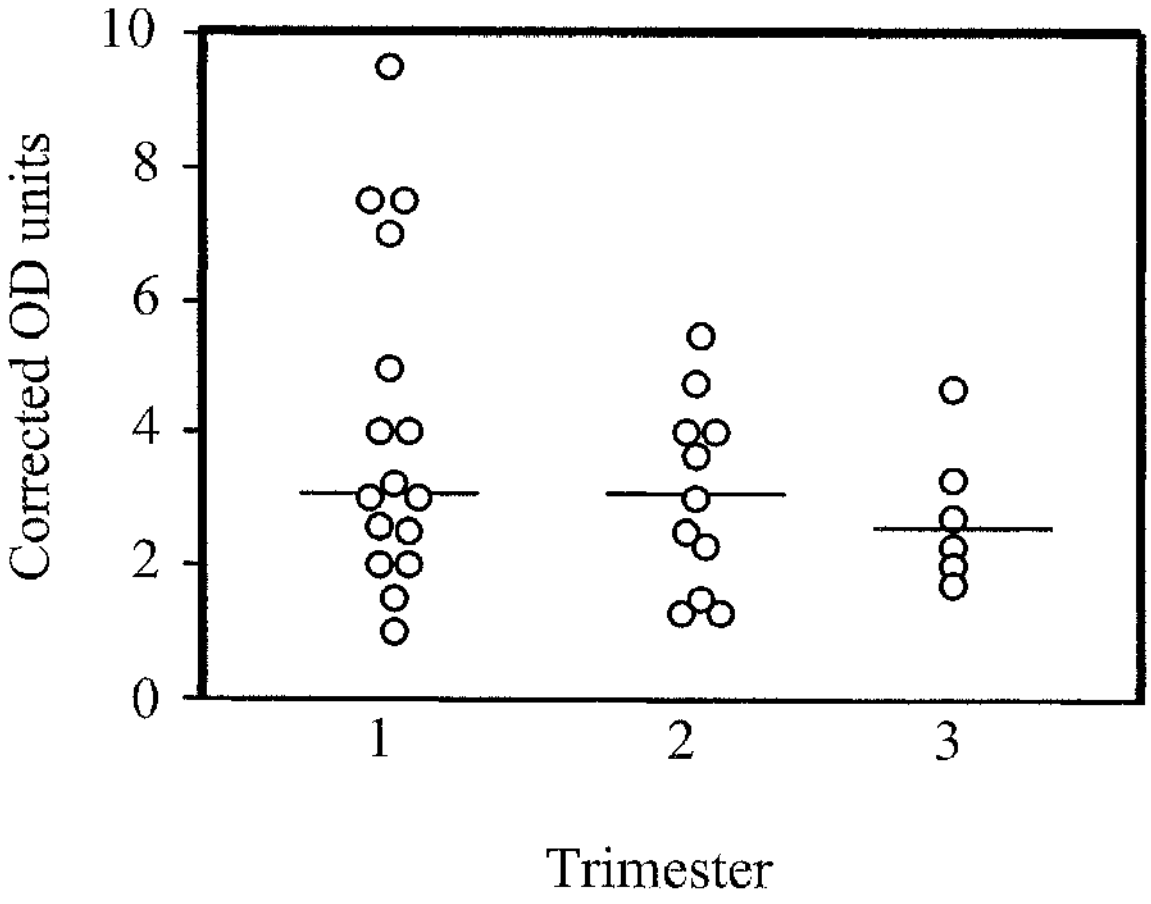


Table 11a **Descriptive statistics for densitometrical analysis of pro-MMP-2 (after correction with control)**

	Mean	Median	Max	Min	Range between min and max values	SE	SD
pro-MMP-2 1st Trimester	1.29	1.21	2.92	0.53	2.39	0.15	0.59
pro-MMP-2 2nd Trimester	0.80	0.92	1.2	0.15	1.05	0.11	0.35
pro-MMP-2 3rd Trimester	0.52	0.46	0.86	0.29	0.57	0.10	0.24

Table 11b **Statistical analysis of the variation in pro-MMP-2 activity between the trimesters**

Test	pro-MMP-2
Trimesters compared	**p-value**
Kruskal-Wallis ($1^{st}/2^{nd}/3^{rd}$)	$p<0.01$
Mann-Whitney ($1^{st}/2^{nd}$)	$p<0.01$
Mann-Whitney ($1^{st}/3^{rd}$)	$p<0.01$
Mann-Whitney ($2^{nd}/3^{rd}$)	N/S

Table 12a **Descriptive statistics for densitometrical analysis of pro-MMP-9 (after correction with control)**

	Mean	Median	Max	Min	Range between min and max values	SE	SD
pro-MMP-9 1st Trimester	4.08	3.13	9.5	1.0	8.5	0.63	2.51
pro-MMP-9 2nd Trimester	3.08	3.00	5.5	1.3	4.20	0.43	1.43
pro-MMP-9 3rd Trimester	2.78	2.50	4.70	1.70	3.0	0.45	1.09

Table 12b **Statistical analysis of the variation in pro-MMP-9 activity between the trimesters**

Test	pro-MMP-9
Trimesters compared	**p-value**
Kruskal-Wallis ($1^{st}/2^{nd}/3^{rd}$)	N/S
Mann-Whitney ($1^{st}/2^{nd}$)	N/S
Mann-Whitney ($1^{st}/3^{rd}$)	N/S
Mann-Whitney ($2^{nd}/3^{rd}$)	N/S

The next stage in the analysis was to determine if there were any correlations in pro-MMP-2 or pro-MMP-9 activity within each trimester. The corrected data was represented on histograms for pro-MMP-2 (figure 38) and pro-MMP-9 (figure 39). The data was then analysed using a Spearman's correlation. Analyses were carried out for both pro-MMP-2 and pro-MMP-9 to determine correlation of MMP activity within the 1st trimester (7-13 weeks), 2nd trimester (14-19 weeks), 1st/2nd trimester (7-19 weeks), 2nd/3rd trimester (14-40 weeks) and 1st/2nd/3rd trimesters (7-40 weeks).

The summary of the statistics for the analysis is shown in table 13. In all significant correlations throughout this thesis the correlation coefficient (R) is stated. A highly significant negative correlation of pro-MMP-2 activity with gestation was found from 7-13 weeks ($p<0.01$), 7-19 weeks ($p<0.01$) and from 7-40 weeks of gestation ($p<0.01$). No significant correlation was found when the 2nd and 3rd trimesters were analysed separately or together.

A significant negative correlation (ie band intensity decreased as gestation increased) of pro-MMP-9 activity with gestation was found from 7-13 weeks ($p<0.05$). No other significant correlations were found.

Figure 38 Histogram of pro-MMP-2 activity throughout gestation

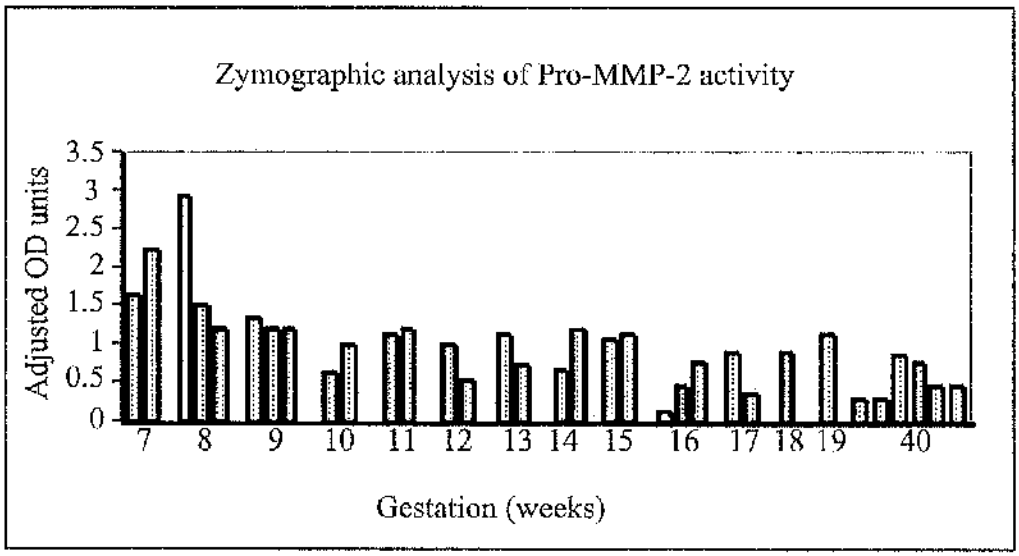


Figure 39 Histogram of pro-MMP-9 activity throughout gestation

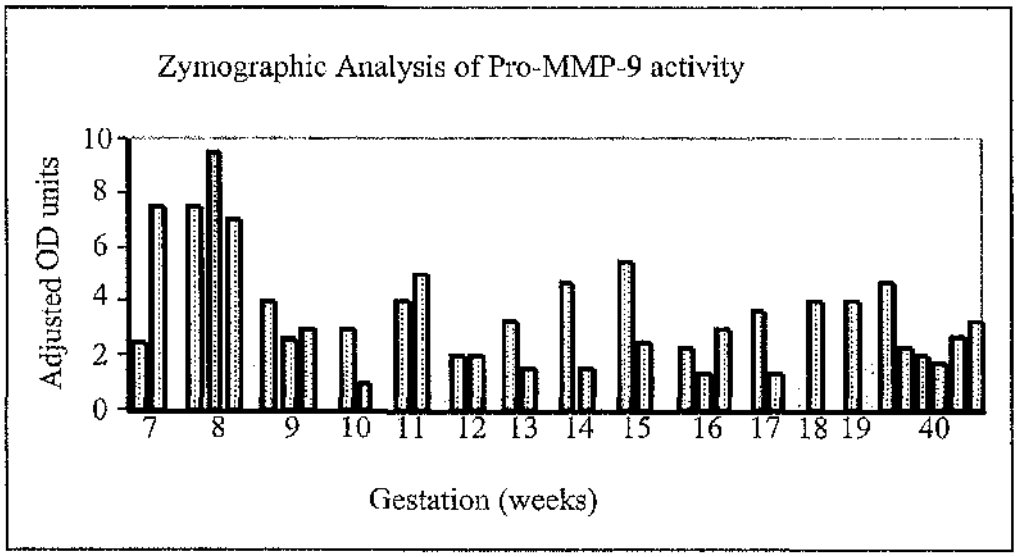


Table 13 Pro-MMP-2 and pro-MMP-9 activity correlation with gestation

Test	pro-MMP-2	pro-MMP-2	pro-MMP-9	pro-MMP-9
	p-value	**R-value**	**p-value**	**R-value**
Spearman's Rank 1st trimester	p<0.01	-0.83	p<0.05	-0.54
Spearman's Rank 2nd trimester	N/S	N/S	N/S	N/S
Spearman's Rank (1st/2nd trimester)	p<0.01	-0.71	N/S	N/S
Spearman's Rank (2nd/3rd trimester)	N/S	N/S	N/S	N/S
Spearman's Rank (1st/2nd/3rd trimester)	p<0.01	-0.76	N/S	N/S

In order to determine if there was any correlation of pro-MMP-9 with pro-MMP-2 activity, the ratio of pro-MMP-9 to pro-MMP-2 was calculated for each sample and then the results were determined using a Spearman's correlation. The uncorrected values obtained by densitometry of the zymograms were used because pro-MMP-9 and pro-MMP-2 were always present on the same gel and indeed in the same lane. The descriptive statistics for this analysis are shown in table 14 for both pro-MMP-2 and pro-MMP-9. The results of the Spearman's correlation of pro-MMP-9 with pro-MMP-2 are shown in table 15. No correlation was found between pro-MMP-9 and pro-MMP-2 activity in any of the groups tested.

For the final analysis of the zymography gels, a comparison was made of band intensity of pro-MMP-2 and pro-MMP-9 in the 1st and 2nd trimesters and at term. Figure 40 shows a comparison of pro-MMP-2 and pro-MMP-9 band intensity throughout gestation. The data was tested using a Wilcoxon signed ranks test. The results of the statistical tests to compare band intensity are shown in table 16. A significantly higher band intensity of pro-MMP-2 than pro-MMP-9 was found ($p<0.01$) in the first trimester. No significant differences were found in the second trimester or at term. When all three trimesters were included in the analysis a significantly higher band intensity of pro-MMP-2 than MMP-9 was found ($p<0.01$).

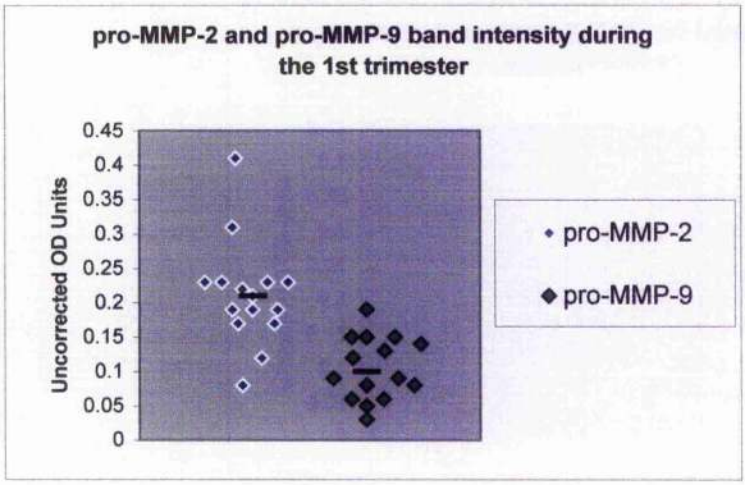
Table 14 **Descriptive statistics for densitometrical analysis of pro-MMP-2 and pro-MMP-9 (before correction with control)**

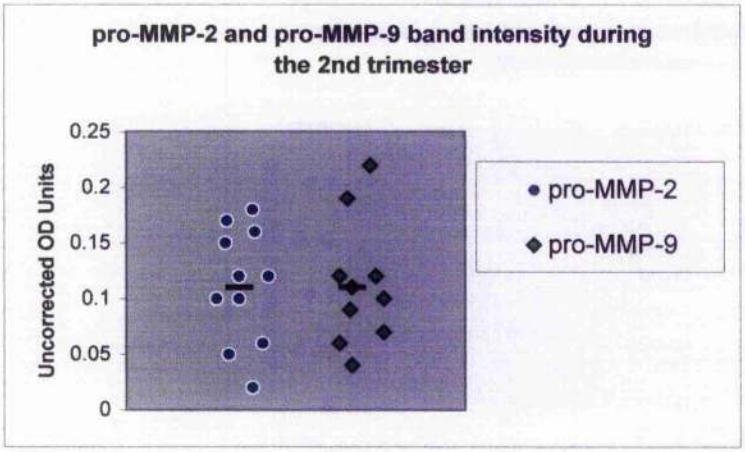
	Mean	Median	Max	Min	Range between min and max values	SE	SD
Pro-MMP-2 1st Trimester	0.21	0.20	0.41	0.08	0.33	0.02	0.08
Pro-MMP-2 2nd Trimester	0.11	0.12	0.18	0.02	0.16	0.02	0.05
Pro-MMP-2 3rd Trimester	0.09	0.11	0.14	0.04	0.10	0.02	0.04
Pro-MMP-9 1st Trimester	0.10	0.09	0.19	0.03	0.16	0.01	0.04
Pro-MMP-9 2nd Trimester	0.11	0.10	0.22	0.04	0.18	0.02	0.06
Pro-MMP-9 3rd Trimester	0.08	0.08	0.14	0.05	0.09	0.01	0.03

Table 15 **Zymography statistical analysis: pro-MMP-2 with pro-MMP-9 correlation throughout gestation**

Test	p-value	R-value
Spearman's Rank 1st trimester	N/S	N/S
Spearman's Rank 2nd trimester	N/S	N/S
Spearman's Rank 3rd trimester	N/S	N/S
Spearman's Rank (1st/2nd trimester)	N/S	N/S
Spearman's Rank (2nd/3rd trimester)	N/S	N/S
Spearman's Rank (1st/2nd/3rd trimester)	N/S	N/S

Figure 40 **Scattergraph comparison of pro-MMP-2 and pro-MMP-9 band intensity throughout gestation**





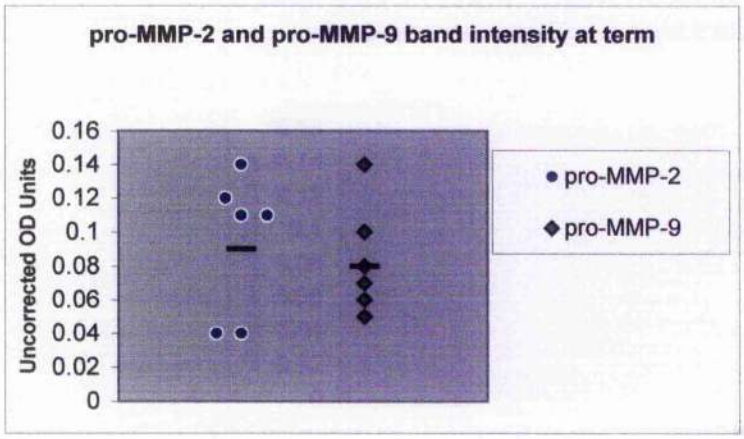


Table 16 **Comparison of pro-MMP-2 with pro-MMP-9 band intensity throughout gestation**

Wilcoxon Signed Ranks test	p-value
All trimesters	p<0.01
1st trimester	p<0.01
2nd trimester	N/S
3rd trimester	N/S

Summary of analysis of pro-MMP-2 and pro-MMP-9 activity in placental homogenates by zymography

The analysis of pro-MMP-9 activity showed a decrease in activity with increasing gestation during the first trimester using a Spearman's correlation. There were no other significant changes.

When pro-MMP-2 activity was analysed, a highly significant decrease was detected when a Spearman's correlation was carried out on homogenates from 7-19 weeks ($1^{st}/2^{nd}$ trimester) and on homogenates from 7-40 weeks ($1^{st}/2^{nd}/3^{rd}$ trimester). No significant correlation was found on homogenates from 14-40 weeks alone ($2^{nd}/3^{rd}$ trimester). When pro-MMP-2 levels were analysed by Kruskal–Wallace and Mann-Whitney testing to determine variation throughout the different trimesters, a significant decrease was found between 1^{st} and 2^{nd} trimesters and a highly significant decrease was found between 1^{st} and 3^{rd} trimesters.

No correlation was found between pro-MMP-2 and pro-MMP-9 levels at any stage in gestation when the data was analysed using Spearman's correlation.

In comparing pro-MMP-2 and pro-MMP-9 band intensity using a Wilcoxon Signed Ranks test, a significantly higher band intensity of pro-MMP-2 than MMP-9 was found ($p<0.01$) in the first trimester and when all three groups were included in the analysis. No significant differences were found in the second trimester or at term. When all three trimesters were included in the analysis a significantly higher band intensity of pro-MMP-2 than MMP-9 was found ($p<0.01$).

4.2 Determination of expression and location of MMP-9 and MMP-2 in placenta throughout pregnancy using immunohistochemistry

4.2.1 MMP-9 immunohistochemistry

Zymography experiments had shown a decrease in pro-MMP-9 activity during the first trimester but no other changes with gestation. Next the expression and location of MMP-9 in placenta throughout gestation by immunohistochemistry were investigated.

Frozen sections were cut from placental blocks from 7-19 weeks of gestation and from the same term placentae which had been used for zymography. The gestations were 1x 7, 2x 8, 2x 9, 2x 10, 2x 11, 2x 12, 2x 13, 3x 14, 2x 15, 2x 16, 1x 17, 2x 18, 1x 19, and 11x 40 weeks of gestation.

Immunohistochemistry was carried out using the Biogenesis MMP-9 antibody which detects both pro and active MMP-9 (section 3.1.7). The MMP-9 antibody was used at a dilution of 1/500 as described previously (section 2.2). The staining was scored on a 0-4 scale as described in section 2.6. Control slides where sheep IgG or antibody diluent was substituted for primary antibody showed no staining (section 2.2). For all immunohistochemistry experiments described in this chapter and in chapter 5, staining was evenly distributed throughout the placental sections from the chorionic to the basal plate. Where EVT was evaluated it was present in the decidua or in cell columns. There was insufficient decidual tissue to perform a comprehensive analysis on decidua alone.

Across gestation MMP-9 was present to a greater or lesser extent in EVT, endothelium, muscle surrounding blood vessels and in the stroma. There was virtually no staining on vCTB or STB. Figure 41 shows photographs of representative MMP-9 staining from selected gestations. Figure 41a shows a placenta at seven weeks of gestation with very little staining except in the intervillous blood space, which is likely to be endogenous peroxidase. Figure 41b shows limited staining in EVT at 8 weeks of gestation. Figure 41c shows a placenta at sixteen weeks of gestation with moderately strong endothelial staining. Figure 41d shows a placenta at term with even stronger endothelial and stronger stromal staining.

The raw data obtained from scoring the stained sections is shown in table 17. The data was grouped into trimesters and statistical analysis was carried out to determine if there were any statistical differences in MMP-9 expression between

trimesters. A summary of the descriptive statistics of the data is given in table 18. No assumptions were made as to the normality of the data and non-parametric testing was used throughout.

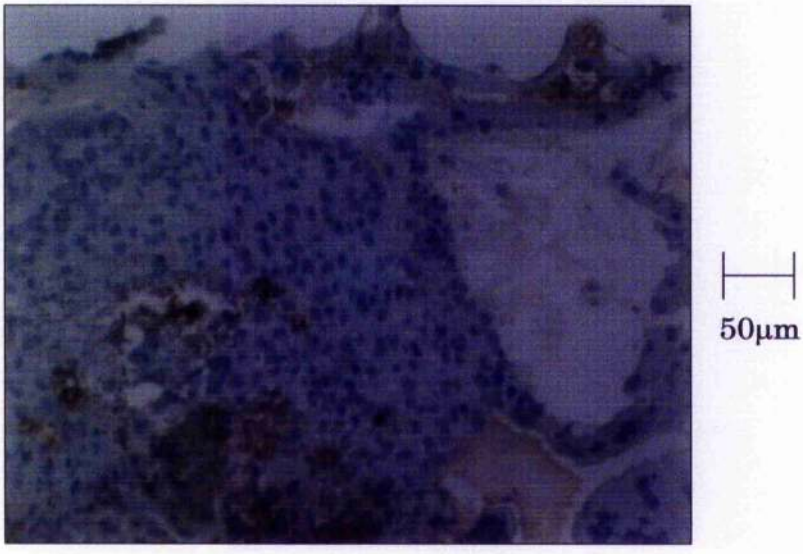
Table 19 shows the results of the statistical analysis for MMP-9. Kruskal-Wallis analysis showed a significant difference between the trimesters in endothelial, muscle and stromal staining ($p<0.01$). When these groups were analysed individually by Mann-Whitney analysis, there were no significant changes between 1st and 2nd trimesters in any cell types.

There was significantly more endothelial staining in the third trimester when compared with the second trimester ($p<0.01$) and in the third trimester when compared with the first trimester ($p<0.01$). There was also significantly more stromal staining in the third trimester when compared with the second trimester ($p<0.01$) and in the third trimester when compared with the first trimester ($p<0.01$). There was significantly more muscle staining in the third trimester when compared with the second trimester ($p<0.05$) and in the third trimester when compared with the first trimester ($p<0.05$). No significant differences in vCTB or EVT staining were found in any of the tests. However the Kruskal-Wallace analysis for EVT almost reached significance as did the Mann-Whitney analysis between the first and second trimester ($p=0.08$) with staining tending to be lower in the second trimester than in the first.

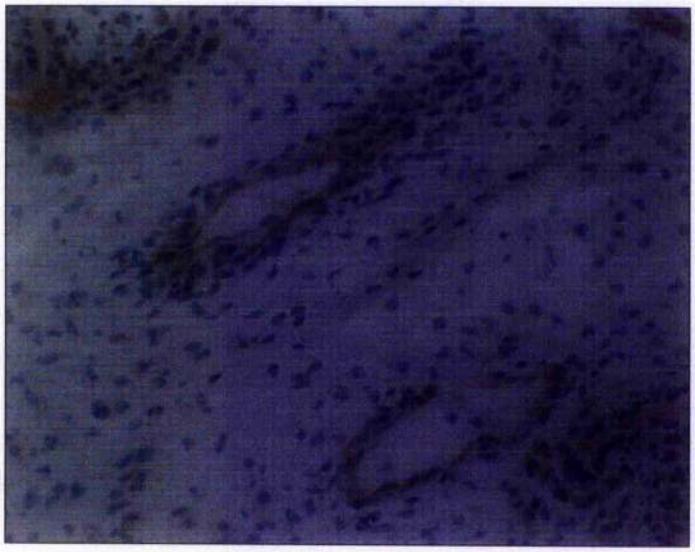
Figure 41 Immunohistochemistry of MMP-9 throughout gestation

a) 7 weeks of gestation

b) 8 weeks of gestation



c) **16 weeks of gestation**



d) **Term**

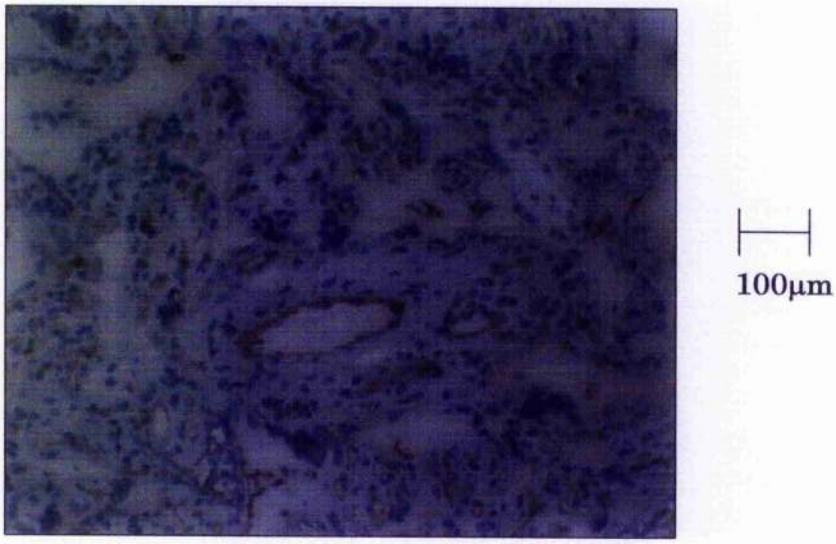


Table 17 Semi-quantitative analysis of MMP-9 immunostaining

Gestation (weeks)	vCTB	EVT	Endothelium	Muscle	Stroma
7	0	2	0	0	0
8	0	N/P*	0	0	0
8	0	2	0	0	2
9	0	2	0	0	0
9	0	N/P	0	0	0
10	0	1.5	0	0	0.5
10	1.5	1.5	0	0	0.5
11	0	3	0	0	0.5
11	0	N/P	0	0	0.5
12	0	0.5	0	0	0.5
12	0	N/P	0	0	0
13	1	1	0	0	0
13	1	N/P	1	0	0
14	0	0.5	0	0	0
14	0	0.5	0	0	0
14	0	0.5	1	0	0
15	0	1.5	0	0	0.5
15	0	1.5	0	0	0
16	0	1	2	0	0.5
16	1	N/P	2	0	0.5
17	0	1.5	0	0	0.5
18	1.5	1.5	0	0.5	0
18	0.5	N/P	1	0	0.5
19	0	N/P	0	0	1
40	0	N/P	2.0	1	2
40	0	0.5	1.5	0	1.5
40	0	N/P	2	0.5	1
40	0	N/P	1.5	0	2
40	0	N/P	2.5	0	1
40	0	N/P	2	0.5	0.5
40	0	N/P	2.5	0	2
40	0	N/P	2	1	1.5
40	0	N/P	2.5	1	1.5
40	1	N/P	3	1.5	2

*N/P = Not Present

Table 18 MMP-9 immunohistochemistry descriptive statistics

	Mean	Median	Max	Min	Range between min and max	SE	SD
vCTB							
1st	0.27	0.0	1.5	0.0	1.5	0.15	0.53
2nd	0.27	0.0	1.5	0.0	1.5	0.16	0.52
3rd trimester	0.10	0.0	1.0	0.0	1.0	0.10	0.32
EVT							
1st	1.69	1.75	3.0	0.5	2.5	0.27	0.75
2nd	1.06	1.25	1.5	0.5	1.0	0.18	0.5
3rd trimester	0.5	0.5	0.5	0.5	0.0	N/A*	N/A*
Endothelium							
1st	0.08	0.0	1.0	0.0	1.0	0.08	0.28
2nd	0.55	0.0	2.0	0.0	2.0	0.25	0.82
3rd trimester	2.15	2.0	3.0	1.5	1.5	0.15	0.47
Muscle							
1st	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2nd	0.05	0.0	0.50	0.0	0.50	0.05	0.15
3rd trimester	0.55	0.50	1.5	0.0	1.5	0.17	0.55
Stroma							
1st	0.35	0.0	2.0	0.0	2.0	0.15	0.55
2nd	0.32	0.50	1.0	0.0	1.0	0.10	0.34
3rd trimester	1.5	1.5	2.0	0.50	1.5	0.17	0.53

N/A* = Not Applicable

Table 19 **Analysis of variation in MMP-9 immunostaining between the three trimesters**

Test	vCTB	EVT	Endothelium	Muscle	Stroma
Kruskal-Wallace	**p-value**				
$1^{st}/2^{nd}/3^{rd}$ trimester	N/S*	N/S	p<0.01	p<0.01	p<0.01
Mann-Whitney					
$1^{st}/2^{nd}$ trimester	N/S	N/S	N/S	N/S	N/S
$2^{nd}/3^{rd}$ trimester	N/S	N/S	p<0.01	p<0.05	p<0.01
$1^{st}/3^{rd}$ trimester	N/S	N/S	p<0.01	p<0.05	p<0.01

*N/S = Non-Significant

The next stage in the analysis was to determine if there were any correlations in MMP-9 expression within each trimester. The data was represented on histograms for each cell type (figure 42). Note that for some cell types many values were zero (no staining) and therefore do not appear as bars on the chart. The data was then analysed using a Spearman's correlation. Analyses were carried out to determine correlation of MMP-9 expression within the 1^{st} trimester (7-13 weeks), 2^{nd} trimester (14-19 weeks), $1^{st}/2^{nd}$ trimester (7-19 weeks), $2^{nd}/3^{rd}$ trimester (14-40 weeks) and $1^{st}/2^{nd}/3^{rd}$ trimesters (7-40 weeks). The results of the analysis are shown in table 20.

The data summarized in table 20 shows a significant positive correlation (staining increased as gestation increased) of MMP-9 staining with gestation on EVT (figure 42a) between 14-19 weeks ($p<0.02$) but a negative correlation (staining decreased as gestation increased) between 7-40 weeks of gestation ($p<0.05$).

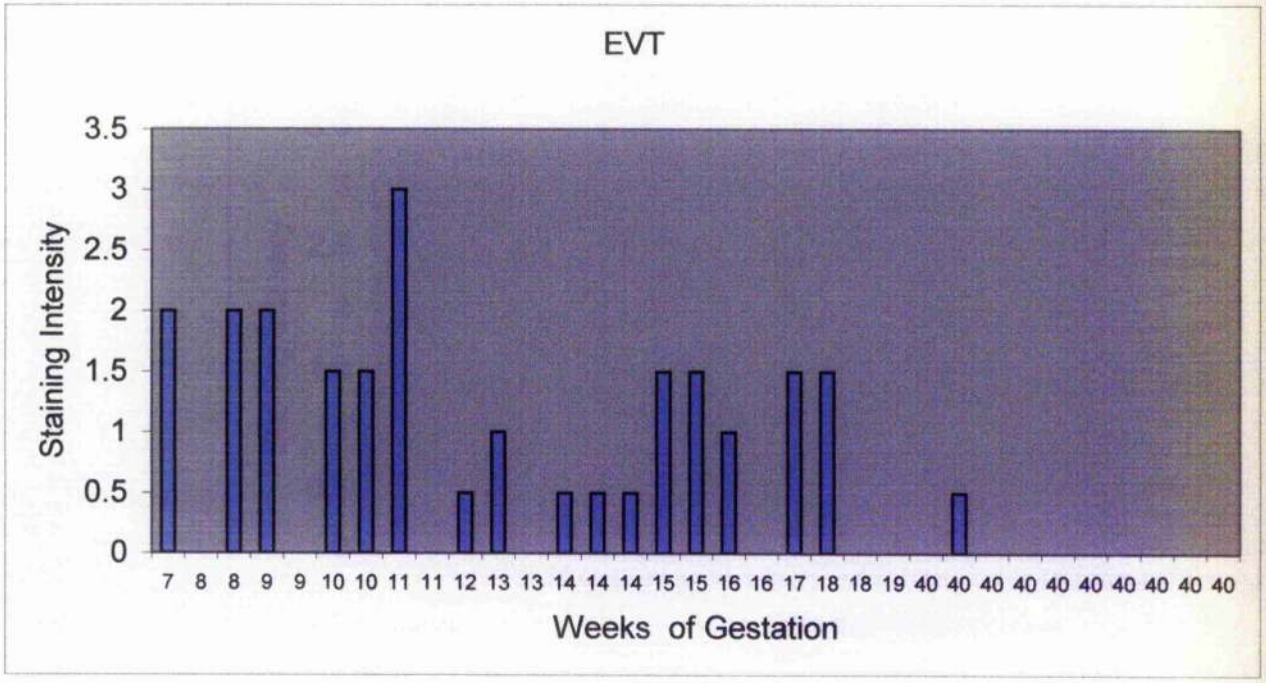
A significant positive correlation (staining increased as gestation increased) of MMP-9 staining with gestation on endothelium (figure 42b) was found between 7-19 weeks ($p<0.05$), between 14-40 weeks ($p<0.01$) and between 7-40 weeks ($p<0.01$). No correlation was found between 7-13 weeks or between 14-19 weeks of gestation.

A significant positive correlation (staining increased as gestation increased) of MMP-9 staining with gestation on muscle (figure 42c) was found between 7-40 weeks ($p<0.01$).

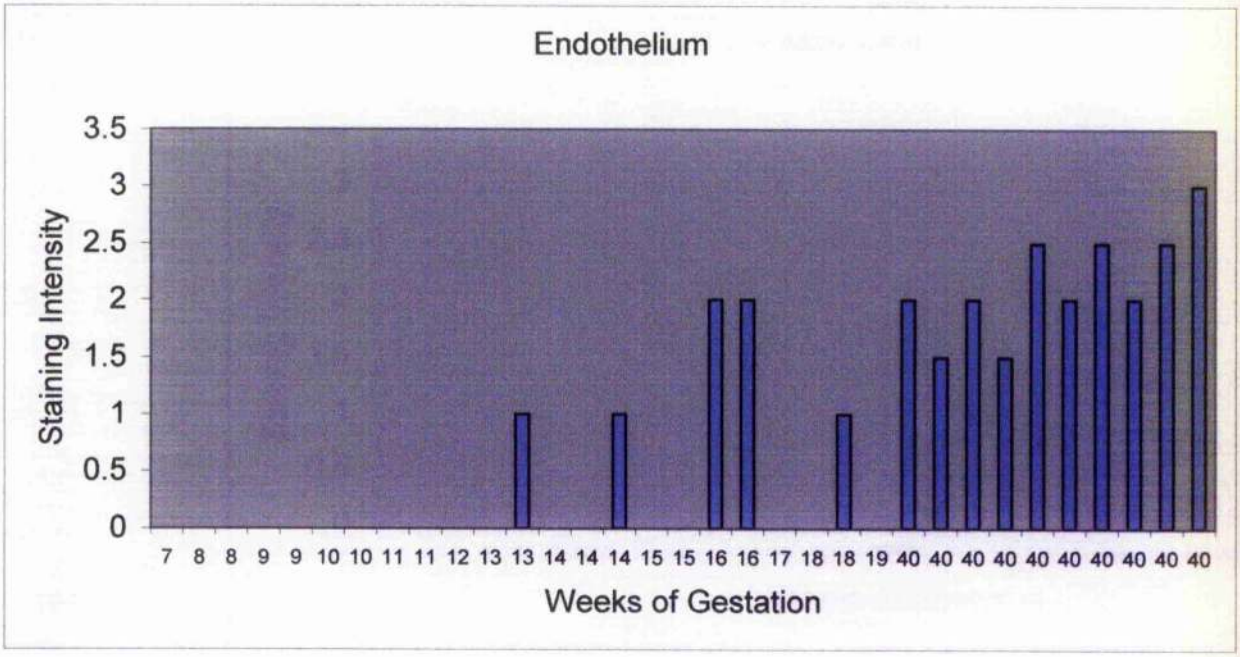
A significant positive correlation (staining increased as gestation increased) of MMP-9 stromal staining (figure 42d) with gestation was found between 14-19 weeks ($p<0.05$), between 14-40 weeks ($p<0.01$) and between 7-40 weeks ($p<0.01$). No correlation was found between 7-13 weeks or between 7-19 weeks of gestation. No significant correlation of vCTB staining (figure 42e) was found in any of the tests.

Figure 42 Histograms of MMP-9 immunostaining throughout gestation

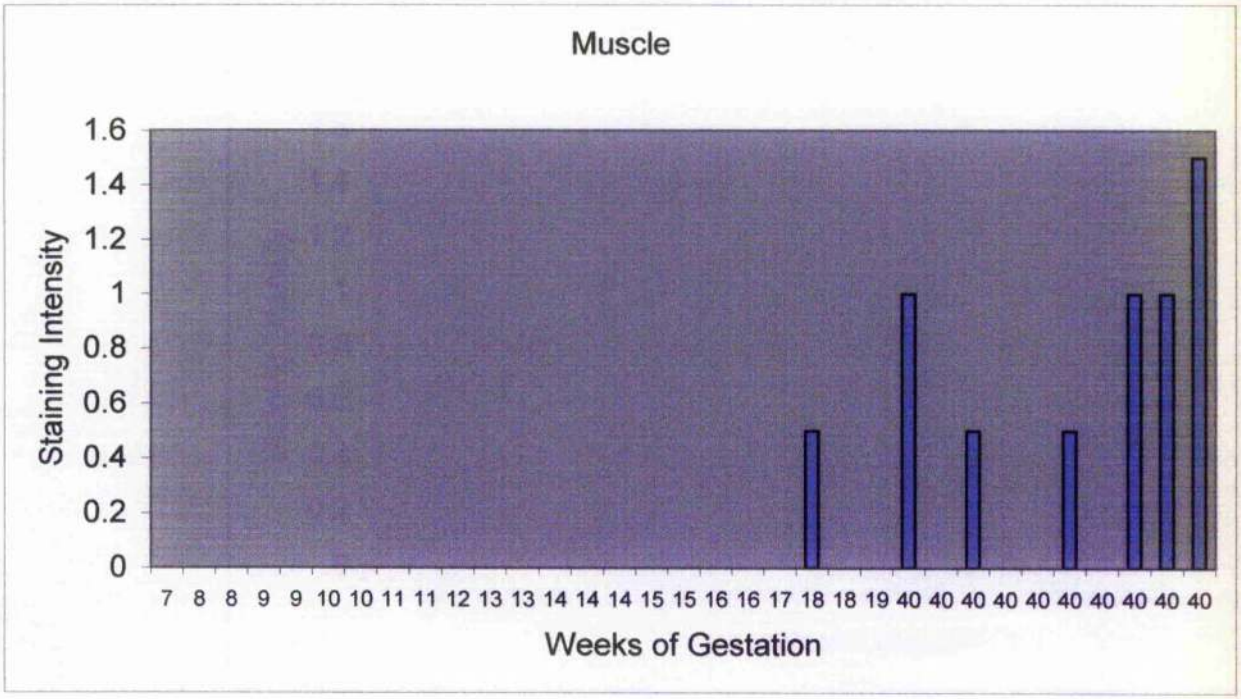
a)



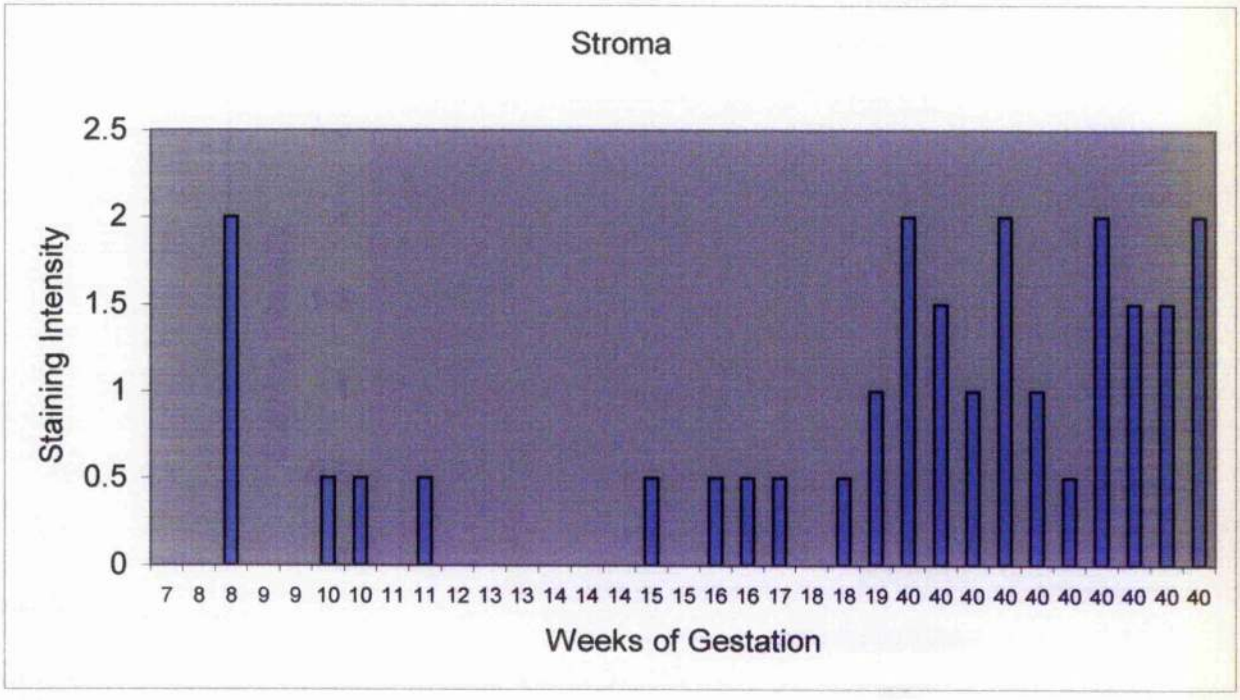
b)



c)


Muscle
Staining Intensity
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
7 8 8 9 9 10 10 11 11 12 13 13 14 14 14 15 15 16 16 17 18 18 19 40 40 40 40 40 40 40 40 40 40
Weeks of Gestation

d)


Stroma
Staining Intensity
2.5
2
1.5
1
0.5
0
7 8 8 9 9 10 10 11 11 12 13 13 14 14 14 15 15 16 16 17 18 18 19 40 40 40 40 40 40 40 40 40 40
Weeks of Gestation

e)

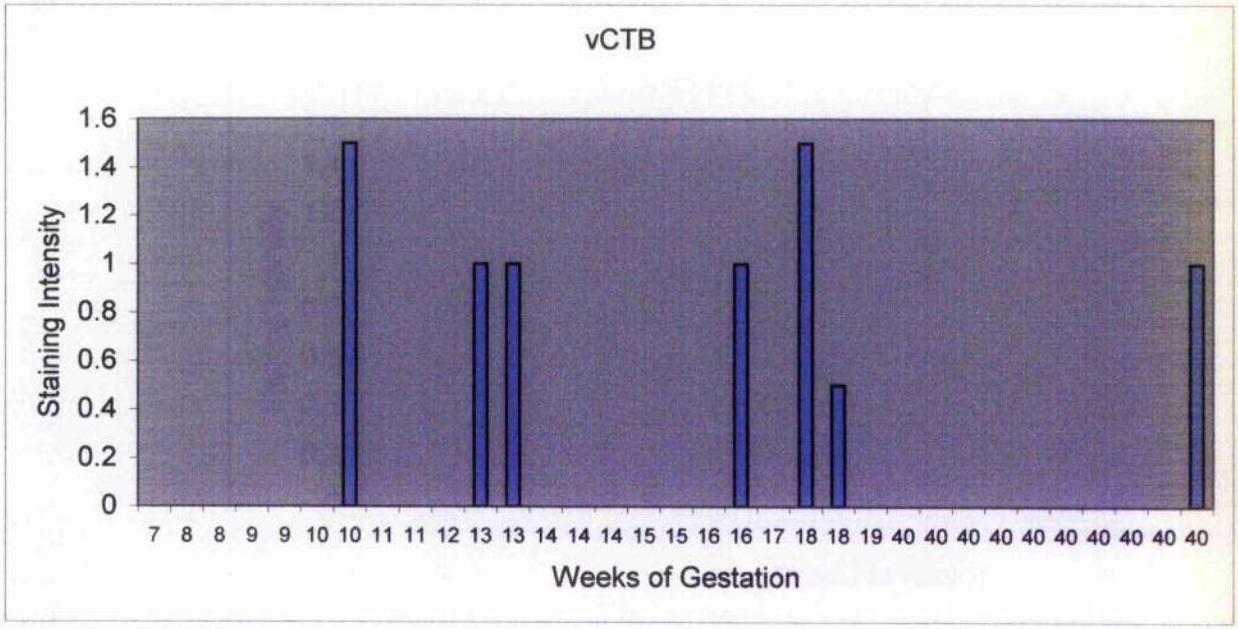

vCTB
Staining Intensity
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
7 8 8 9 9 10 10 11 11 12 13 13 14 14 14 15 15 16 16 17 18 18 19 40 40 40 40 40 40 40 40 40
Weeks of Gestation

Table 20 Correlation of MMP-9 immunostaining with gestation

Spearman's Rank	vCTB	EVT	Endothelium	Muscle	Stroma
	p-value and R-value				
1st trimester	N/S*	N/S	N/S	N/S	N/S
2nd trimester	N/S	p<0.02 R=0.81	N/S	N/S	p<0.05 R=0.66
1st/2nd trimester	N/S	N/S	p<0.05 R=0.43	N/S	N/S
2nd/3rd trimester	N/S	N/S	p<0.01 R=0.71	N/S	p<0.01 R=0.87
1st/2nd/3rd trimester	N/S	p<0.05 R=-0.51	p<0.01 R=0.80	p<0.01 R=0.51	p<0.01 R=0.62

*N/S = Non-Significant

4.2.2 MMP-2 immunohistochemistry

Zymography experiments had shown a decrease in pro-MMP-2 activity with gestation throughout pregnancy. Next the expression and location of MMP-2 in placenta throughout gestation by immunohistochemistry were investigated. The same placental sections were used as for MMP-9 immunohistochemistry (section 4.2.1).

Immunohistochemistry was carried out using a Biogenesis antibody against MMP-2, which detects both pro and active MMP-2 (section 3.1.3). The MMP-2 antibody was used at a dilution of 1/250 (section 2.2) and the staining was scored on a 0-4 scale as described previously (section 2.6). Control slides where sheep IgG or antibody diluent was substituted for primary antibody showed no staining (section 2.2).

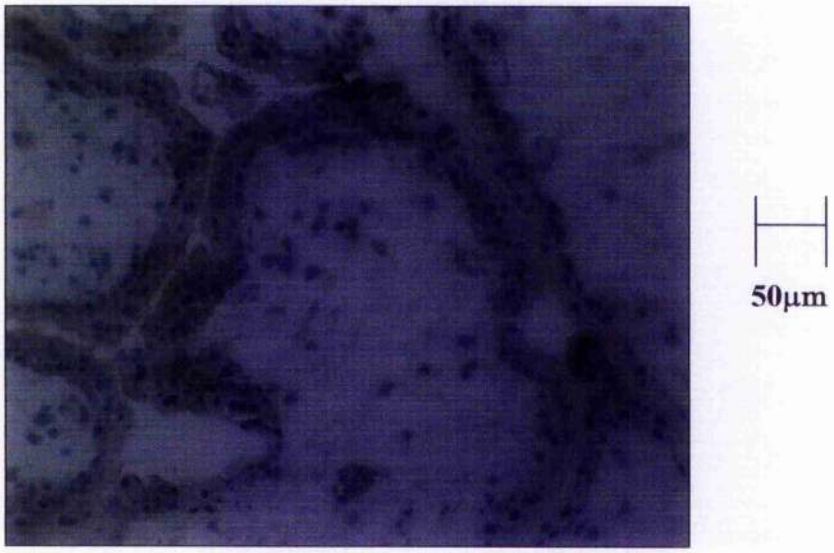
Across gestation MMP-2 was present to a greater or lesser extent in vCTB and STB, EVT, endothelium, muscle surrounding blood vessels and in the stroma. Figure 43 shows photographs of representative MMP-2 staining from selected gestations. Figure 43a shows a placenta at seven weeks of gestation with moderate vCTB staining. Figure 43b shows EVT staining at 8 weeks of gestation. Figure 43c shows a placenta at fifteen weeks of gestation with moderately strong endothelial staining. Figure 43d shows a placenta at term with stronger endothelial staining.

The raw data obtained from scoring the stained sections is shown in table 21. Next the data was grouped into trimesters and statistical analysis was carried out to determine if there were any statistical differences in MMP-2 expression between trimesters. A summary of the descriptive statistics of the data is given in table 22. No assumptions were made as to the normality of the data and non-parametric testing was used throughout. Table 23 shows the results of the statistical analysis for MMP-2. Kruskal-Wallis analysis showed a highly significant difference between the trimesters in vCTB, endothelial and muscle staining but not in EVT or stromal staining.

Mann-Whitney analysis detected that staining of vCTB was lower in the third trimester compared with the first trimester and in the second trimester compared with the first trimester. There was significantly more endothelial staining in the third trimester compared with the second trimester ($p<0.01$), in the second trimester compared with the first trimester and in the third trimester compared with the second trimester. No significant differences were found in EVT, muscle or stromal staining.

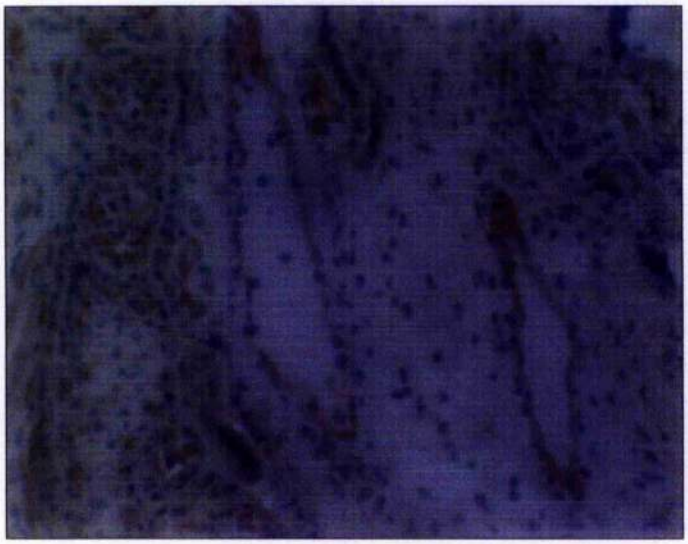
Figure 43 Immunohistochemistry of MMP-2 throughout gestation

a) 7 weeks of gestation



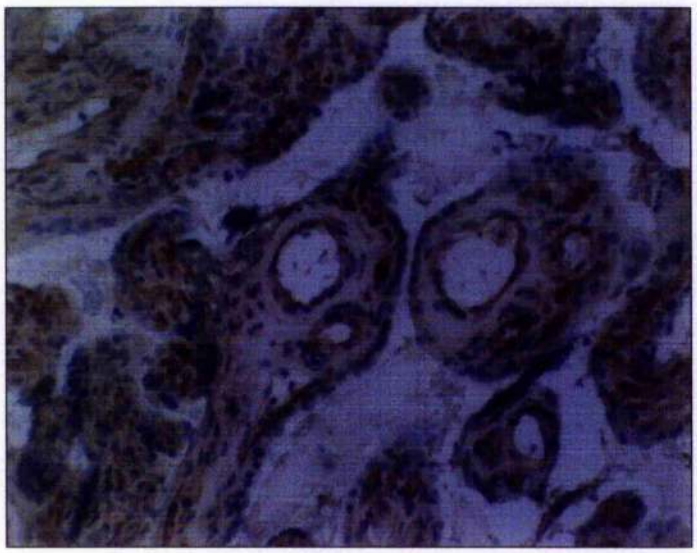
b) 8 weeks of gestation

c) **15 weeks of gestation**



50μm

d) **Term**



100μm

Table 21 Semi-quantitative analysis of MMP-2 immunostaining

Gestation (Weeks)	vCTB	EVT	Endothelium	Muscle	Stroma
7	1.5	2	0	0	0.5
8	0.5	N/P*	0	0	0
8	2	2	0	0	1.5
9	0.5	1.5	0	0	0.5
9	0.5	N/P	0	0	0.5
10	1	1.5	0	0	0.5
10	2.5	2	2.5	2.5	2
11	1	2	0	0	1
11	0.5	N/P	0	0	0.5
12	1.5	2.5	0	0	1
13	1.5	2	2	0	0.5
13	2	1.5	2.5	0	1
14	0.5	2.5	1	0	0.5
14	1.5	2.5	2	0	1.5
14	0.5	0.5	2.5	0	0.5
15	1	0.5	1.5	1.5	1.5
15	0	N/P	1.5	0	0
16	0	0.5	2.5	0	0
16	0	N/P	2.5	0	0
17	0	N/P	2	0	0
18	1	0.5	1.5	0	0.5
18	0	N/P	2.5	0	0
19	0	1.5	2	0	1.5
40	0	N/P	2.5	0	1
40	0	N/P	2.5	1.5	1.5
40	1.5	0.5	2.5	0	0.5
40	0	N/P	2.5	0	2
40	2	N/P	3.5	2.5	2.5
40	0	N/P	2.5	1.5	1
40	0	N/P	2.5	0	1
40	0	N/P	2.5	0	0.5
40	0	N/P	2.5	1	0
40	0	N/P	2.5	1	0
40	0	N/P	2.5	0	0.5

*NP = Not present

Table 22 MMP-2 immunohistochemistry descriptive statistics

	Mean	Median	Max	Min	Range between min and max	SE	SD
vCTB							
1st	1.19	1.0	2.5	0.50	2.0	0.19	0.69
2nd	0.36	0.0	1.5	0.0	1.5	0.17	0.55
3rd trimester	0.35	0.0	2.0	0.0	2.0	0.24	0.75
EVT							
1st	1.89	2.0	2.5	1.5	1.0	0.11	0.33
2nd	1.21	0.5	2.5	0.5	2.0	0.36	0.95
3rd trimester	0.5	0.5	0.5	0.5	0.0	N/A*	N/A*
Endothelium							
1st	0.62	0.0	2.5	0.0	2.5	0.28	1.02
2nd	2.09	2.0	2.5	1.5	1.0	0.13	0.44
3rd trimester	2.60	2.5	3.5	2.5	1.0	0.10	0.32
Muscle							
1st	0.19	0.0	2.5	0.0	2.5	0.19	0.69
2nd	0.14	0.0	1.5	0.0	1.5	0.14	0.45
3rd trimester	0.94	1.0	2.5	0.0	2.5	0.32	0.90
Stroma							
1st	0.77	0.50	2.0	0.0	2.0	0.15	0.53
2nd	0.59	0.50	1.5	0.0	1.5	0.20	0.66
3rd trimester	0.95	0.75	2.5	0.0	2.5	0.26	0.83

N/A* = Not Applicable

Table 23 **Analysis of variation in MMP-2 immunostaining between the three trimesters**

Test	vCTB	EVT	Endothelium	Muscle	Stroma
	p-value				
Kruskal-Wallace ($1^{st}/2^{nd}/3^{rd}$ trimester)	p<0.01	N/S*	p<0.01	p<0.05	N/S
Mann-Whitney					
$1^{st}/2^{nd}$ trimester	p<0.01	N/S	p<0.02	N/S	N/S
$2^{nd}/3^{rd}$ trimester	N/S	N/S	p<0.02	N/S	N/S
$1^{st}/3^{rd}$ trimester	p<0.01	N/S	p<0.01	N/S	N/S

*N/S = Non-Significant

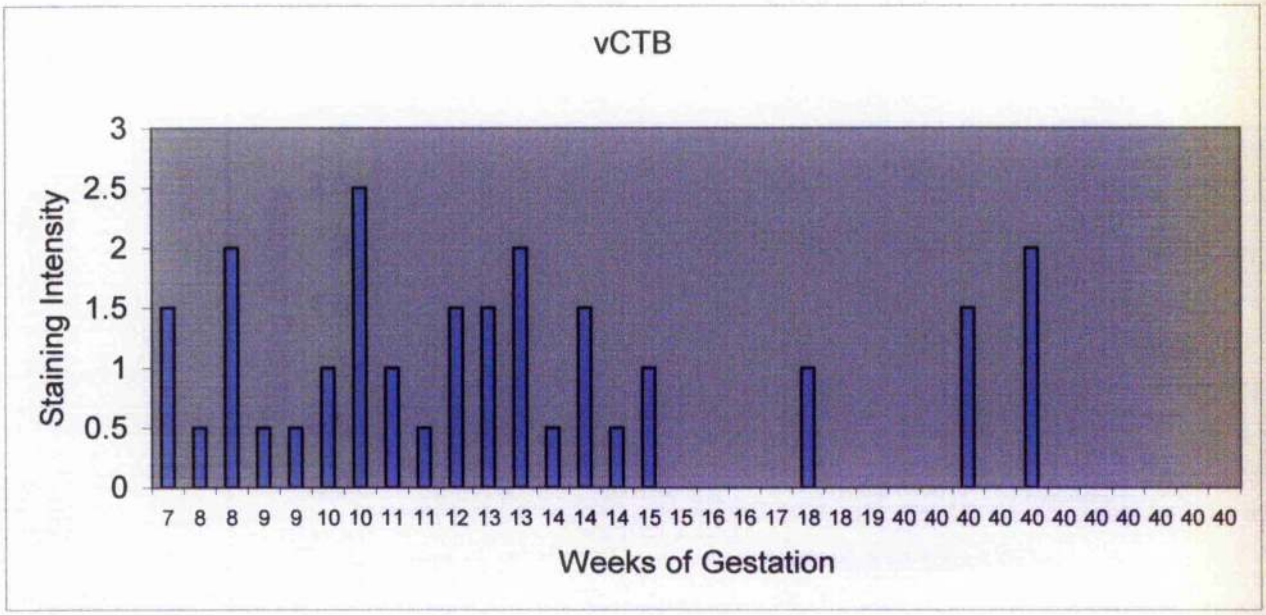
Following this the data was analysed to determine if there were any correlations in MMP-2 expression within each trimester. The data was represented on histograms for each cell type (figure 44). Note that for some cell types many values were zero (no staining) and therefore do not appear as bars on the chart. The data was then analysed using a Spearman's correlation. Analyses were carried out to determine correlation of MMP-2 expression within the 1st trimester (7-13 weeks), 2nd trimester (14-19 weeks), 1st/2nd trimester (7-19 weeks), 2nd/3rd trimester (14-40 weeks) and 1st/2nd/3rd trimesters (7-40 weeks). The results of the analysis are shown in table 24.

The data summarized in table 24 shows vCTB staining (figure 44a) was negatively correlated (staining decreased as gestation increased) with gestation between 7-19 weeks and between 7-40 weeks of gestation. EVT staining (figure 44b) was negatively correlated (staining decreased as gestation increased) with gestation between 7-40 weeks of gestation.

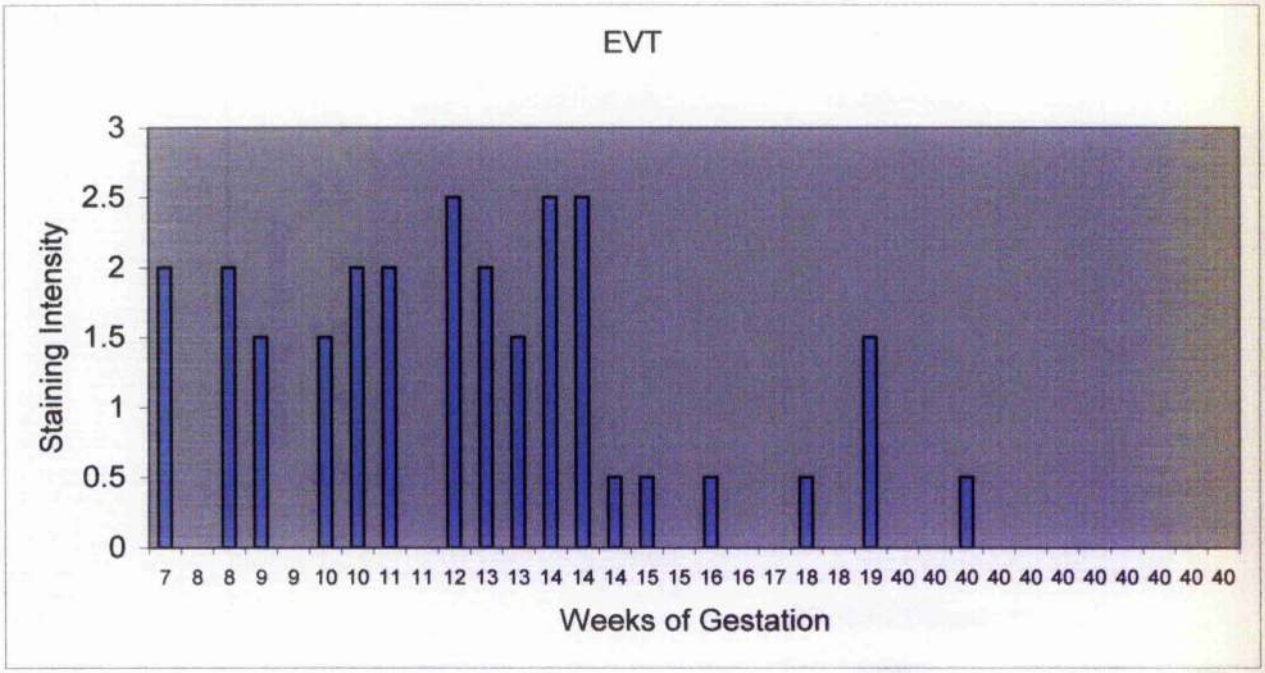
A significant positive correlation (staining increased as gestation increased) of endothelial MMP-2 staining (figure 34c) with gestation was found between 7-13 weeks, between 7-19 weeks, between 14-40 weeks and between 7-40 weeks of gestation. No significant correlations with gestation were found in muscle (figure 44d) or stromal staining (figure 44c).

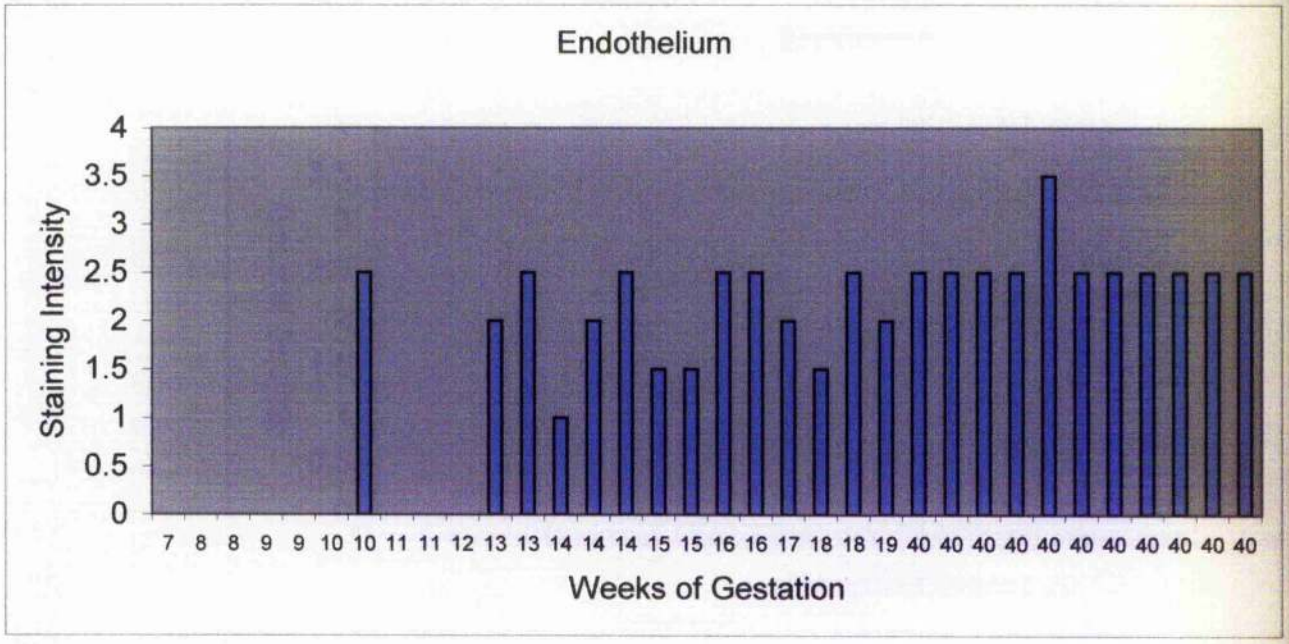
Figure 44 Histograms of MMP-2 immunostaining throughout gestation

a)

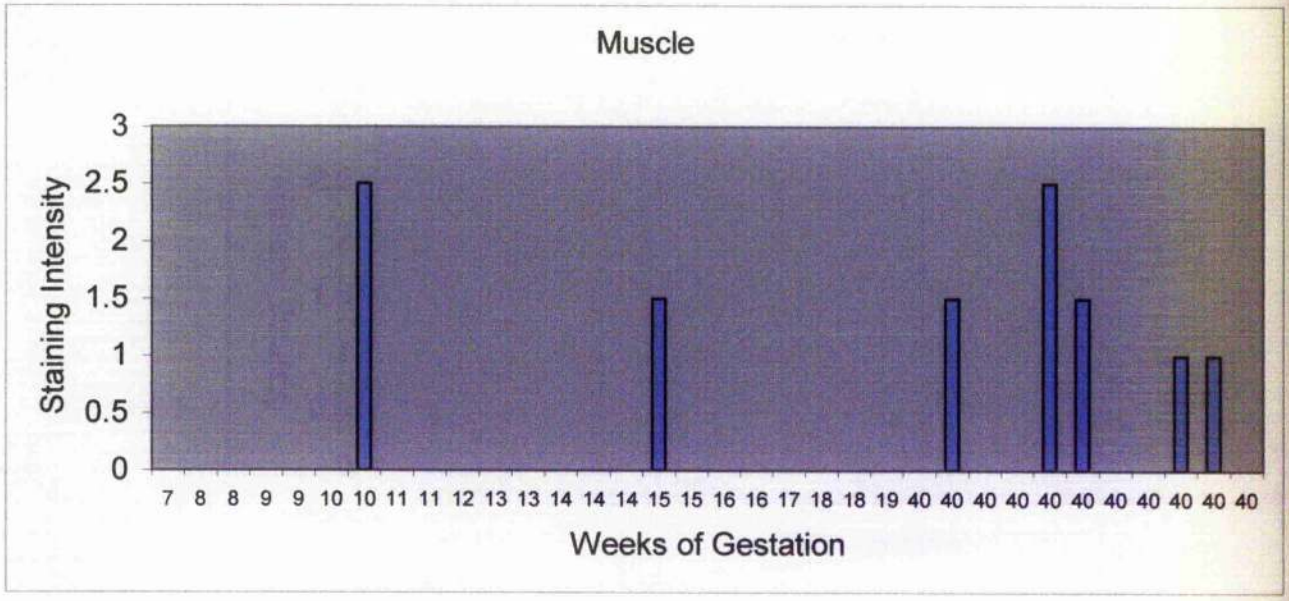


b)




Endothelium
Staining Intensity
4
3.5
3
2.5
2
1.5
1
0.5
0
7 8 8 9 9 10 10 11 11 12 13 13 14 14 14 15 15 16 16 17 18 18 19 40 40 40 40 40 40 40 40 40 40 40
Weeks of Gestation

d)


Muscle
Staining Intensity
3
2.5
2
1.5
1
0.5
0
7 8 8 9 9 10 10 11 11 12 13 13 14 14 14 15 15 16 16 17 18 18 19 40 40 40 40 40 40 40 40 40 40 40
Weeks of Gestation

e)

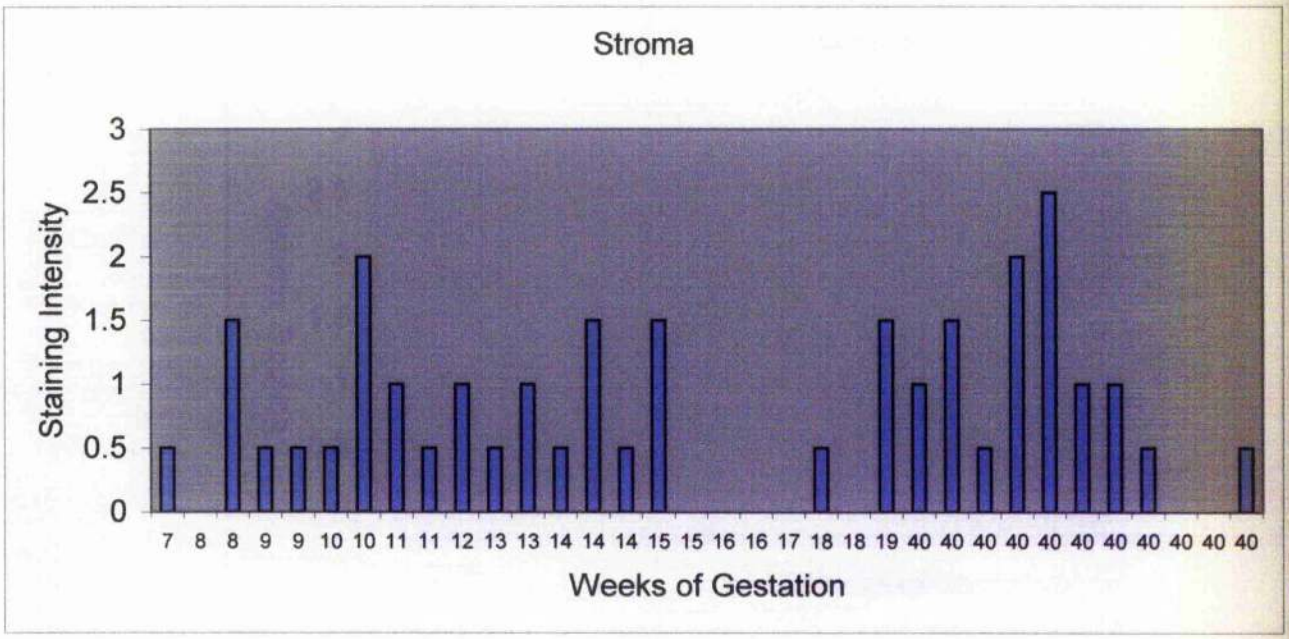

Stroma
Staining Intensity
3
2.5
2
1.5
1
0.5
0
7 8 8 9 9 10 10 11 11 12 13 13 14 14 14 15 15 16 16 17 18 18 19 40 40 40 40 40 40 40 40 40 40
Weeks of Gestation

Table 24 Correlation of MMP-2 immunostaining with gestation

Test	vCTB	EVT	Endothelium	Muscle	Stroma
Spearman's Rank	**p-value and R-value**				
1st trimester	N/S*	N/S	p<0.05 R=0.58	N/S	N/S
2nd trimester	N/S	N/S	N/S	N/S	N/S
1st/2nd trimester	p<0.01 R=-0.59	N/S	p<0.01 R=0.67	N/S	N/S
2nd/3rd trimester	N/S	N/S	p<0.01 R=0.54	N/S	N/S
1st/2nd/3rd trimester	p<0.01 R=-0.56	p<0.05 R=-0.51	p<0.01 R=0.77	N/S	N/S

*N/S = Non-Significant

4.3 Discussion

4.3.1 MMP-9: Summary of results

4.3.1.1 Zymography

Analysis of pro-MMP-9 zymography band intensity (and hence activity) showed no significant differences between the three trimesters. A significant negative correlation (i.e. band intensity decreased as gestation increased) of pro-MMP-9 activity with gestation was found from 7-13 weeks. No other significant correlations were found. The band intensity of pro-MMP-9 was significantly lower than pro-MMP-2 in the first trimester. No significant differences were found in the second trimester or at term. When all three trimesters were included in the analysis the band intensity of pro-MMP-9 was significantly lower than pro-MMP-2. No correlation was found between pro-MMP-9 and pro-MMP-2 band intensity at any stage in gestation.

4.3.1.2 Immunohistochemistry

Immunohistochemical analysis across gestation found that MMP-9 was present to a greater or lesser extent in EVT, endothelium, muscle surrounding blood vessels and in the villous stroma. There was virtually no staining on vCTB or STB. There were no significant changes in MMP-9 expression between 1st and 2nd trimesters in any cell types. There was significantly more endothelial, stromal and muscle staining in the third trimester when compared with the second trimester and in the third trimester when compared with the first trimester. MMP-9 staining on endothelium increased from 7-19 weeks, 14-40 weeks and 7-40 weeks and on the muscle surrounding blood vessels from 7-40 weeks. MMP-9 stromal staining also increased from 14-19 weeks, 14-40 weeks and 7-40 weeks.

No significant differences between the trimesters in vCTB or EVT staining were found. However the analysis for EVT almost reached significance between the first and second trimester (expression tended to be higher during the first trimester). Staining for MMP-9 increased on EVT from 14-19 weeks but was negatively correlated on EVT between 7-40 weeks of gestation.

4.3.1.3 Comparison of MMP-9 zymography and immunohistochemistry results

When the zymography data was split into three trimesters and analysed, no significant differences in pro-MMP-9 activity were found between any of the trimesters. However, when zymography data was correlated with gestation a significant decrease in MMP-9 activity during the first trimester was detected. Analysis of the immunohistochemical data showed a significant increase in endothelium, muscle and stromal MMP-9 staining in the second and third trimesters compared with the first trimester. The decrease in MMP-9 expression in EVT almost reached significance and when immunohistochemical data was correlated with gestation EVT expression of MMP-9 decreased overall from 7-40 weeks of gestation. If more EVT had been present in the tissue it is possible a greater negative correlation would have been found. As such the increase in endothelial, muscle and stromal expression of MMP-9 and the decrease in EVT expression may in effect cancel each other and account for the inability of zymography to detect any changes in MMP-9 activity between the trimesters. The fact that zymography did detect a decrease in MMP-9 activity during the first trimester suggests that any decrease in EVT expression may be fairly substantial.

4.3.2 MMP-2: Summary of results

4.3.2.1 Zymography

When pro-MMP-2 expression was analysed by zymography there was significantly less pro-MMP-2 activity in the 2nd trimester compared with the 1st trimester. There was also significantly less pro-MMP-2 activity in the 3rd trimester compared with the 1st trimester. There were no significant differences in pro-MMP-2 activity between the 2nd and 3rd trimesters.

A significant negative correlation of pro-MMP-2 activity with gestation was found from 7-13 weeks, 7-19 weeks and from 7-40 weeks of gestation. No significant correlation was found when the 2nd and 3rd trimesters were analysed separately or together without the first trimester samples.

4.3.2.2 Immunohistochemistry

Immunohistochemical analysis across gestation found that MMP-2 was present to a greater or lesser extent in vCTB and STB, EVT, endothelium, muscle surrounding blood vessels and in the stroma.

MMP-2 staining on vCTB was lower in the third trimester compared with the first trimester and was also in lower in the second trimester compared with the first trimester. vCTB staining decreased from 7-19 weeks and from 7-40 weeks of gestation. There was significantly more endothelial staining in the third trimester compared with the second trimester, in the second trimester compared with the first trimester and in the third trimester compared with the second trimester. Endothelial MMP-2 staining increased from 7-13 weeks, 7-19 weeks, 14-40 weeks and from 7-40 weeks of gestation. No significant differences were found between individual trimesters in EVT, muscle or stromal staining although muscle staining did increase significantly overall. However EVT staining was negatively correlated with gestation between 7-40 weeks of gestation.

4.3.3 Comparison of MMP-2 zymography and immunohistochemistry results

When the MMP-2 zymography data was split into three trimesters and analysed, pro-MMP-2 activity decreased significantly overall between the three trimesters, specifically declining from 1st to the 2nd trimester and from 1st to the 3rd trimester. When the MMP-2 zymography data was correlated with gestation a significant decrease in MMP-2 was found between 7-13 weeks, 7-19 weeks and 7-40 weeks of gestation.

Immunohistochemical results showed a significant decrease in MMP-2 expression in vCTB overall between the three trimesters, specifically declining from 1st to 2nd trimesters and from 1st to 3rd trimesters. Both vCTB and EVT expression of MMP-2 decreased overall from 7-40 weeks of gestation. An increase in MMP-2 expression in the endothelium and in muscle through pregnancy was also detected with the staining on the endothelium increasing significantly from 1st to 2nd trimesters, 1st to 3rd trimesters and from 2nd to 3rd trimesters. It is likely that the decrease in vCTB

expression of MMP-2 is sufficiently large enough to be detected despite the increase in endothelial and muscle expression of MMP-2, which may be smaller. It is interesting to note in parallel with the MMP-2 results that MMP-9 expression in vCTB was very low throughout pregnancy and zymography did not detect any changes overall in MMP-9 expression.

4.3.3 Comparison of the current study with previously published studies

Before meaningful comparison of the results of the current study with others can occur it is necessary to understand that the study found that the pro forms of both MMP-9 and MMP-2 were predominant over the active forms and thus all discussion predominantly refers to the pro forms of the enzymes. The MMP-9 assay, although only performed on a few samples would seem to support this argument. In other studies it is generally clear which form of the enzyme is referred to if zymography is the method used. However some published studies which use immunohistochemistry are unclear as to what forms of the enzymes the antibodies used detect and thus care must be taken although many of the antibodies on the market do detect both pro and active MMP-2 and MMP-9.

Clearly, in an environment where degradation of the ECM must be tightly regulated it could be expected that an abundance of the pro-form of an enzyme would be available to be converted to the active form when required. Hence the mechanisms for regulating the conversion of the pro to active forms of MMPs may be as critical as the MMPs themselves. This will be discussed in chapter 5.

An early study of MMPs and CTB invasion by Fisher et al. (1989) demonstrated, by zymography, the presence of several metalloproteinases in cellular extracts of CTB which had been cultured on matrices. Several unique proteinases were present in the 1st trimester extract (20–200kDa) compared with the second and third trimester extracts, in which a 92kDa proteinase was pre-dominant. This decreased from second to third trimester. Culture medium from the same cells predominantly showed 92kDa activity. Since then, many other studies have aimed to determine when and where MMPs are produced in the placenta. These will be discussed in the following section. MMP-9 and MMP-2 have become the focus for many of these studies, which have generally taken one of two forms – non-quantitative immunohistochemistry of placental sections or semi-quantitative analysis

of MMP activity in the culture medium from cultured trophoblasts or villous tissue from various stages in pregnancy. There is no general consensus on MMP expression throughout pregnancy and a primary aim of the current study was to attempt to undertake a semi-quantitative immunohistochemical analysis of MMP-9 and MMP-2 expression in conjunction with zymographical analysis of MMP-9 and MMP-2 expression in placental homogenates. Clearly zymographical analysis of MMP secretion into culture medium from cultured trophoblasts and villous tissue from comparable gestations would also be desirable for comparison and indeed may perhaps show discrepancies between cell culture and *in vivo* studies, however this was deemed out-with the scope of the current study. The study is most readily compared to others initially by direct comparison of the immunohistochemical data and subsequently by comparing the zymography results with the published studies on CTB culture.

4.3.3.1 MMP-2 immunohistochemistry

A summary of previously published studies which have used immunohistochemistry and other techniques to examine cellular distribution of MMP-2 and MMP-9 in the placenta at various stages of pregnancy is shown in table 25. This table also shows the number of samples from each gestation used in the studies. As can be seen, many studies have used only a few samples from limited gestations hence emphasizing the need for a more comprehensive analysis.

Two early immunohistochemical and in situ hybridisation studies showed expression of MMP-2 in normal placentae from 8-11 weeks of gestation. The first of these reported that MMP-2 mRNA and protein were present in EVT. MMP-2 protein but not mRNA was present on vCTB and STB. Little MMP-2 mRNA or protein were present on the endothelium (Autio-Harmainen et al. 1992). In the same study it was also reported that type IV collagen was secreted by CTB and it was suggested that this may reflect a mechanism by which trophoblast cells modulate basement membrane deposition. An immunohistochemical study by Fernandez et al. (1992) also found MMP-2 protein expressed on vCTB and EVT and suggested that the vCTB may be involved in the initial degradation of the superficial endometrium during the first trimester. MMP-2 expression was also found in the decidua and villous stroma in this study. Polette et al. (1994) found using in situ hybridisation and

immunohistochemistry that during the first trimester EVT produced MMP-2 protein and mRNA but they also reported MMP-2 expression in the villous stromal compartment and underlying endothelium at term. The authors suggested this may implicate MMP-2 in other processes such as extracellular matrix remodelling or vascular network establishment. A further immunohistochemical study (Huppertz et al. 1998) found that MMP-2 expression was fairly weak during the first and second trimesters in EVT but fairly strong in EVT in the third trimester in frozen placental sections. Sawicki et al. (2000) localised pro-MMP-2 protein to the stromal vascular endothelium and vCTB at term. They also reported that vCTB expression of pro-MMP-2 was not as pronounced as pro-MMP-9 expression at term.

None of the previous studies have attempted any sort of quantitative analysis and no study has analysed both MMP-2 and MMP-9 expression systematically throughout pregnancy in EVT, vCTB, endothelium and stroma. The current immunohistochemical study agrees with previous findings by Polette et al. that EVT expression of MMP-2 tends to decrease with gestation, being strongest during the first trimester and declining towards term. Since not all of the placental sections studied contained EVT it is possible that if there had been more data, the analysis between the individual trimesters would have reached significance. The earlier studies by Autio-Harmainen et al. and Fernandez et al. also agree with the data presented herein on the EVT expression of MMP-2 early in gestation. These studies did not examine placenta from later than 11 weeks of gestation so no comparison with the current study can be made.

The findings of the current study are also in agreement with those which show vCTB MMP-2 expression early in pregnancy (Autio-Harmainen et al. 1992 and Fernandez et al.1992) and further this by showing that this decreases towards term. The study by Sawicki et al. (2000) found low levels of vCTB MMP-2 expression at term but did not study earlier placentae.

The data reported herein for endothelial MMP-2 expression supports the results reported by Autio-Harmainen et al. (1992) who found no endothelial expression of MMP-2 protein or mRNA in early gestation placentae and Polette et al. (1994) who found endothelial expression of MMP-2 protein in term placentae.

Thus taking all the studies into consideration it may be suggested that MMP-2 is primarily expressed in the vCTB and EVT early in pregnancy around the time of invasion. Later in gestation it seems that expression of both vCTB and EVT is down-

regulated and endothelial and stromal expression increases. This would support a hypothesis that MMP-2 may be important in invasion in early pregnancy but may also have a role in vascular and matrix remodelling in the villi later in pregnancy.

4.3.3.2 MMP-9 immunohistochemistry

The most marked difference in the current study between MMP-9 and MMP-2 expression was in the lack of vCTB expression of MMP-9 compared with MMP-2 expression. Throughout gestation there was virtually no vCTB MMP-9 expression. These findings agree with the study by Vettraino et al. (1996) who reported no MMP-9 expression on vCTB although they found strong MMP-9 expression on neutrophils throughout the placenta. However, while Sawicki et al. (2000) reported that pro-MMP-9 protein was expressed strongly in the vascular endothelium at term as was found in the current study, they found that it was also expressed consistently in STB and weakly in CTB. Other previous studies on MMP-9 localisation using immunohistochemistry or in situ hybridization (Polette et al. 1994; Hurskainen et al. 1996) have investigated EVT expression. Both of these studies are in agreement that MMP-9 is expressed by EVT in early pregnancy but is downregulated towards term, which agrees with the results presented herein. Polette also reported MMP-9 expression in vCTB and in the decidua during the first trimester and endothelial and stromal expression at term. Hurskainen et al. found both MMP-9 protein and mRNA on villous endothelium and stroma. Interestingly, while MMP-9 mRNA was present on vCTB the protein was not reliably present. Huppertz et al. (1998) also found MMP-9 immunoreactivity in EVT in the first trimester. In cryostat but not paraffin sections this was reduced in the second trimester. In term placenta the situation was unclear since strong EVT staining was found in cryostat but not in paraffin sections. This study illustrates one cause of discrepancies between studies - the type of section used (paraffin/cryostat). Other methodological problems may be caused by differences in tissue preparation and antigen retrieval techniques. In croystat sections missing reactivity may be caused by diffusion, although the antigenicity is better preserved than in paraffin sections. Furthermore, different antibodies may have different reaction patterns. Confounding data may also result from choice of placental material - in the current study all cases were normal whereas some studies have allowed abnormal placentae (eg blighted ouvum) to be included. One other source of

difference may be the time taken between placental collection and specimen preparation which may result in partial degradation of proteins within the tissue. It is interesting to note the increase in MMP-9 expression in EVT between 14-19 weeks of gestation which may correspond with the proposed second wave of invasion.

Table 25 Summary of published immunohistochemistry studies on MMP-9 and MMP-2 expression

Authors	Date Published	Type of Study	Number of samples studied	Gestation of samples studied	MMP-2 distribution	MMP-9 distribution	Notes
Autio-Harmainen et al.	1992	Immunohistochemistry (paraffin/cryostat sections) In situ hybridisation	7	8-11 weeks of gestation	mRNA and protein : EVT, villous stroma, decidua Protein: vCTB/STB	Not studied	Blighted ovum/tubal pregnancy included
Fernandez et al.	1994	Immunohistochemistry (paraffin sections)	17	First trimester	Protein: EVT, vCTB, decidua, villous stoma	Not studied	
Polette et al.	1994	Immunohistochemistry (paraffin sections) In situ hybridisation	14	First trimester and term	Protein and mRNA: EVT and decidua in 1st trimester, less at term	Protein and mRNA: EVT, vCTB and decidua in 1st trimester only	

					Stroma and endothelium at term		
Hurskainen et al.	1996	Immunohistochemistry (paraffin/frozen sections) In situ hybridisation	8	8-10 weeks of gestation	Not studied	mRNA and protein: EVT, stroma, endothelium mRNA only on vCTB	
Vettraino	1996	Immunohistochemistry (paraffin sections) In situ hybridisation	8	8 weeks of gestation (2 samples) and term (6 samples)	Not studied	Protein and mRNA: only in granulocytes of maternal sinusoidal spaces, fetal capillaries and EVT	
Huppertz et al.	1998	Immunohistochemistry (paraffin/frozen sections)	14	9, 10, 12, 16 and 38-41	Protein: EVT throughout	Protein: EVT in 1st trimester,	Major differences

				weeks of gestation	gestation	unclear in later stages	between paraffin/cryostat sections
Sawicki et al.	2000	Immunohistochemistry (paraffin)	Unknown	Term	Protein: vCTB, endothelium	Protein: vCTB, endothelium	Lower expression of MMP-2 than MMP-9
Isaka et al.	2003	Immunohistochemistry In situ hybridisation Film in situ zymography	30	First trimester/term	mRNA and protein: EVT throughout	mRNA and protein: EVT, vCTB, stroma throughout but neither on EVT at term	Gelatinase activity in 1st trimester but not at term

4.3.3.3 Studies of MMP secretion – MMP-2 and MMP-9

Although the current study did not examine secretion of MMPs by cultured CTB, it is informative to compare the results obtained from the zymography of placental homogenates with published studies on CTB secretion of MMPs.

Librach et al. (1991) reported that purified first trimester trophoblasts secreted MMP-9 and this was required for invasiveness *in vitro*. The same study supported previous data which showed that purified third trimester villous trophoblasts secreted MMP-2 but not MMP-9 (Fisher et al. 1989). However, a more recent study (Graham and McCrae, 1996) reported that cultured third trimester trophoblasts secrete primarily MMP-9 and Sawicki et al. (2000) reported that third trimester cultured CTB secreted MMP-9 and some MMP-2. Shimonovitz et al. (1994) also reported that cultured first trimester trophoblasts secreted both MMP-2 and MMP-9 in large amounts. By the third trimester only MMP-9 was secreted in large amounts although considerably less was secreted than during the first trimester.

Lim et al. (1997) studied MMP expression in culture medium from cultured CTB from normal and pre-eclamptic third trimester placentae. They found that pre-eclamptic pregnancies were associated with both reduced invasion and decreased MMP-9 secretion. Hence this study supports the reports that MMP-9 is still expressed in the third trimester.

These four latter studies tend to agree with the current study in finding that MMP-9 is not exclusive to the first trimester but rather continues to be expressed until term. The study by Shimonovitz et al. (1994) also agrees with the findings presented here in the respect that MMP-2 secretion declined between the first to the third trimester. It is possible that different trophoblast isolation protocols may account for some of the differences in *in vitro* data.

Using ELISA and zymography Niu et al. (2000) found that 1st trimester cultured villous tissue secreted around 10x more pro-MMP-2 than pro-MMP-9, and that pro-MMP-2 levels decreased dramatically in the 2nd and 3rd trimesters. MMP-9 levels however did not decrease significantly. Active MMP-2 was also secreted from villous tissue in the first trimester in smaller quantities than pro-MMP-2. These results are comparable to those presented herein. In some of the zymography gels in the current study it was possible to see faint bands of active MMP-2 although these were not large enough to be quantified by zymography. On the other hand, Niu et al.

reported that 1st trimester CTB cultured in the absence of stromal cells had pro-MMP-9 levels 10x those of pro-MMP-2. Many studies which have cultured CTB alone have emphasized the importance of the role of MMP-9 in invasion as opposed to MMP-2. This study, in conjunction with the previous one suggests that care should be taken in interpreting these results as the absence of stromal cells may influence production of MMPs dramatically. Moreover, Campbell et al. (2003) cultured CTB alone and with decidual endothelial cells (DEC) and found that coculture of term CTB with DEC resulted in reduced pro-MMP-9 expression and reduced CTB invasion compared to CTB cultured alone. This further supports the concept that MMP secretion profiles of CTB cultured alone may not accurately reflect the *in vivo* situation and that other cells may secrete critical regulators of MMP expression and activity. Niu et al. also examined MMP-9 and MMP-2 levels in placental extracts and found no significant decrease in either MMP-2 or MMP-9 with gestation. We have found however that the presence of EDTA in the homogenisation buffer causes inhibition of MMP activity and in the study by Niu et al. EDTA was present. The homogenisations in this thesis were performed in the absence of EDTA.

Xu et al. (2000) were the first to study the dynamics of MMP secretion within the first trimester and reported a gradual increase in MMP-9 secretion from week 7 – 11 cultured CTB and a corresponding decline in MMP-2 secretion. mRNA expression corresponded with the protein expression studies which used ELISA and zymographical methods. This finding is not in agreement with the zymography results herein, which showed that in placental homogenates MMP-9 activity decreased through the first trimester. The differences may again arise from the fact that the tissues used in this study were whole placenta and therefore contain many cell types. However the current study did not find vCTB expression of MMP-9 in the first trimester and it is unlikely that all of the isolated cells in the study by Xu et al. were EVT. Therefore it may be possible that the matrix on which CTB were cultured may affect MMP production (Bichof et al. 1991).

Sawicki et al. (2000) proposed that it was the differentiated STB as opposed to undifferentiated CTB which released MMP-9 and MMP-2, in particular the latter and also reported that >90% of the secretion was in a basolateral direction. Basal release from STB, they suggested, would eject the gelatinases onto the trophoblast basal lamina in the third trimester when there are few underlying CTB and thus enable increased villous umbilical angiogenesis, growth of villous STB and of vascular

endothelium. In this study the homogenates of cultured term CTB had high levels of MMP-9 but this was not reflected in the culture medium in the first 24 hours. No MMP-2 was secreted or was present in cell homogenates after 24 hours. On the contrary STB homogenates (7 day culture period) and culture medium had high MMP-9 levels and MMP-2 was present in the culture supernatant. The current study, in contrast, found very little vCTB or STB expression of MMP-9 although MMP-2 expression was present in small amounts at term.

Caniggia et al. have also studied the expression of MMP-9 and MMP-2 in early pregnancy (Caniggia et al. 1999; Caniggia et al. 2000b). Explant studies by this group have led to the hypothesis that HIF-1α expression is high between 5-8 weeks of gestation, resulting in high TGF-β_3 levels and consequent inhibition of MMP-9 expression. MMP-2 expression was reported to be high during early gestation during the low oxygen stage. The group reported that HIF-1α expression fell around 9 weeks of gestation as did TGF-β_3 and suggested that this led to increased MMP-9 expression and invasion. MMP-2 expression decreased at the same stage. The authors concluded that MMP-2 is involved in CTB proliferation and MMP-9 in EVT invasion.

Difficulties in comparing the current study or other studies to those performed by Caniggia and colleagues arise from the different classification systems for gestation used and furthermore there may be inherent problems with the explant culture model used by the group (chapter 8). The current study did find MMP-2 expression in vCTB particularly in the first trimester but the expression did not seem to be particularly associated with proliferation nor was there a sudden reduction at the end of the first trimester although expression did decrease with increasing gestation. MMP-2 expression was also present on EVT which suggests it may also be involved in invasion. MMP-9 expression was present on EVT which supports the previous study in the role of this molecule in invasion. The work of Caniggia et al. will be discussed further in chapter 5 in relation to hypoxia and HIF-1α.

A very recent study (Isaka et al. 2003) studied expression of MMP-2 and MMP-9 protein and mRNA and also examined gelatinase activity on placental sections using film in situ zymography. In first trimester placental sections, MMP-2 expression and activity was present in EVT and MMP-9 in vCTB. At term, although MMP-2 expression was seen in EVT, gelatinase activity was not. In purified CTB gelatinase activity was present in the first trimester but not at term. The invasive

ability of early CTB was inhibited by TIMP-2 or by MMP-2 antibody but not by TIMP-1 or by MMP-9 antibody to as great a degree, suggesting that MMP-2 may be important in invasion. The role of TIMPs is considered in chapter 5.

In summary the current study supports several others previous studies in finding that MMP-9 continues to be expressed through the 2nd trimester and at term and hence may have other roles in addition to being involved in trophoblast invasion. The decrease in MMP-2 throughout pregnancy presented in this thesis is also supported by several other studies. The present study suggests MMP-2 may also be important during the first trimester in trophoblast invasion and that levels of MMP-2 may vary more with gestation than MMP-9 levels, in particular declining in the vCTB and EVT whilst increasing in the endothelium.

While the results presented herein on the location of MMP-2 in the placenta tend to agree with published studies, discrepancies exist between studies as to where MMP-9 is expressed. The conclusion that vCTB expression of MMP-9 is low throughout pregnancy is different from some other studies, although it is in agreement with the work of Vettraino et al. (1996) and Hurskainen et al. (1996). However the results herein show decreasing EVT expression with progression of pregnancy and increasing endothelial expression, which agrees with the findings of several published studies. It thus seems likely that both MMP-2 and MMP-9 may be important in various processes during placentation in several different cell types.

Chapter 5: Detection and quantification of TIMP-1, TIMP-2 and HIF-1α in placenta throughout pregnancy using immunohistochemistry

In the previous chapter the expression and activity of MMP-2 and MMP-9 were studied in placenta throughout gestation. While MMPs are thought to be important in controlling aspects of placental development, they themselves are subject to complex regulatory processes which may also have a role in placental development. Therefore the expression of several regulators of MMPs in sections of the same placentae as had been used in MMP-2 and MMP-9 immunohistochemistry (chapter 4) was investigated next.

Thus the aims of this section were three-fold:

- Firstly to examine the cellular distribution of TIMP-1, TIMP-2 and HIF-1α in placentae from 7-19 weeks of gestation and from term placentae
- Secondly to perform a semi-quantitative analysis of the expression of TIMP-1, TIMP-2 and HIF-1α in placentae from 7-19 weeks of gestation and from term placentae
- Thirdly to compare the spatial and temporal distribution of these molecules to the distribution of MMP-2 and MMP-9 as reported in chapter 4

5.1 TIMP-1 immunohistochemistry

Immunohistochemistry was performed using a TIMP-1 antibody (Biogenesis) as described in section 2.2. The antibody was used at a dilution of 1/250 and controls where rabbit IgG or antibody diluent was substituted for the primary antibody were negative (section 2.2).

Across gestation TIMP-1 was present to a greater or lesser extent on vCTB and STB, EVT, stroma and on muscle surrounding the endothelium but not on the endothelium itself.

Figure 45 shows photographs of representative TIMP-1 staining from selected gestations. Figure 45a shows a placenta at seven weeks of gestation with very little staining except in the intervillous space, which is likely to be endogenous peroxidase. Figure 45b shows moderately stained EVT at 13 weeks of gestation. Figure 45c shows a placenta at nineteen weeks of gestation with strong stromal and muscle staining.

Figure 45d shows a placenta at term again with strong stromal and muscle staining. The raw data obtained from scoring the stained sections is shown in table 26.

The data was grouped into trimesters and statistical analysis was carried out to determine if there were any statistical differences in TIMP-1 expression between trimesters. A summary of the descriptive statistics of the data is given in table 27 and table 28 shows the results of the statistical analysis for TIMP-1. No assumptions were made as to the normality of the distribution of the data and non-parametric testing was used throughout.

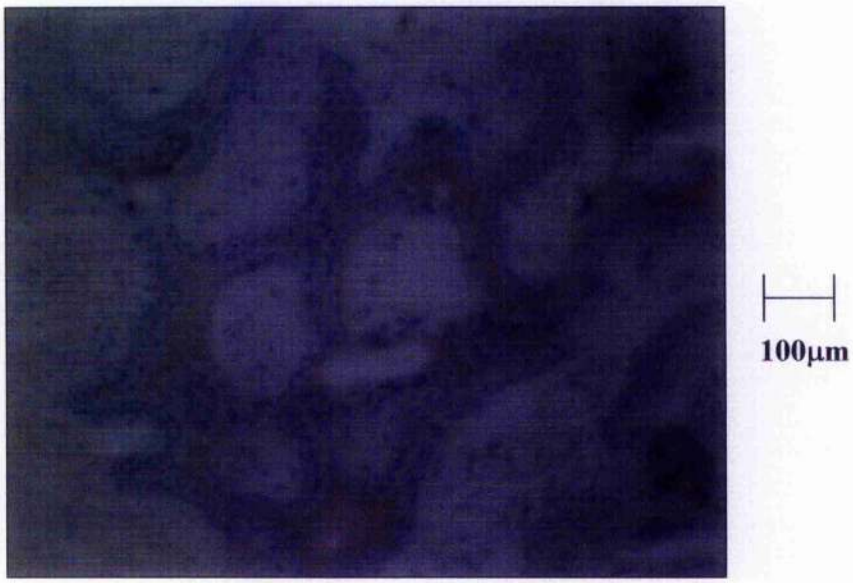
Kruskal-Wallis analysis showed a highly significant change in muscle staining between the three trimesters ($p<0.01$). Mann-Whitney analysis subsequently showed significantly lower TIMP-1 expression in the first trimester compared with the third trimester ($p<0.05$) and significantly lower expression in the first trimester compared with the second trimester ($p<0.05$).

Kruskal-Wallis analysis also showed a significant change in stromal staining between the three trimesters ($p<0.02$). Mann-Whitney analysis subsequently showed significantly lower TIMP-1 expression in the second trimester compared with the third trimester ($p<0.02$) and significantly lower expression in the first trimester compared with the third trimester ($p<0.01$).

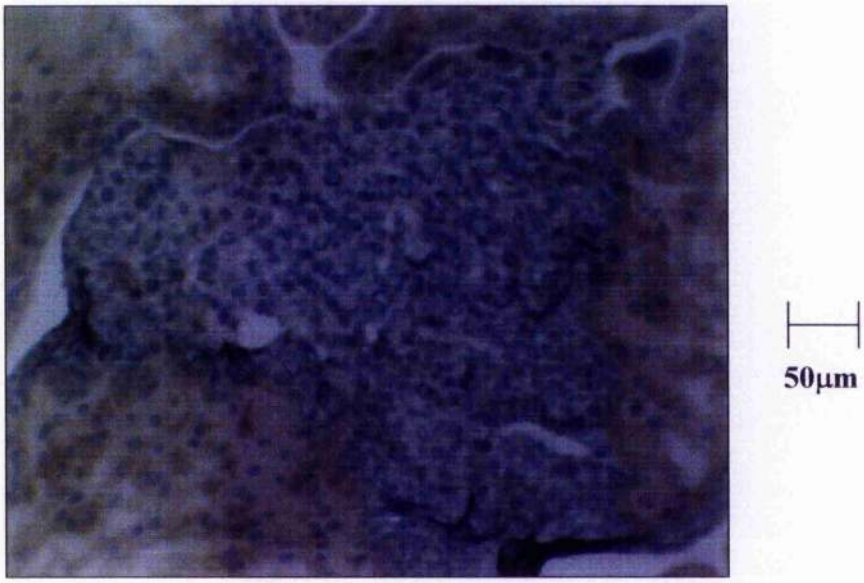
No significant changes in TIMP-1 expression were found in any of the tests on vCTB, EVT or on the endothelium.

Figure 45 Immunohistochemistry of TIMP-1 throughout gestation

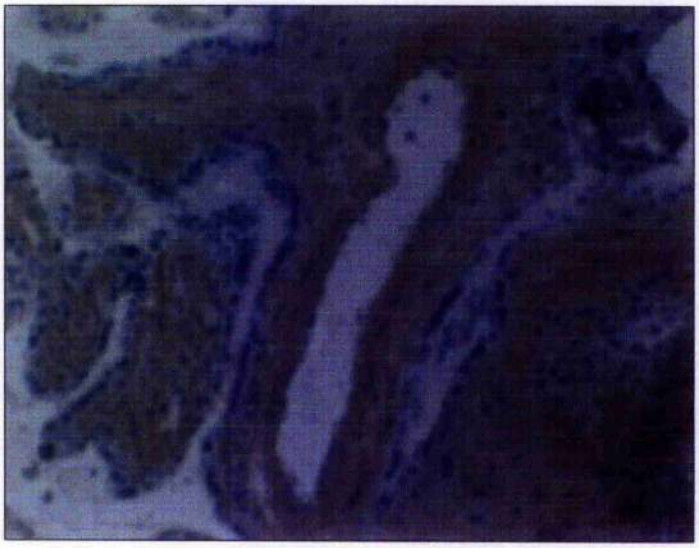
a) 7 weeks of gestation



b) 13 weeks of gestation

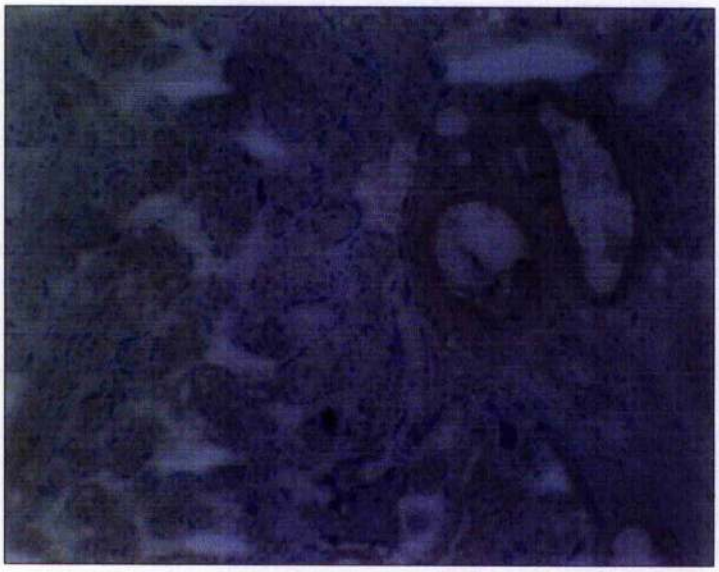


c) **19 weeks of gestation**



50μm

d) **Term**



100μm

Table 26 Semi-quantitative analysis of TIMP-1 immunostaining

Gestation (weeks)	vCTB	EVT	Endothelium	Muscle	Stroma
7	0	0.5	0	0	0.5
7	2	2	0	0	2
8	0	2	0	0	0.5
9	1	1	0	0	2
11	0	N/P*	0	0	1
11	0.5	1.5	0	0	1.5
12	0.5	1.5	0	0	1.5
13	0	1.5	0	0	1.5
14	0.5	0.5	0	1.5	1.5
14	1	N/P	0	1.5	1.5
14	0	N/P	0	0	1.5
15	0	N/P	0	0	1
15	0	N/P	0	2	1.5
15	0	0.5	0	0	2
16	0	1	0	0	1
16	0	N/P	0	1	2.5
16	0	1	0	2	3
17	0	N/P	0	0	1
17	0	N/P	0	2	2
17	0	N/P	0	1.5	2
17	0	1	0	2	0.5
18	1.5	1.5	2	0	1.5
18	0	N/P	0	0	0.5
19	0	N/P	0	1.5	2
40	1	N/P	0	3	3
40	0.5	N/P	0	2.5	3.5
40	0	N/P	0	0	2
40	0	N/P	0	2.5	2.5

*NP = Not present

Table 27 TIMP-1 immunohistochemistry descriptive statistics

	Mean	Median	Max	Min	Range between min and max	SE	SD
vCTB							
1st	0.5	0.25	2.0	0.0	2.0	0.25	0.71
2nd	0.19	0.0	1.5	0.0	1.5	0.11	0.44
3rd trimester	0.38	0.25	1.0	0.0	1.0	0.24	0.48
EVT							
1st	1.43	1.5	2.0	0.5	1.5	0.2	0.53
2nd	0.92	1.0	1.5	0.5	1.0	0.15	0.38
3rd trimester	N/A*	N/A	N/A	N/A	N/A	N/A	N/A
Endothelium							
1st	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2nd	0.13	0.0	2.0	0.0	2.0	0.13	0.5
3rd trimester	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Muscle							
1st	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2nd	0.94	1.25	2.0	0.0	2.0	0.22	0.89
3rd trimester	2.0	2.5	3.0	0.0	3.0	0.68	1.35
Stroma							
1st	1.31	1.50	2.0	0.50	1.50	0.21	0.59
2nd	1.56	1.50	3.0	0.50	2.5	0.17	0.68
3rd trimester	2.75	2.75	3.5	2.0	1.5	0.32	0.65

*N/A = Not Applicable

Table 28 **Analysis of variation in TIMP-1 immunostaining between the three trimesters**

Test	vCTB	EVT	Endothelium	Muscle	Stroma
	p-value				
Kruskal-Wallace $1^{st}/2^{nd}/3^{rd}$ trimester	N/S*	N/S	N/S	p<0.01	p<0.02
Mann-Whitney					
$1^{st}/2^{nd}$ trimester	N/S	N/S	N/S	p<0.05	N/S
$2^{nd}/3^{rd}$ trimester	N/S	N/A*[1]	N/S	N/S	p<0.02
$1^{st}/3^{rd}$ trimester	N/S	N/A	N/S	p<0.05	p<0.01

*N/S = Non Significant

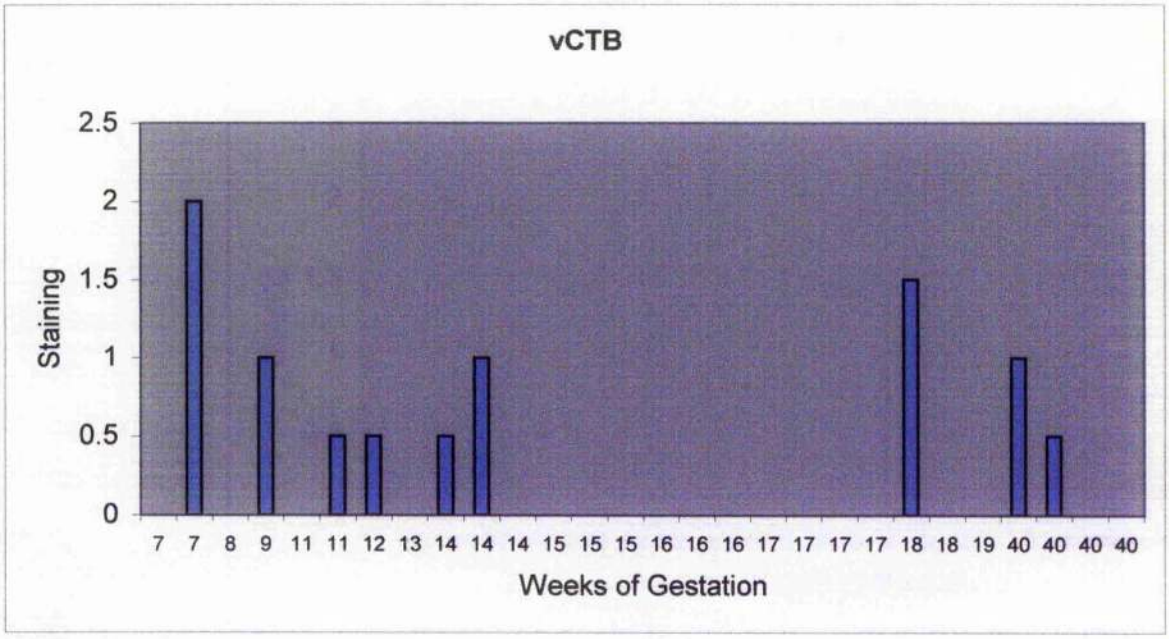
*[1] N/A = test not applicable (too few samples)

Correlation analysis was then performed to determine if there were any correlations in TIMP-1 expression within each trimester. The data was represented on histograms for each cell type (figure 46). Note that for some cell types many values were zero (no staining) and therefore do not appear as bars on the graph. The data was then analyzed using a Spearman's correlation. Analyses were carried out to determine correlation of TIMP-1 expression within the 1^{st} trimester (7-13 weeks), 2^{nd} trimester (14-19 weeks), $1^{st}/2^{nd}$ trimester (7-19 weeks), $2^{nd}/3^{rd}$ trimester (14-40 weeks) and $1^{st}/2^{nd}/3^{rd}$ trimesters (7-40 weeks). The results of the analysis are shown in table 29.

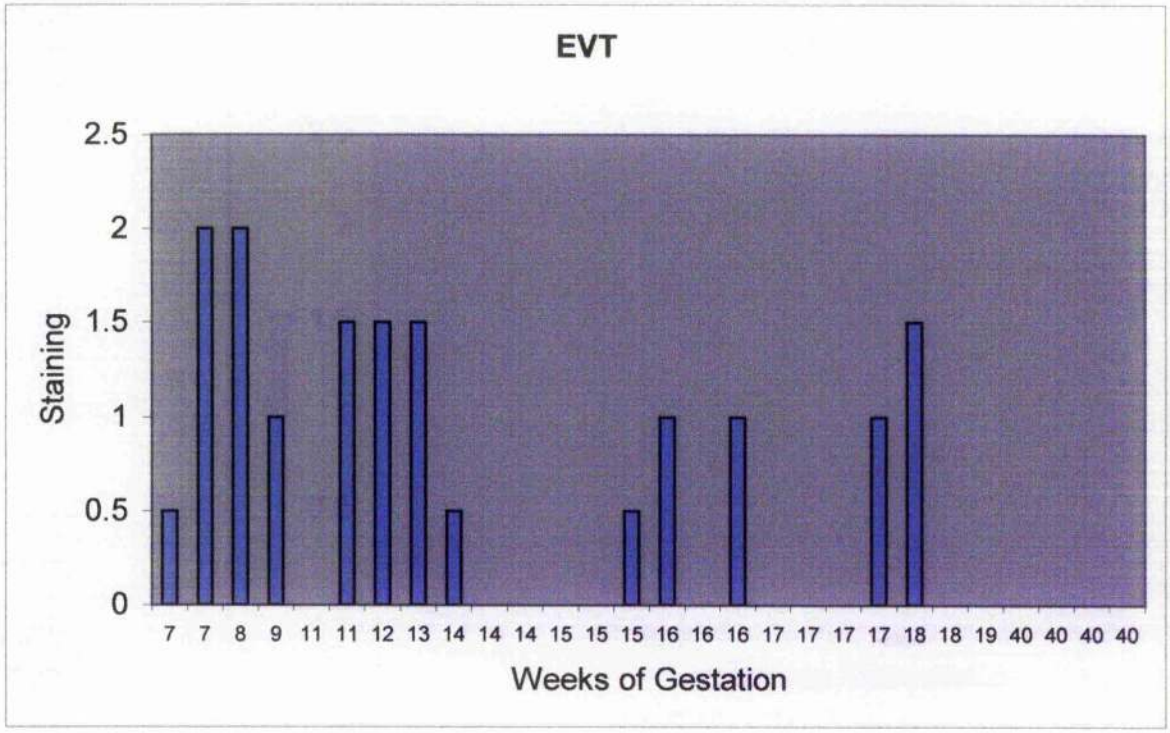
No significant correlations in TIMP-1 expression were found in any of the tests on vCTB (figure 46a) or on the endothelium (not shown as virtually all values were = 0). TIMP-1 staining on EVT (figure 46b) was positively correlated with gestation (staining increased as gestation increased) between 14-19 weeks ($p<0.01$). TIMP-1 staining on muscle (figure 46c) was positively correlated with gestation (staining increased as gestation increased) between 7-19 weeks ($p<0.05$) and between 7-40 weeks ($p<0.01$) of gestation. TIMP-1 staining on the stroma (figure 46d) was positively correlated with gestation (staining increased as gestation increased) between 7-40 weeks of gestation ($p<0.05$).

Figure 46 Histograms of TIMP-1 immunostaining throughout gestation

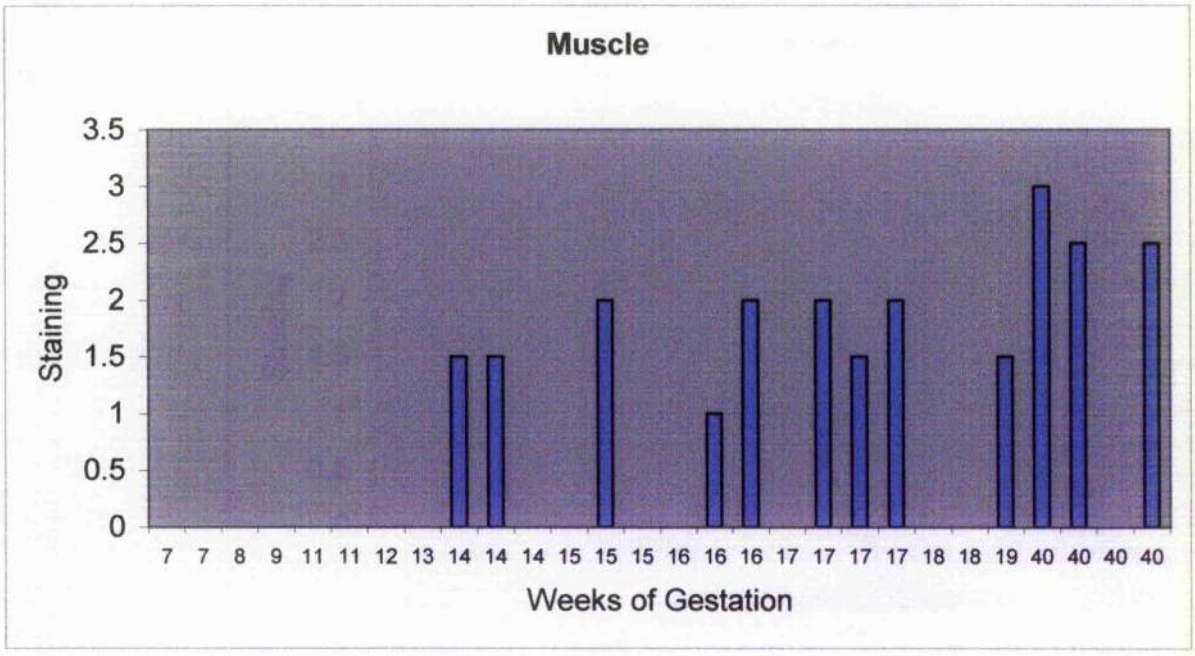
a)



b)



c)


Muscle
Staining
3.5
3
2.5
2
1.5
1
0.5
0
7 7 8 9 11 11 12 13 14 14 14 15 15 15 16 16 16 17 17 17 17 18 18 19 40 40 40 40
Weeks of Gestation

d)

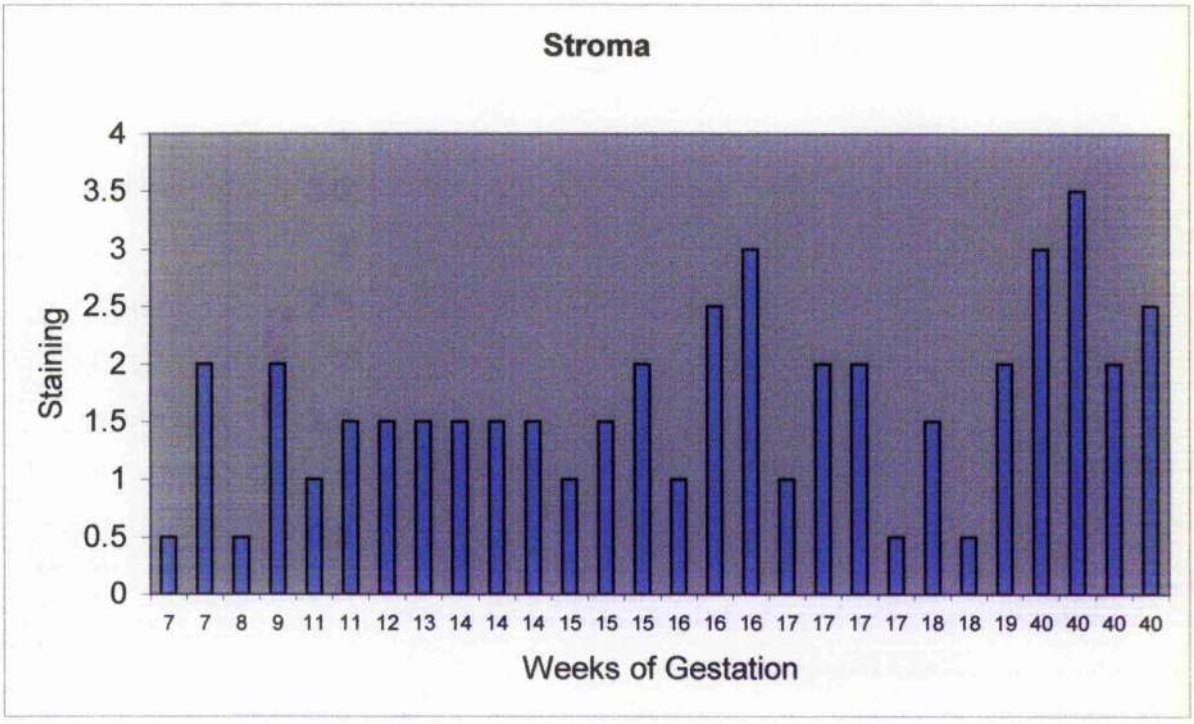

Stroma
Staining
4
3.5
3
2.5
2
1.5
1
0.5
0
7 7 8 9 11 11 12 13 14 14 14 15 15 15 16 16 16 17 17 17 17 18 18 19 40 40 40 40
Weeks of Gestation

Table 29 Correlation of TIMP-1 immunostaining with gestation

Test	vCTB	EVT	Endothelium	Muscle	Stroma
Spearman's Rank	p-value and R-value				
1st trimester	N/S*	N/S	N/S	N/S	N/S
2nd trimester	N/S	p<0.01 R=0.93	N/S	N/S	N/S
1st/2nd trimester	N/S	N/S	N/S	p<0.05 R=0.44	N/S
2nd/3rd trimester	N/S	N/S	N/S	N/S	N/S
1st/2nd/3rd trimester	N/S	N/S	N/S	p<0.01 R=0.54	p<0.05 R=0.41

* N/S = Non-Significant

5.2 TIMP-2 immunohistochemistry

Immunohistochemistry was subsequently performed using a TIMP-2 antibody (Biogenesis) as described in section 2.2. The antibody was used at a dilution of 1/300. The staining was scored on a 0-4 scale (section 2.6). Control slides where sheep IgG or antibody diluent was substituted for primary antibody showed no staining (section 2.2).

Across gestation TIMP-2 staining was present to a greater or lesser extent on vCTB and STB, EVT, stroma, endothelium, and on the muscle surrounding the endothelium.

Figure 47 shows photographs of representative TIMP-2 staining from selected gestations. Figure 47a shows a placenta at seven weeks of gestation with strong vCTB staining but little other staining. Figure 47b shows a placenta at nine weeks of gestation with stronger endothelial staining. Figure 47c shows moderately stained EVT at 16 weeks of gestation. Figure 47d shows a placenta at term with strong endothelial staining, some muscle staining but little vCTB staining.

The raw data obtained from scoring the stained sections is shown in table 30. The data was grouped into trimesters and statistical analysis was carried out to determine if there were any statistical differences in TIMP-2 expression between trimesters. A summary of the descriptive statistics of the data is given in table 31 and table 32 shows the results of the statistical analysis for TIMP-2. No assumptions were made as to the normality of the distribution of the data and non-parametric testing was used throughout.

Kruskal-Wallis analysis showed a significant change in vCTB and EVT staining between the three trimesters ($p<0.05$) and a highly significant change in endothelial staining between the three trimesters ($p<0.01$) but no differences in muscle or stromal staining.

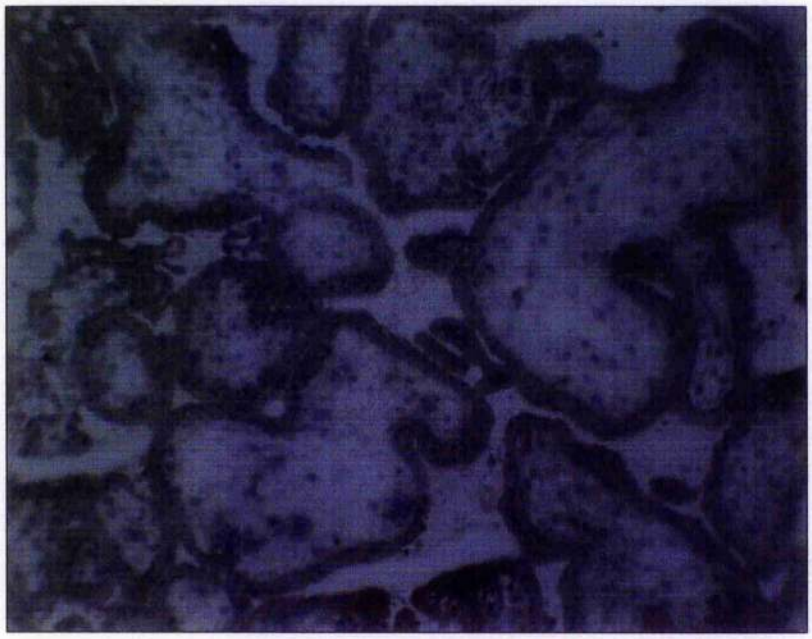
Mann-Whitney analysis showed significantly lower TIMP-2 expression in the first trimester compared with the third trimester on the vCTB ($p<0.01$), muscle ($p<0.05$) and stroma ($p<0.05$).

Mann-Whitney analysis also showed significantly lower TIMP-2 expression in the endothelium in the first trimester compared with the third trimester ($p<0.02$) and in the first trimester compared with the second trimester ($p<0.02$).

There were no significant differences in EVT scoring although the $1^{st}/2^{nd}$ trimester comparison almost reached significance.

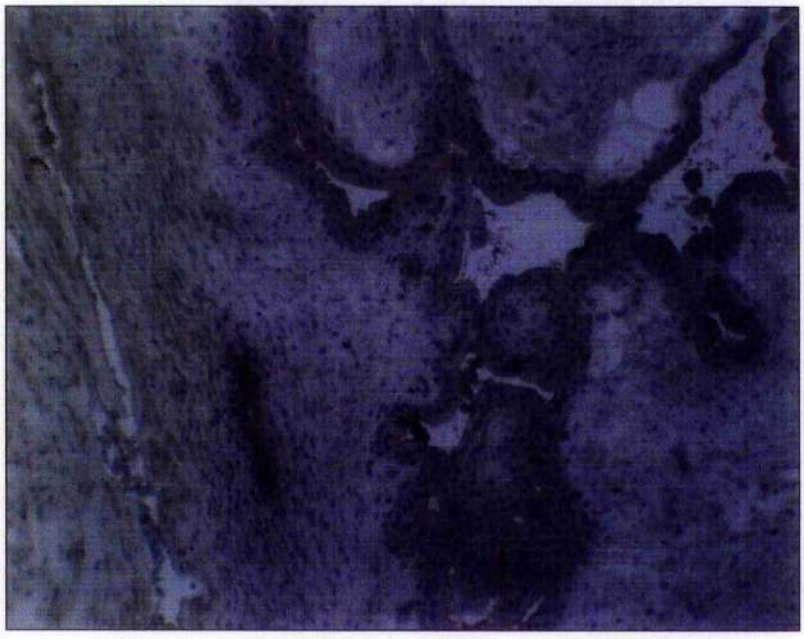
Figure 47 Immunohistochemistry of TIMP-2 throughout gestation

a) 7 weeks of gestation



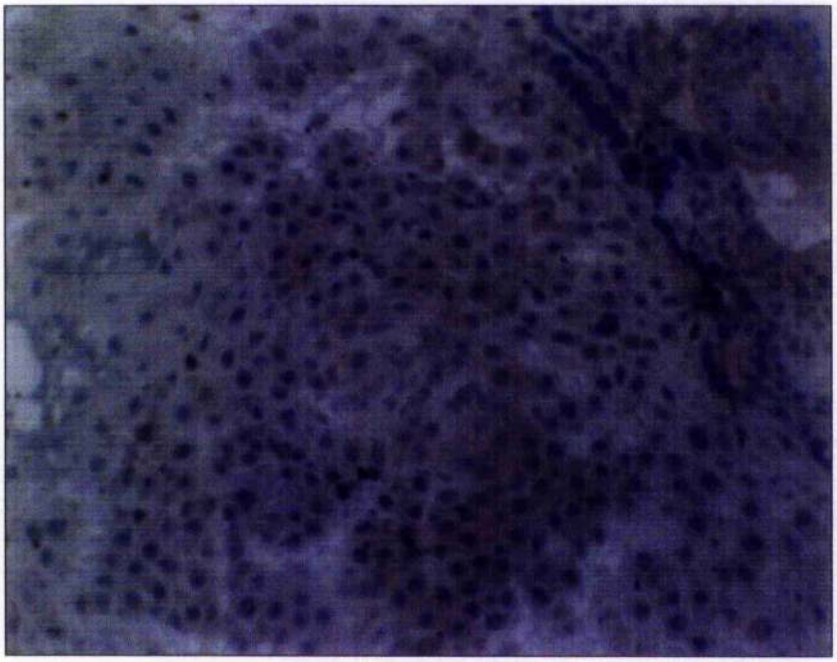
100μm

b) 9 weeks of gestation



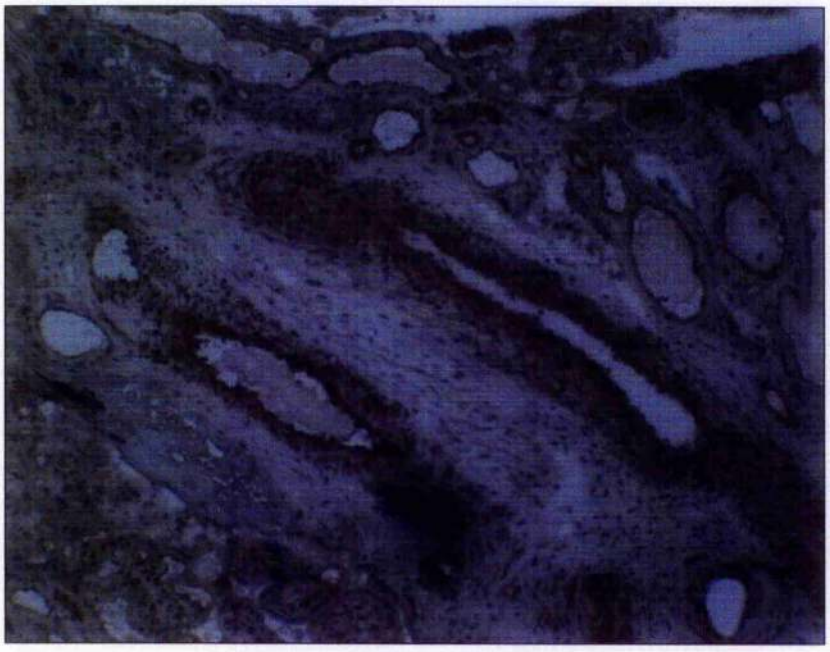
100μm

c) **16 weeks of gestation**



50μm

d) **Term**



100μm

Table 30 Semi-quantitative analysis of TIMP-2 immunostaining

Gestation (weeks)	vCTB	EVT	Endothelium	Muscle	Stroma
7	2	2	0	0	0
7	1.5	N/P*	0	0	0
8	2	2	0	0	0
9	2	2	0	0	0.5
9	2.5	N/P	2.5	1	2
9	1.5	2	2	1	1
10	1.5	1.5	0	0	0.5
10	2.5	2.5	0	0	0.5
11	1.5	2	1.5	0.5	0.5
11	1	N/P	1	0	0.5
12	0	N/P	1	0	0.5
13	0.5	0.5	1.5	0.5	0.5
13	1.5	1.5	1.5	0.5	0.5
14	2	N/P	1.5	0	0.5
15	0.5	0.5	2	0	0
16	1.5	1.5	2	1.5	1
16	0	N/P	1.5	0	0.5
17	1	0.5	2.5	1.5	1.5
17	0	N/P	2	0.5	0.5
40	0	N/P	2.5	1	1.5
40	0.5	N/P	2	1	1
40	0.5	N/P	2.5	1	1

* NP –Not present

Table 31 TIMP-2 immunohistochemistry descriptive statistics

	Mean	Median	Max	Min	Range between min and max	SE	SD
vCTB							
1st	1.54	1.5	2.5	0.0	2.5	0.20	0.72
2nd	0.83	0.75	2.0	0.0	2.0	0.33	0.82
3rd trimester	0.33	0.50	0.50	0.0	0.50	0.17	0.29
EVT							
1st	1.78	2.0	2.5	0.5	2.0	0.19	0.57
2nd	0.83	0.5	1.5	0.5	1.0	0.33	0.58
3rd trimester	N/A*	N/A	N/A	N/A	N/A	N/A	N/A
Endothelium							
1st	0.85	1.0	2.5	0.0	2.5	0.25	0.90
2nd	1.92	2.0	2.5	1.5	1.0	0.15	0.38
3rd trimester	2.33	2.5	2.5	2.0	0.5	0.17	0.29
Muscle							
1st	0.27	0.0	1.0	0.0	1.0	0.11	0.39
2nd	0.58	0.25	1.5	0.0	1.5	0.30	0.74
3rd trimester	1.0	1.0	1.0	1.0	0.0	0.0	0.0
Stroma							
1st	0.54	0.50	2.0	0.0	2.0	0.14	0.52
2nd	0.67	0.50	1.5	0.0	1.5	0.21	0.52
3rd trimester	1.17	1.0	1.5	1.0	0.5	0.17	0.29

*N/A = Not Applicable

Table 32 **Analysis of variation in TIMP-2 immunostaining between the three trimesters**

Test	vCTB	EVT	Endothelium	Muscle	Stroma
Kruskal-Wallace	**p-value**				
$1^{st}/2^{nd}/3^{rd}$ trimester	p<0.05	p<0.05	p<0.01	N/S*	N/S
Mann-Whitney					
$1^{st}/2^{nd}$ trimester	N/S	N/S	p<0.02	N/S	N/S
$2^{nd}/3^{rd}$ trimester	N/S	N/A*[1]	N/S	N/S	N/S
$1^{st}/3^{rd}$ trimester	p<0.01	N/A	p<0.02	p<0.05	p<0.05

*N/S = Non Significant

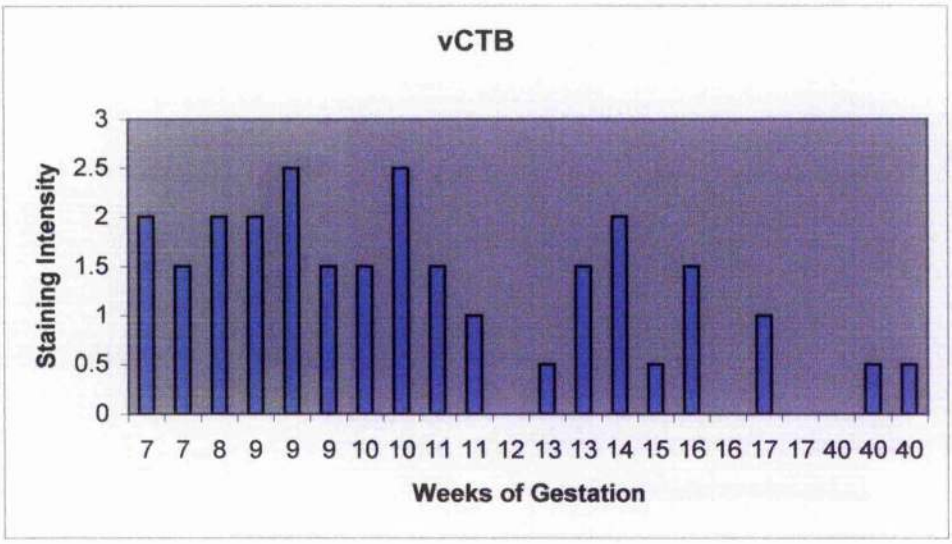
*[1] N/A = test not applicable (too few samples)

The data was then analysed to determine if there were any correlations in TIMP-2 expression within each trimester. The data was represented on histograms for each cell type (figure 48). Note that for some cell types many values were zero (no staining) and therefore do not appear as bars on the chart. The data was then analyzed using a Spearman's correlation. Analyses were carried out to determine correlation of TIMP-2 expression within the 1^{st} trimester (7-13 weeks), 2^{nd} trimester (14-19 weeks), $1^{st}/2^{nd}$ trimester (7-19 weeks), $2^{nd}/3^{rd}$ trimester (14-40 weeks) and $1^{st}/2^{nd}/3^{rd}$ trimesters (7-40 weeks). The results of the analysis are shown in table 33.

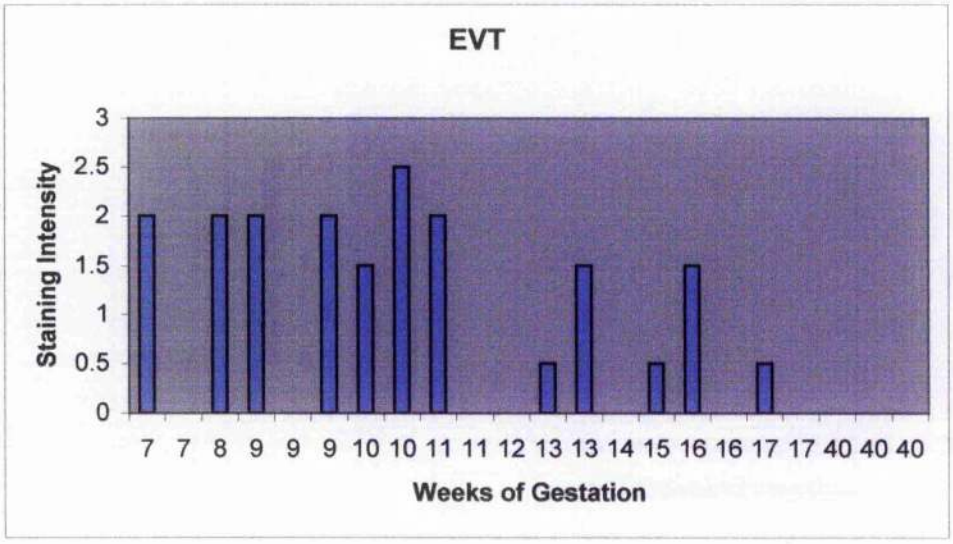
TIMP-2 staining on vCTB (figure 48a) was negatively correlated with gestation (staining decreased as gestation increased) between 7-13 weeks ($p<0.05$), between 7-19 weeks ($p<0.01$) and between 7-40 weeks ($p<0.01$) of gestation. TIMP-2 staining on EVT (figure 48b) was negatively correlated with gestation (staining decreased as gestation increased) between 7-19 weeks ($p<0.01$) and between 7-40 weeks ($p<0.01$) of gestation. TIMP-2 staining on endothelium (figure 48c) was positively correlated with gestation (staining increased as gestation increased) between 7-19 weeks ($p<0.01$), between 14-40 weeks ($p<0.05$) and between 7-40 weeks ($p<0.01$) of gestation. TIMP-2 staining on the stroma (figure 48d) was positively correlated with gestation (staining increased as gestation increased) between 14-40 weeks ($p<0.05$) and between 7-40 weeks ($p<0.02$) of gestation. No significant correlations in TIMP-2 expression were found in any of the tests on muscle (figure 48e) staining.

Figure 48 Histograms of TIMP-2 immunostaining throughout gestation

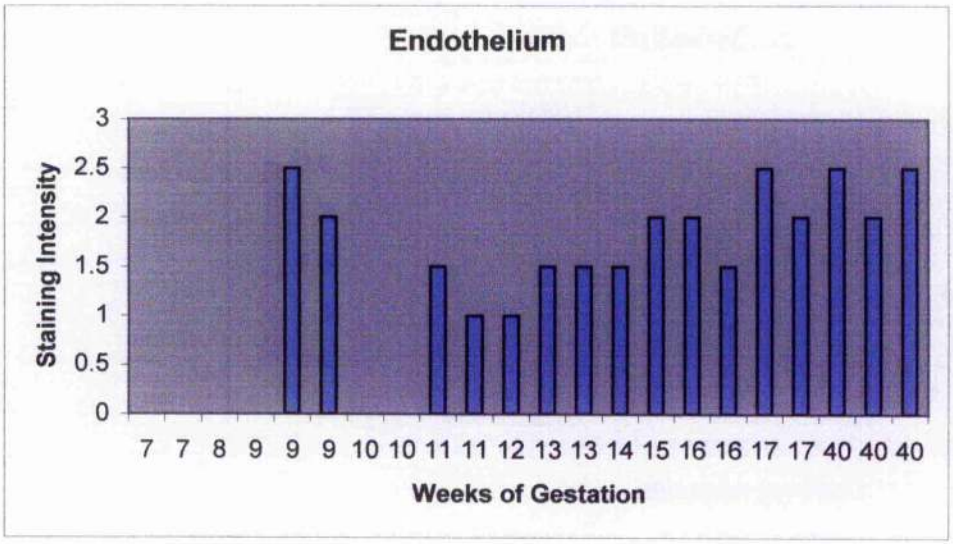
a)



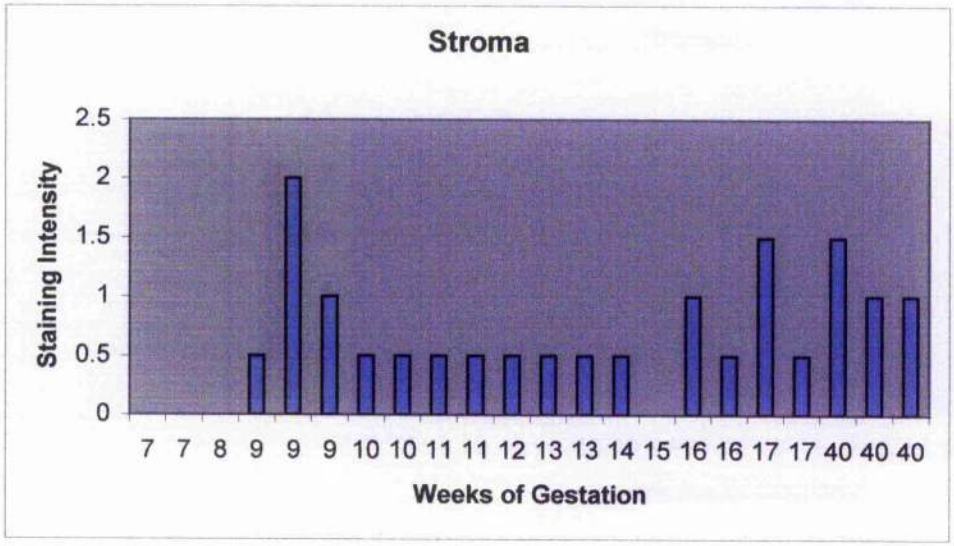
b)



c)


Endothelium
Staining Intensity
3
2.5
2
1.5
1
0.5
0
7 7 8 9 9 9 10 10 11 11 12 13 13 14 15 16 16 17 17 40 40 40
Weeks of Gestation

d)


Stroma
Staining Intensity
2.5
2
1.5
1
0.5
0
7 7 8 9 9 9 10 10 11 11 12 13 13 14 15 16 16 17 17 40 40 40
Weeks of Gestation

e)

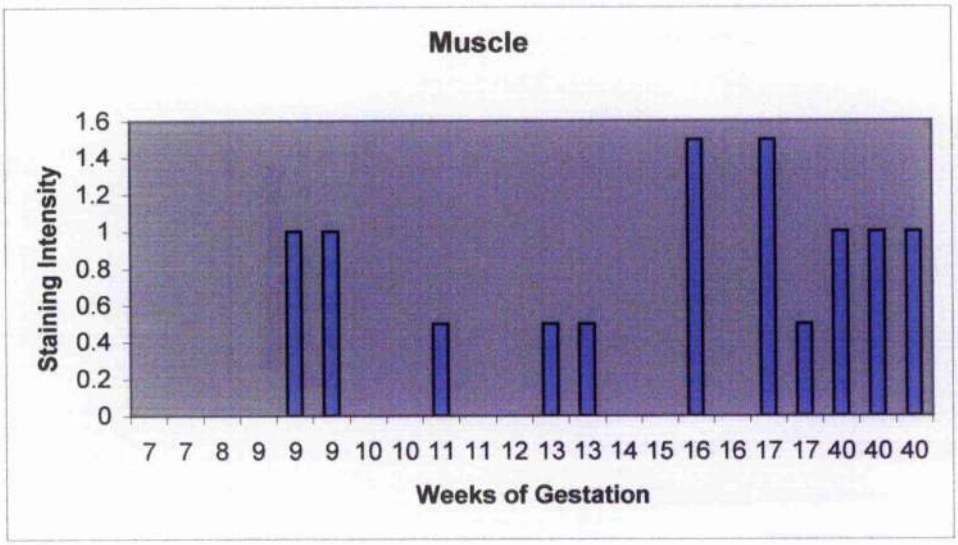

Muscle
Staining Intensity
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
7 7 8 9 9 9 10 10 11 11 12 13 13 14 15 16 16 17 17 40 40 40
Weeks of Gestation

Table 33 Correlation of TIMP-2 immunostaining with gestation

Test	vCTB	EVT	Endothelium	Muscle	Stroma
Spearman's Rank	p-value and R-value				
1st trimester	p<0.05 R=-0.61	N/S*	N/S	N/S	N/S
2nd trimester	N/S	N/S	N/S	N/S	N/S
1st/2nd trimester	p<0.01 R=-0.64	p<0.01 R=-0.76	p<0.01 R=0.63	N/S	N/S
2nd/3rd trimester	N/S	N/S	p<0.05 R=0.72	N/S	p<0.05 R=0.69
1st/2nd/3rd trimester	p<0.01 R=-0.71	p<0.01 R=-0.76	p<0.01 R=0.71	N/S	p<0.02 R=0.51

*N/S = Non-Significant

5.3 Discussion

5.3.1 Detection and quantification of TIMP-1 and TIMP-2 in placental sections using immunohistochemistry

TIMPs are well-documented regulators of MMP activity throughout mammalian systems (section 1.4). TIMPs inhibit the activity of MMPs by binding to activated MMPs in a 1:1 ratio to form a high affinity, essentially irreversible complex. While TIMPs bind most MMPs at the highly conserved active zinc-binding site, they can also bind other domains of both MMP-9 and MMP-2 (Birkedal-Hansen, 1995). TIMP-1 can bind to and inhibit both active and pro-MMP-9 (Goldberg et al. 1992) although it can also inhibit the active forms of most other MMPs. TIMP-2 binds preferentially to active and pro-MMP-2. It is the balance between activated MMP and free TIMP that determines overall MMP activity. TIMP-1 is responsive to a variety of factors such as cytokines, growth factors and phorbol esters, whereas TIMP-2 is largely constitutively expressed in mammalian tissue (reviewed in Woessner and Nagase, 2000).

It is unknown whether the MMP-9 and MMP-2 antibodies which were used in the immunohistochemistry experiments in chapter 4 detect only free MMPs or if they also detect MMPs bound to TIMPs. It may be that bound MMP-TIMP complexes show partial cross-reactivity with the antibodies. Zymography does not detect bound MMP-TIMP complexes as these are unable to degrade the gelatin and thus the experiments in section 4.1 should provide an accurate summary of MMP activity across gestation. It is also possible to perform reverse zymography to quantify levels of TIMPs in a sample or to perform film in-situ zymography, a technique which detects gelatinase activity in a placental section. Additionally TIMP expression could possibly be assessed semi-quantitatively by western blotting. Due to time constraints none of these experiments was undertaken but would be interest for further study. This current study undertook to examine the distribution of TIMP-1 and TIMP-2 within the placenta to determine whether either of the TIMPs were co-distributed with MMP-9 or MMP-2 in the hope that this would provide further insight into the mechanisms which govern placental development. For such potentially important regulators of MMP activity and thus of placental development there are surprisingly few studies which have previously examined TIMP distribution by

immunohistochemistry in the placenta and these will be discussed in relation to the results presented in this thesis.

5.3.2 TIMP-1: Summary of immunohistochemical analysis

Across gestation TIMP-1 was present to a greater or lesser extent on vCTB and STB, EVT, stroma and on muscle surrounding the endothelium but not on the endothelium itself. Statistical analysis showed no significant changes in TIMP-1 expression between the three trimesters on vCTB, EVT or on the endothelium. However, TIMP-1 staining on EVT was positively correlated with gestation between 14-19 weeks. Analysis showed significantly lower TIMP-1 expression in muscle in the first trimester compared with the third trimester and significantly lower expression in the first trimester compared with the second trimester. TIMP-1 staining on muscle was positively correlated with gestation between 7-19 weeks and between 7-40 weeks of gestation. It was also found that stromal TIMP-1 expression was significantly lower in the second trimester compared with the third trimester and significantly lower expression in the first trimester compared with the third trimester. TIMP-1 staining on the stroma was positively correlated with gestation between 7-40 weeks of gestation.

5.3.3 Comparison with MMP-9 immunohistochemical analysis

Since TIMP-1 is known to specifically inhibit both pro and active MMP-9 the distributions of TIMP-1 with MMP-9 were compared. While MMP-9 was localised to EVT, endothelium, muscle and stroma, TIMP-1 was localised to vCTB, EVT, muscle and stroma. The main change in MMP-9 staining with increasing gestation was an increase in endothelial staining. A significant positive correlation of MMP-9 staining with gestation on EVT between 14-19 weeks but a negative correlation overall between 7-40 weeks of gestation was also found. Interestingly TIMP-1 expression on EVT was also positively correlated with gestation between 14-19 weeks although no correlation in EVT expression was found at any other stage in gestation. This may correspond with the proposed second wave of trophoblast invasion. TIMP-1 expression decreased in the muscle and stroma with increasing gestation while MMP-9 expression did not change. Thus the only cell type where MMP-9 and TIMP-1 seemed to have a degree of co-distribution spatially and temporally was on the EVT.

Since tight control of trophoblast is very important this may reflect a protective mechanism. TIMP-1 may also have roles in regulating other MMPs in vCTB, while it may be that endothelial expression of MMP-9 during the third trimester is not subject to control by TIMP-1 and is perhaps regulated by other mechanisms.

5.3.4 TIMP-2: Summary of immunohistochemical analysis

Across gestation TIMP-2 staining was present to a greater or lesser extent on vCTB and STB, EVT, stroma, endothelium, and on the muscle surrounding the endothelium. There was significantly lower TIMP-2 expression in the first trimester compared with the third trimester on the endothelium, muscle and stroma. TIMP-2 staining on the stroma was positively correlated with gestation between 14-40 weeks and between 7-40 weeks of gestation. There was also lower TIMP-2 expression in the endothelium in the first trimester compared with the second trimester. TIMP-2 staining on endothelium was positively correlated with gestation between 7-19 weeks, between 14-40 weeks and between 7-40 weeks of gestation. vCTB staining was significantly higher in the first trimester compared with the second and third trimesters. TIMP-2 staining on vCTB was negatively correlated with gestation between 7-13 weeks, between 7-19 weeks and between 7-40 weeks of gestation. An overall analysis of TIMP-2 expression in EVT across gestation was significant although there were no differences between the individual trimesters. TIMP-2 staining on EVT was negatively correlated with gestation between 7-19 weeks and between 7-40 weeks of gestation.

5.3.5 Comparison with MMP-2 immunohistochemical analysis

Since TIMP-2 is known to specifically inhibit both pro and active MMP-2 the distributions of TIMP-2 and MMP-2 as determined by immunohistochemistry were compared. MMP-2 was localised to vCTB, EVT, muscle and stroma and endothelium. TIMP-2 staining was present on the same cells. The main changes in MMP-2 expression with gestation were an increase in endothelial staining with gestation and a decrease in vCTB staining. EVT staining was negatively correlated with gestation. TIMP-2 staining in vCTB also decreased with gestation while staining on endothelium, muscle and stroma increased. EVT expression of TIMP-2 was

negatively correlated with gestation. From the results of this analysis it appears that TIMP-2 and MMP-2 are co-distributed within the placenta and their temporal expression patterns are very similar. With regard to the pattern of MMP-2 and TIMP-2 expression, it was found that staining was highest in vCTB and EVT in the early stages of gestation. This expression decreased and endothelial, muscle and stromal expression of TIMP-2 rose, while only MMP-2 expression rose only in endothelial cells. This may indicate the involvement of TIMP-2 in regulating MMP-2 participation in vascular remodelling later in pregnancy. It is interesting that while many studies have suggested that MMP-9 may be regulated by oxygen tension, the same has not been reported for MMP-2. Thus TIMP-2 may be of vital significance in regulating MMP-2 activity and as such be an important factor in various aspects of placental development.

5.3.6 Comparison to previous studies

Several immunohistochemical studies of TIMP expression in placenta have focused on whether trophoblast invasion is regulated in an autocrine or a paracrine manner. Nomura et al. (1989) and Werb et al. (1992) observed that murine decidua expressed high levels of TIMP-1 and TIMP-2 *in vivo*. Polette et al. (1994) made similar observations in human decidua using in situ and immunohistochemical techniques to investigate protein and mRNA distribution. They found that MMP-2 and TIMP-2 were expressed at comparable levels throughout pregnancy in the placental bed. TIMP-1 expression was highest in the placental bed at term whilst MMP-9 expression in the placental bed was highest in the first trimester. The same study further reported that the mRNAs of MMP-2 and TIMP-1 were co-distributed in the villous stroma and endothelial cells throughout pregnancy whilst MMP-9 and TIMP-2 were co-distributed in the vCTB. This study only involved two placental bed biopsies from first and third trimester however, although it included seven placentae from each trimester, hence the placental bed results need to be interpreted with care. The current study found conflicting results in that no co-distribution of MMP-9 with TIMP-2 in the vCTB was present, while MMP-2 and TIMP-2 were co-distributed in these cells. Moreover, no TIMP-1 was present on the endothelium. These differences may arise from various factors discussed in section 4.3.3.2 with regard to the MMP immunohistochemical studies. Interestingly Ruck et al. (1996) also found TIMP-2

expression in vCTB particularly in the first trimester and suggested it may be co-distributed with MMP-2.

Several other studies have examined TIMP distribution in the placenta and placental bed. Damsky et al. (1993) and Nawrocki et al. (1997) both reported TIMP expression in decidual cells. Further in situ and immunohistochemical data showed TIMP 1-3 expression in cell columns, endothelium, fibroblast stroma and decidua in early pregnancy (Hurskainen et al. 1996). Of particular note in relation to the current study was strong TIMP-2 but not TIMP-1 mRNA expression on the STB and CTB of the villi. An immunohistochemical study by Huppertz et al. (1998) supported the concept that trophoblast expression of TIMP-1 and TIMP-2 may be important in autocrine regulation of trophoblast invasion and reported TIMP-1 expression in EVT throughout pregnancy. In the first and second trimester TIMP-2 was strongly expressed on EVT that had invaded deeply, while in the third trimester TIMP-2 was present in the basement membrane dividing CTB from the villous stroma. Ruck et al. (1996) also reported EVT expression of TIMP-2. Interestingly, an immunohistochemical study performed by Vegh et al. (1999) showed decreased levels of TIMP-1 expression in the STB in choriocarcinoma compared with normal pregnancies at term, suggesting that a decrease in TIMP expression may lead to conditions whereby trophoblast invasion proceeds in an uncontrolled manner.

5.3.7 *In vitro* studies of TIMP expression

Various *in vitro* studies of cultured CTB have reported conflicting results as to the role of TIMPs in the placenta. The presence of TIMP-1 (Lala and Graham, 1990; Graham and Lala, 1991; Librach et al. 1991 and Behrendtsen et al. 1992) and TIMP-2 (Librach et al. 1991; Isaka et al. 2003) have been found to completely inhibit invasion *in vitro* in several studies.

Niu et al. (2000) found constant levels of both TIMPs throughout gestation in tissue extracts and cell culture medium and a concurrent immunohistochemical study showed particularly strong immunoreactivity for TIMP-2 in vCTB at term. In contrast the current study found that vCTB expression of TIMP-2 had decreased to a low level by term. Xu et al. (2000) found no TIMP-2 expression in culture medium from 1st trimester CTB using ELISA but reported that TIMP-1 expression increased from weeks 6-11. The immunohistochemical data herein, however, shows no change in

TIMP-1 and a decrease in TIMP-2 expression in vCTB staining during the first trimester. Differences are likely to arise from the fact that cultured cells may behave differently from those *in vivo*. Bischof et al. (1998a; 1998b) reported that culture medium conditioned by decidualised stromal cells stimulated trophoblastic secretion of TIMP-1 further supporting the paracrine role of the decidua in regulating trophoblast invasion.

5.3.8 Summary of TIMP analysis

In summary this current study found evidence to support the theory that some autocrine regulation of MMPs by TIMPs in EVT may occur in addition to paracrine regulation by the decidua. A larger amount of EVT present in the samples used in the study would have allowed a more comprehensive analysis to be undertaken. Furthermore, this study also presents evidence that TIMPs may have a role in regulation of MMP activity in a variety of other cell types within the placenta, in particular in the endothelium and vCTB. The co-distribution of TIMP-2 with MMP-2 was particularly interesting with regard to the results discussed in the previous chapter where MMP-2 expression varied considerably throughout pregnancy in vCTB and EVT. The potential role of MMPs in vascular and villous remodelling later in pregnancy may thus be connected with the expression and activity of TIMPs.

5.4 Determination of expression and location of HIF-1α in placenta throughout pregnancy using immunohistochemistry

Immunohistochemistry was performed using a HIF-1α antibody (Serotec) as described in section 2.2. The antibody was used at a dilution of 1/250 and control slides where goat IgG or antibody diluent was substituted for primary antibody showed no staining (section 2.2). The staining was scored on a 0-4 scale (section 2.6).

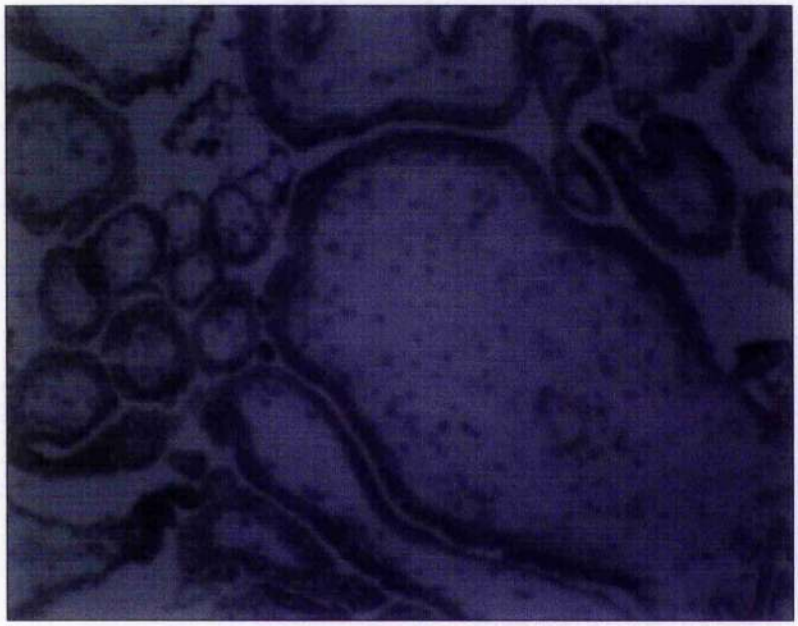
Across gestation HIF-1α staining was present to a greater or lesser extent on vCTB and STB, EVT and on the endothelium of blood vessels and very lightly and variably on the stroma. There was no staining on the muscle surrounding the blood vessels. Figure 49 shows HIF-1α expression at various stages of gestation. Figure 49a shows a placenta at seven weeks of gestation with strong vCTB staining. Figure 49b shows moderately stained EVT at 15 weeks of gestation. Figure 49c shows a placenta at sixteen weeks of gestation with stronger endothelial staining. Figure 49d shows a placenta at term with even stronger endothelial staining but less vCTB staining.

The raw data obtained from scoring the stained sections is shown in table 34. The data was grouped into trimesters and statistical analysis was carried out to determine if there were any statistical differences in HIF-1α expression between trimesters. A summary of the descriptive statistics of the data is given in table 35 and table 36 shows the results of the statistical analysis for HIF-1α. No assumptions were made as to the normality of the distribution of the data and non-parametric testing was used throughout.

Kruskal-Wallis analysis showed a significant change in vCTB staining between the three trimesters ($p<0.01$). Mann-Whitney analysis showed significantly higher HIF-1α expression in vCTB in the first trimester compared with the third trimester ($p<0.02$) and in the first trimester compared with the second trimester ($p<0.02$). Kruskal-Wallis analysis showed a highly significant change in EVT staining between the three trimesters ($p<0.01$). Mann-Whitney analysis showed significantly higher HIF-1α expression in EVT in the first trimester compared with the second trimester ($p<0.02$). No differences in staining were found in muscle, endothelium or stroma.

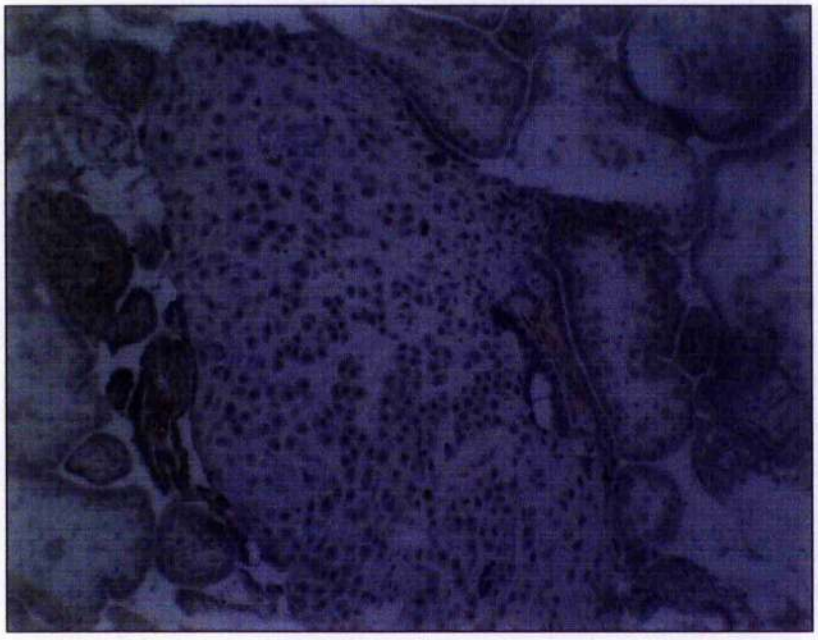
Figure 49 Immunohistochemistry of HIF-1α throughout gestation

a) 7 weeks of gestation



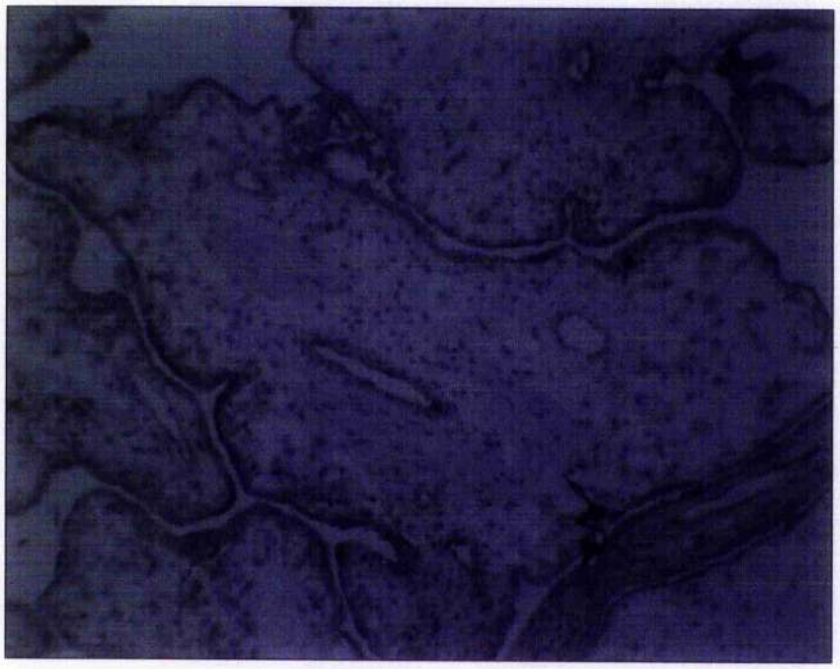
100μm

b) 15 weeks of gestation



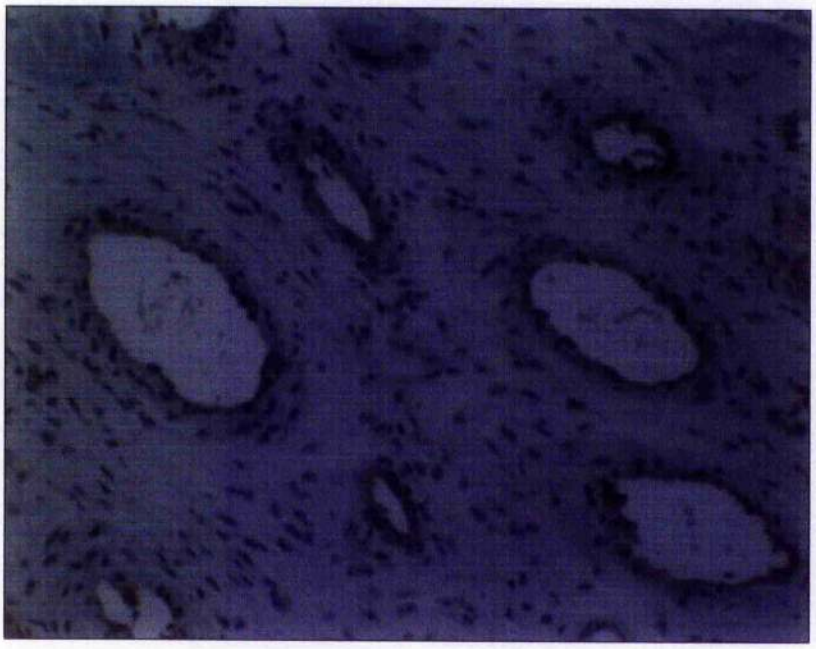
100μm

c) **16 weeks of gestation**



100μm

d) **Term**



50μm

Table 34 Semi-quantitative analysis of HIF-1α immunostaining

Gestation (weeks)	vCTB	EVT	Endothelium	Muscle	Stroma
7	2.5	2.5	0	0	0
8	2	2	1	0	0
9	2	2	1.5	0	0.5
10	2	N/P*	1	0	0
10	2	2	1	0	0
11	2	N/P	1.5	0	0
11	1.5	2	1	0	0.5
14	1.5	1.5	0	0	0
15	2	N/P	1.5	0	0.5
15	1.5	1.5	0.5	0	0
16	1.5	N/P	1.5	0	0
17	1	1.5	2.5	0	0.5
17	1	0.5	1.5	0	0.5
18	1.5	N/P	1.5	0	0
40	1.5	N/P	2	0	1
40	0.5	N/P	1	0	0
40	1.5	N/P	2.5	0	0.5
40	1	N/P	2	0	0.5

*NP = Not present

Table 35 HIF-1α immunohistochemistry descriptive statistics

	Mean	Median	Max	Min	Range between min and max	SE	SD
vCTB							
1st	2.0	2.0	2.5	1.5	1.0	0.11	0.29
2nd	1.43	1.5	2.0	1.0	1.0	0.13	0.35
3rd trimester	1.13	1.25	1.50	0.5	1.0	0.24	0.48
EVT							
1st	2.1	2.0	2.5	2.0	2.0	0.1	0.22
2nd	1.25	1.5	1.5	1.5	0.5	0.25	0.5
3rd trimester	N/A*	N/A	N/A	N/A	N/A	N/A	N/A
Endothelium							
1st	1.0	1.0	1.5	0.0	1.5	0.19	0.50
2nd	1.29	1.5	2.5	0.0	2.5	0.31	0.81
3rd trimester	1.88	2.0	2.5	1.0	1.5	0.31	0.16
Muscle							
1st	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2nd	0.0	0.0	0.0	0.0	0.0	0.0	0.0
3rd trimester	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Stroma							
1st	0.14	0.0	0.5	0.0	0.5	0.09	0.24
2nd	0.21	0.0	0.5	0.0	0.5	0.1	0.27
3rd trimester	0.5	0.5	1.0	0.0	1.0	0.20	0.41

*N/A = Not Applicable

Table 36 **Analysis of variation in HIF-1α immunostaining between the three trimesters**

Test	vCTB	EVT	Endothelium	Muscle	Stroma
Kruskal-Wallace	p-value				
1st/2nd/3rd trimester	p<0.01	p<0.01	N/S*	N/S	N/S
Mann-Whitney					
1st/2nd trimester	p<0.02	p<0.02	N/S	N/S	N/S
2nd/3rd trimester	N/S	N/A*[1]	N/S	N/S	N/S
1st/3rd trimester	p<0.02	N/A	N/S	N/S	N/S

*N/S = Non Significant

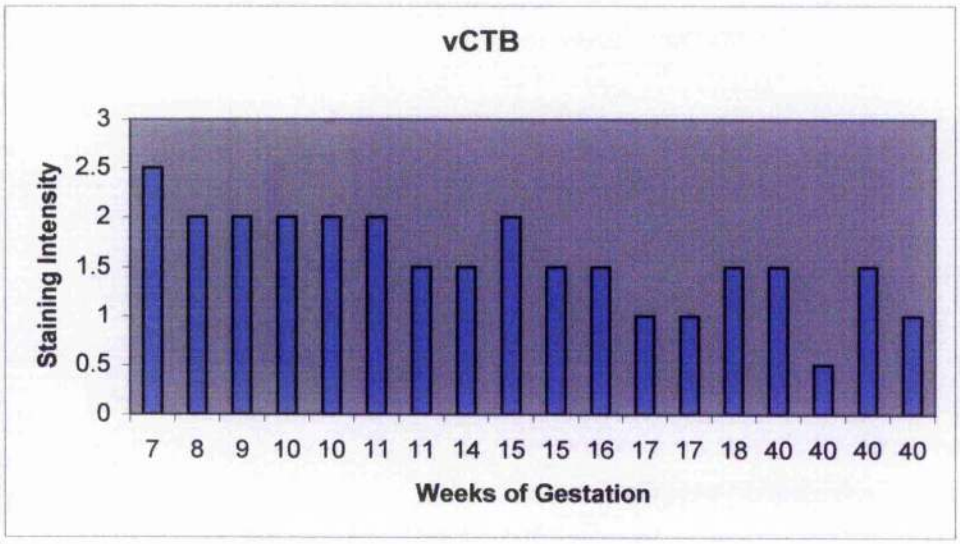
*[1] N/A = test not applicable (too few samples)

The data was then analysed to determine if there were any correlations in HIF-1α expression within each trimester. The data was represented on histograms for each cell type (figure 50). Note that for some cell types many values were zero (no staining) and therefore do not appear as bars on the chart. The data was then analyzed using a Spearman's correlation. Analyses were carried out to determine correlation of HIF-1α expression within the 1st trimester (7-13 weeks), 2nd trimester (14-19 weeks), 1st/2nd trimester (7-19 weeks), 2nd/3rd trimester (14-40 weeks) and 1st/2nd/3rd trimesters (7-40 weeks). The results of the analysis are shown in table 37.

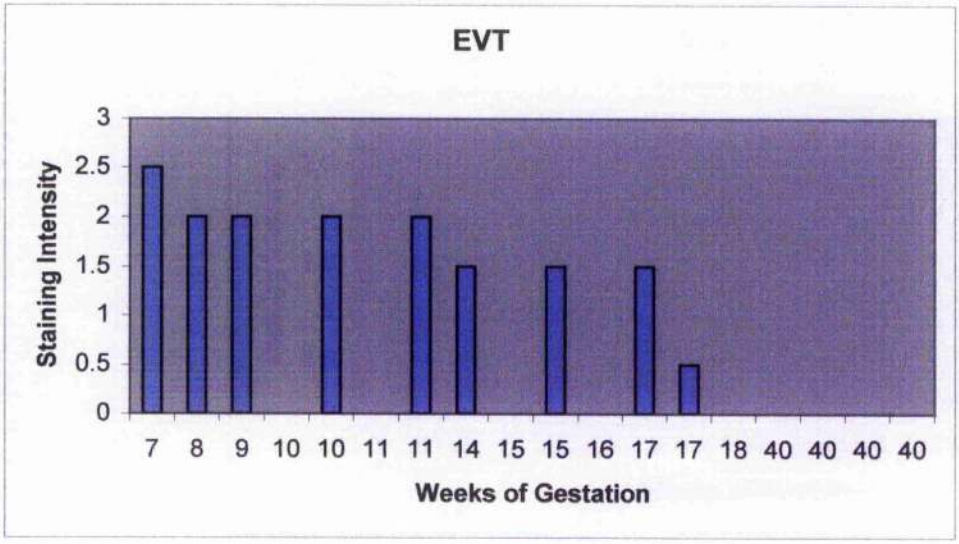
HIF-1α staining on vCTB (figure 50a) was negatively correlated with gestation (staining decreased as gestation increased) between 7-13 weeks ($p<0.05$), between 7-19 weeks ($p<0.01$) and between 7-40 weeks ($p<0.01$) of gestation. HIF-1α staining on EVT (figure 50b) was negatively correlated with gestation (staining decreased as gestation increased) between 7-19 weeks ($p<0.01$) and between 7-40 weeks ($p<0.01$) of gestation. HIF-1α staining on endothelium (figure 50c) was positively correlated (staining increased as gestation increased) with gestation between 7-19 weeks ($p<0.05$), between 14-40 weeks ($p<0.05$) and between 7-40 weeks ($p<0.01$) of gestation. No significant correlations in HIF-1α expression were found in any of the tests on muscle or stromal (figure 50d) staining. No histogram of muscle staining is shown as all of the values were zero.

Figure 50 Histograms of HIF-1α immunostaining throughout gestation

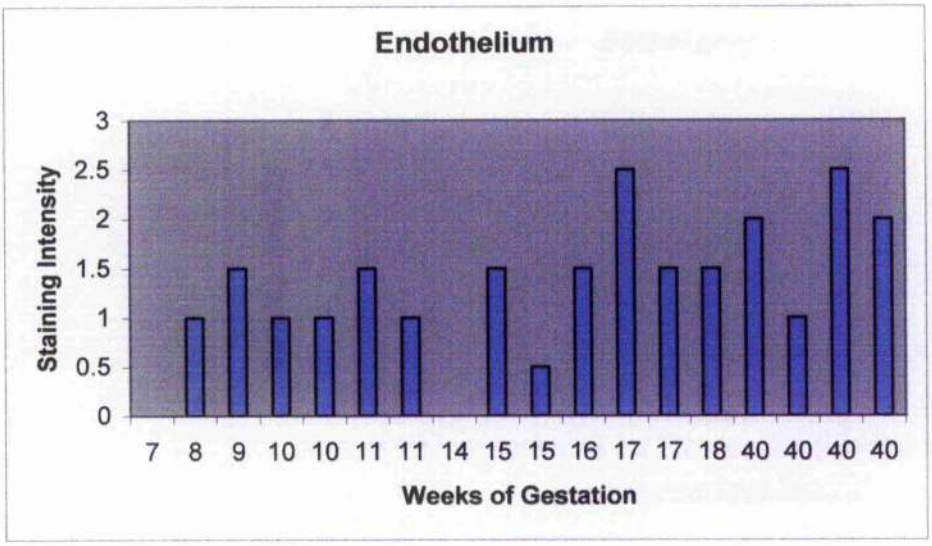
a)



b)



c)


Endothelium
Staining Intensity
3
2.5
2
1.5
1
0.5
0
7 8 9 10 10 11 11 14 15 15 16 17 17 18 40 40 40 40
Weeks of Gestation

d)

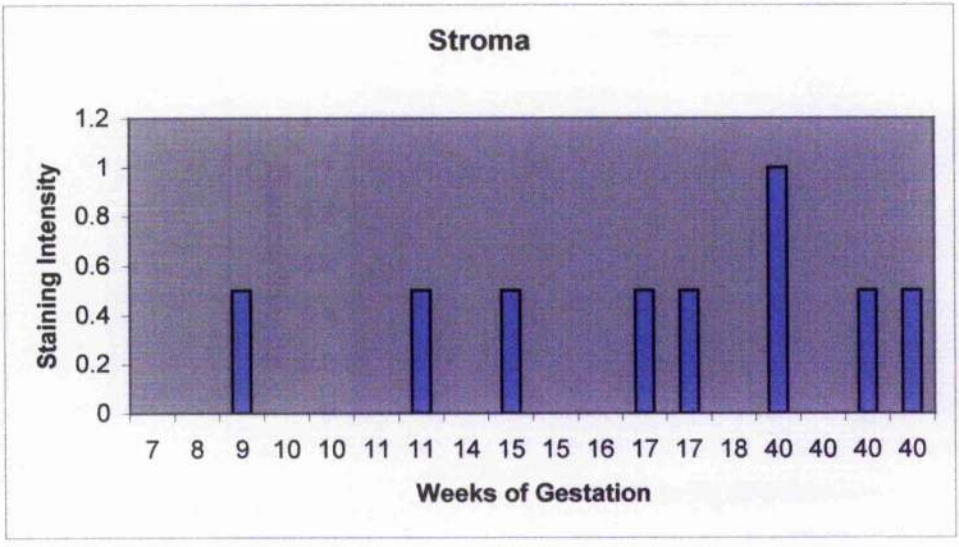

Stroma
Staining Intensity
1.2
1
0.8
0.6
0.4
0.2
0
7 8 9 10 10 11 11 14 15 15 16 17 17 18 40 40 40 40
Weeks of Gestation

Table 37 Correlation of HIF-1α immunostaining with gestation

Test	vCTB	EVT	Endothelium	Muscle	Stroma
Spearman's Rank	p-value and R-value				
1st	p=0.05 R=-0.75	N/S*	N/S	N/S	N/S
2nd	N/S	N/S	N/S	N/S	N/S
1st/2nd trimester	p<0.01 R=-0.83	p<0.01 R=-0.93	p<0.05 R=0.55	N/S	N/S
2nd/3rd trimester	N/S	N/S	p=0.05 R=0.60	N/S	N/S
1st/2nd/3rd trimester	p<0.01 R=-0.82	p<0.01 R=-0.93	p<0.01 R=0.62	N/S	N/S

*N/S = Non-Significant

5.5 HIF-1α immunohistochemistry: discussion

Unlike TIMPs, which are specific regulators of MMPs, HIF is a transcription factor that is thought to control the expression of many factors in response to hypoxia (section 1.5). As discussed in section 1.5.4, HIF-1 is a heteromeric complex that is composed of two subunits, HIF-1β which is constitutively expressed and HIF-1α which is inducible. Previous published studies have examined immunohistochemically the distribution of HIF in placental sections throughout gestation and in placental explants cultured in varying oxygen tensions. The aim of the current study was to carry out semi-quantitative immunohistochemical analysis on HIF-1α distribution in the placenta from 7-19 weeks of gestation and at term and to compare this to the previous data.

5.5.1 Summary of immunohistochemical analysis

Across gestation HIF-1α staining was present to a greater or lesser extent on vCTB and STB, EVT, on the endothelium of blood vessels and very lightly and variably on the stroma. There was no staining on the muscle surrounding the blood vessels. Analysis showed significantly higher HIF-1α expression in vCTB in the first trimester compared with the third trimester and in the first trimester compared with the second trimester. HIF-1α staining on vCTB was negatively correlated with gestation between 7-13 weeks, 7-19 weeks and between 7-40 weeks of gestation. There was also a significantly higher level of HIF-1α expression on EVT in the first trimester compared with the second trimester. HIF-1α staining on EVT was negatively correlated with gestation between 7-19 weeks and between 7-40 weeks of gestation. No differences in staining were found in muscle, endothelium or stroma. However HIF-1α staining on endothelium was positively correlated with gestation between 7-19 weeks, between 14-40 weeks and between 7-40 weeks of gestation.

5.5.2 Comparison to previous studies

In a survey of various human tissues, Weiner et al. (1996) demonstrated the presence of HIF-1α and HIF 1β in mRNA in the human placenta by northern blotting. The presence of HIF-1 DNA binding activity in CTB isolated from term placenta has also been reported (Seligman et al. 1997). A further study reported HIF-1α, HIF-1β and the related subunit HIF-2α mRNA expression in isolated CTB from first trimester placentae (Jantapour et al. 1999).

However, only two groups have studied the expression of HIF-1 within the placenta in further detail. Rajakumar and Conrad, (2000) examined HIF expression in placentae from first trimester to term using a variety of techniques and reported that overall HIF-1α mRNA was expressed at constant levels throughout pregnancy while protein levels varied. HIF-1α expression declined with gestation and was expressed in vCTB and STB, and in the villous endothelium. These findings were comparable to the current study. Interestingly the same group also reported that HIF-1α and HIF-2α protein but not HIF-1β were over-expressed in placentae from women with pre-eclampsia (Rajakumar et al. 2001) and showed that explants from placentae from pre-eclamptic pregnancies failed to adequately down regulate HIF protein upon oxygenation.

However, studies by Cannigia and co-workers have reported different conclusions from the results presented herein and from those studies outlined above. Initial studies by this group showed high HIF-1α mRNA expression in vCTB between 5-8 weeks of gestation in both placental sections and in explants. This expression decreased dramatically between 10-12 weeks around the time that placental oxygenation is reported to occur and staining was virtually non-existent by the second trimester (Caniggia et al. 1996b). In contrast the current study found that HIF-1α was still expressed in the second trimester albeit at reduced levels. Antisense inhibition of HIF-1α resulted in an increase in EVT invasion in explants cultured in 3% oxygen (Caniggia et al. 1996b). Difficulties in interpreting studies by this group arise from the dating system used for estimating the gestational age of placentae collected which is not consistent with that used by many other groups. Moreover, there may be a number of difficulties with regard to the explant model favoured by Caniggia and co-workers. This is discussed in chapter 8. A further study connected the role of HIF with the

regulation of TGFβ_3 and subsequently with MMP-9 and MMP-2 and other markers of trophoblast invasion and postulated that if the increase in oxygen does not occur, or is not detected, then HIF-1α and TGFβ_3 remain high and MMP-9 expression will not increase. The involvement of TGFβ in defective invasion is not consistent with studies by Lyall et al. (2001a) who showed no change in this protein in pre-eclampsia. The current study however did find expression of HIF-1α on EVT and this expression decreased with gestation. This supports the hypothesis that HIF-1α may have a role in preventing invasion in the very early stages of pregnancy.

It is interesting to note the strong expression of HIF-1α on vCTB early in pregnancy at the same time that high expression of MMP-2 and TIMP-2 was found. In contrast MMP-9 was not observed on the vCTB in early pregnancy, suggesting its expression may be tightly controlled and that HIF-1α may have a role in this regulation. The reason for the increase in the expression of HIF-1α on the endothelium through gestation is unknown but it may be involved in the regulation of vascular development.

Thus while HIF-1α may have a role in regulating the response of the placenta to oxygen, the pathways by which it does so are likely to be complex. Control of trophoblast invasion in the first trimester may only be part of a wider role in regulation of vascular remodeling and villous development.

Chapter 6: MMP-9 and MMP-2 expression in placenta from high, moderate and low altitude

It has been reported that women living at high altitude are more likely to develop pre-eclampsia and that even women residing at high altitude who have normal pregnancy outcomes may have many physiological changes similar to those seen in pre-eclampsia (section 1.6). MMP-9 expression has been reported to be decreased in placentae obtained from women with pregnancies complicated by pre-eclampsia (section 1.7) and it has also been reported that its expression is regulated by oxygen (section 1.5). Hence the hypothesis that MMP-9 expression may be altered in placentae from women residing at high altitude, even in pregnancies with normal outcomes, was tested. MMP-2 expression at high altitude was also studied. Placentae were collected and processed as described in section 2.4.2. MMP-9 and MMP-2 expression in full thickness placental sections was analysed by immunohistochemistry using the antibodies described in section 2.2. Immunohistochemistry, rather than zymography, was used to enable MMP expression in specific cell types to be examined.

6.1 MMP-9 expression in placental sections from varying altitudes

Immunohistochemistry was carried out to detect MMP-9 expression and location in the term placental sections from low (Glasgow), moderate (Denver) and high (Leadville) altitudes. The procedure was as described previously (section 2.6). Staining was scored with the identity of the tissues concealed to avoid bias, on a 0-4 scale where 0 = no staining and 4 = very strong staining. Since some of the sections were very large 10 fields of view were scored on each section and an average taken. Control slides where sheep IgG or antibody diluent was substituted for primary antibody showed no staining (section 2.2).

The raw data for scoring the sections is shown in table 38 and the descriptive statistics are shown in table 39. Staining was present in endothelium, muscle and stroma at all altitudes (the amount of EVT and decidua present were not large enough for analysis). The vCTB was only very lightly stained similar to the staining observed in section 4.2. Figure 51 shows a representative section from each altitude. Figure 51a and 51b show placentae from high and moderate altitudes respectively with stromal, endothelial and muscle staining. Figure 51c shows a placenta from low altitude with

stromal staining and with stronger endothelial staining. Figure 52 shows the data in scattergram form for a) vCTB, b) endothelium, c) muscle and d) stroma. The mean value for each cell type is illustrated by a bar.

Table 38 Semi-quantitative analysis of MMP-9 immunostaining at various altitudes

Patient Number	vCTB	Endothelium	Muscle	Stroma
High Altitude				
1	0	1.8	1.7	2.5
2	0.7	0	0.2	0.8
3	1	0	0.8	2.5
4	0	1.3	2.5	3
5	1.4	1.7	1.5	2.2
6	0	0	0.2	1.8
7	0.2	0.6	1	2
8	1.2	1	1.9	2
9	0	2.3	1.45	1.7
10	0	0.8	1.9	2.1
Moderate Altitude				
1	0	1.1	1	2.3
2	0.4	1.4	1	2.1
3	0.4	1.9	1.7	2.3
4	0.05	1.1	1.2	2.5
5	0.2	1.8	0.9	1.8
6	1.3	1.7	0.5	2.5
Low Altitude				
1	0	1.9	1.1	1.4
2	0	1.65	0.54	1.2
3	0.4	1.35	0.85	1.2
4	0.15	2	1.4	1.6
5	0.8	1.68	0.6	1
6	0.45	0.3	0.65	1.85
7	0.25	2.55	2.35	2.1
8	0.05	1.9	1.1	1.8
9	0	1.4	1.2	2
10	0	1.7	1.2	1.85

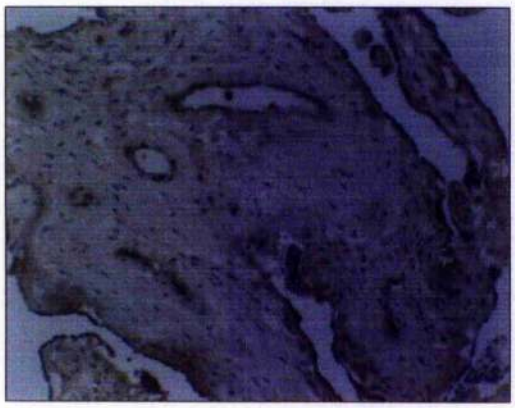
Note that the scores shown denote the average score from 10 fields of view

Table 39 **Descriptive statistics for MMP-9 immunostaining at various altitudes**

Altitude	Cell Type	Max	Min	Range between min and max	Mean	Median	SE	SD
High	vCTB	1.4	0	1.4	0.45	0.1	0.17	0.57
Moderate	vCTB	1.3	0	1.3	0.39	0.3	0.26	0.48
Low	vCTB	0.8	0	0.8	0.21	0.1	0.24	0.27
High	Endothelium	2.3	0	2.3	0.95	0.9	0.19	0.82
Moderate	Endothelium	1.9	1.1	0.8	1.5	1.6	0.19	0.35
Low	Endothelium	2.55	0.3	2.25	1.64	1.7	0.14	0.58
High	Muscle	2.5	0.2	2.3	1.3	1.47	0.16	0.76
Moderate	Muscle	1.7	0.5	1.2	1.1	1.0	0.11	0.39
Low	Muscle	2.35	0.54	1.81	1.1	1.1	0.08	0.53
High	Stroma	3.0	0.8	2.2	2.1	2.1	0.18	0.59
Moderate	Stroma	2.5	1.8	0.7	2.25	2.3	0.17	0.27
Low	Stroma	2.1	1.0	1.1	1.6	1.7	0.12	0.38

Figure 51 MMP-9 expression in placentae from various altitudes

a) MMP-9 expression at high altitude (Leadville = 3100m)



b) MMP-9 expression at moderate altitude (Denver = 1600m)

c) MMP-9 expression at low altitude (Glasgow = sea level)

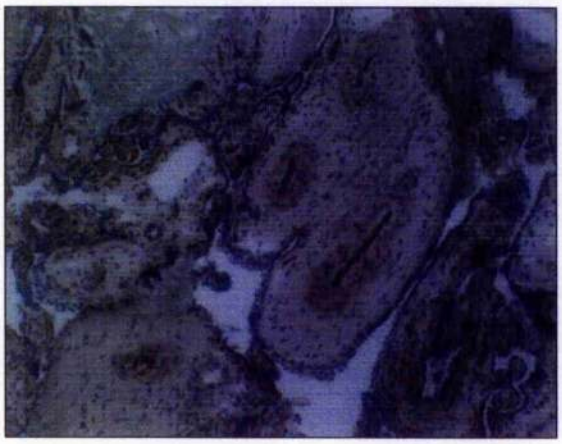
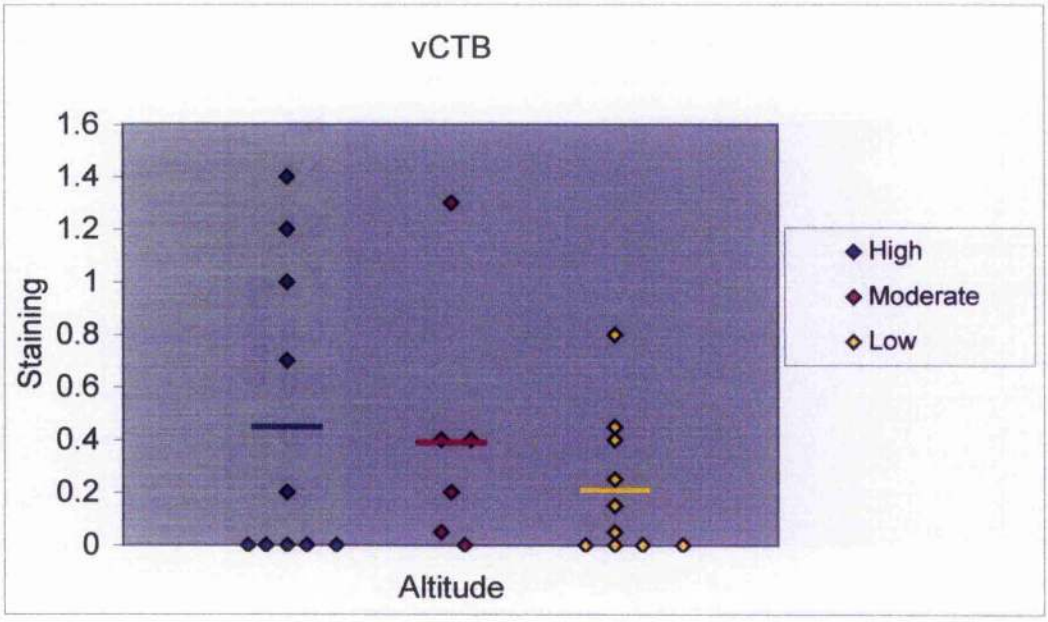
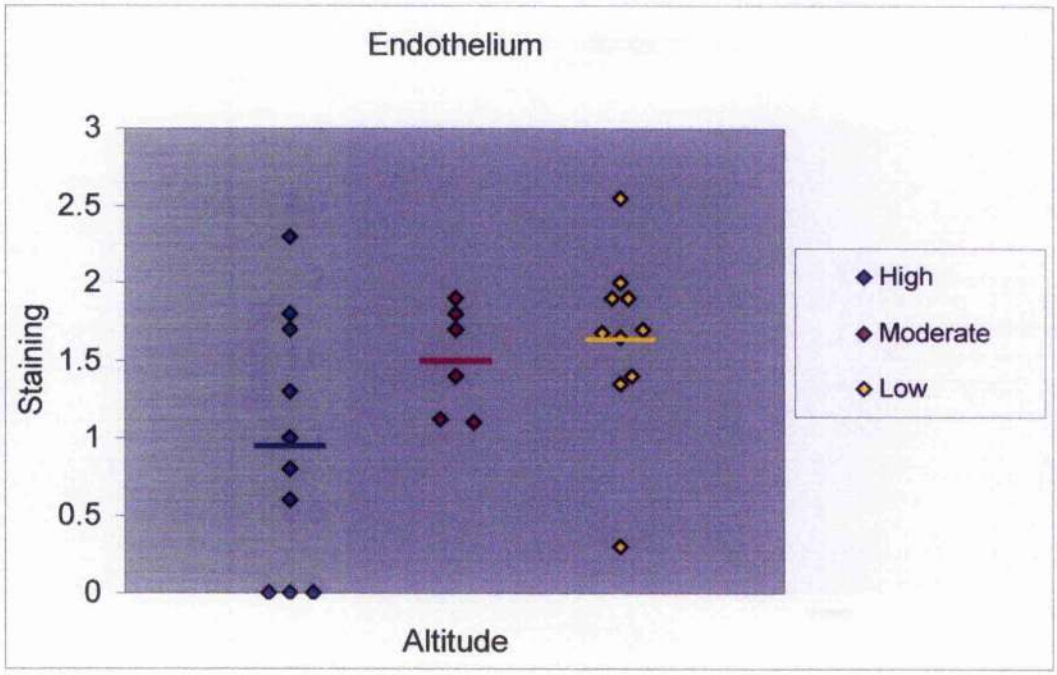


Figure 52 Scattergrams of MMP-9 expression at various altitudes

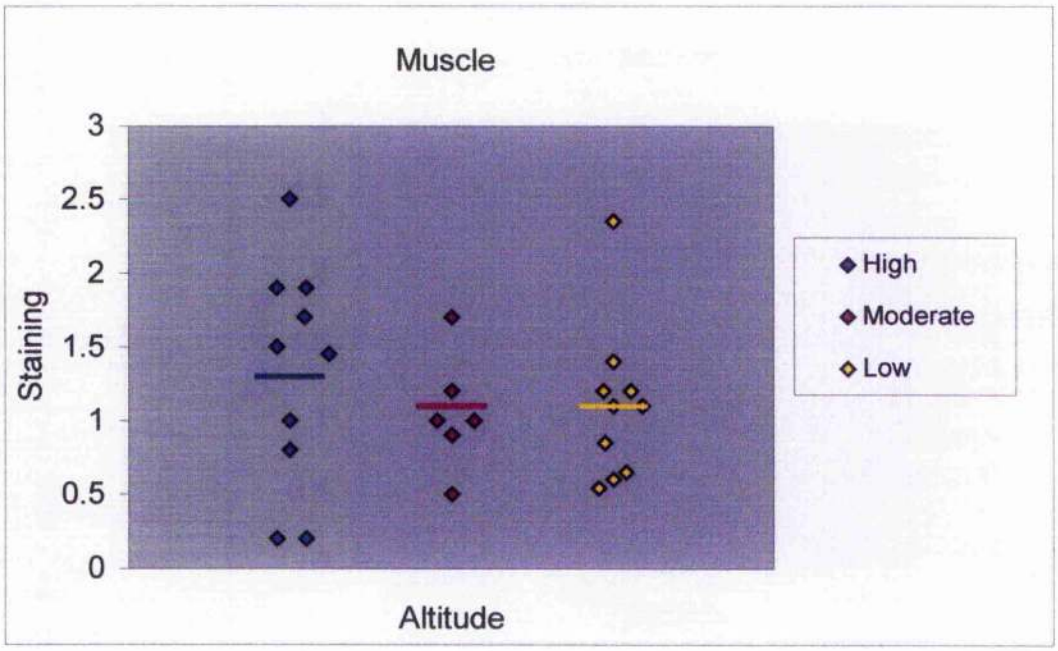
a)



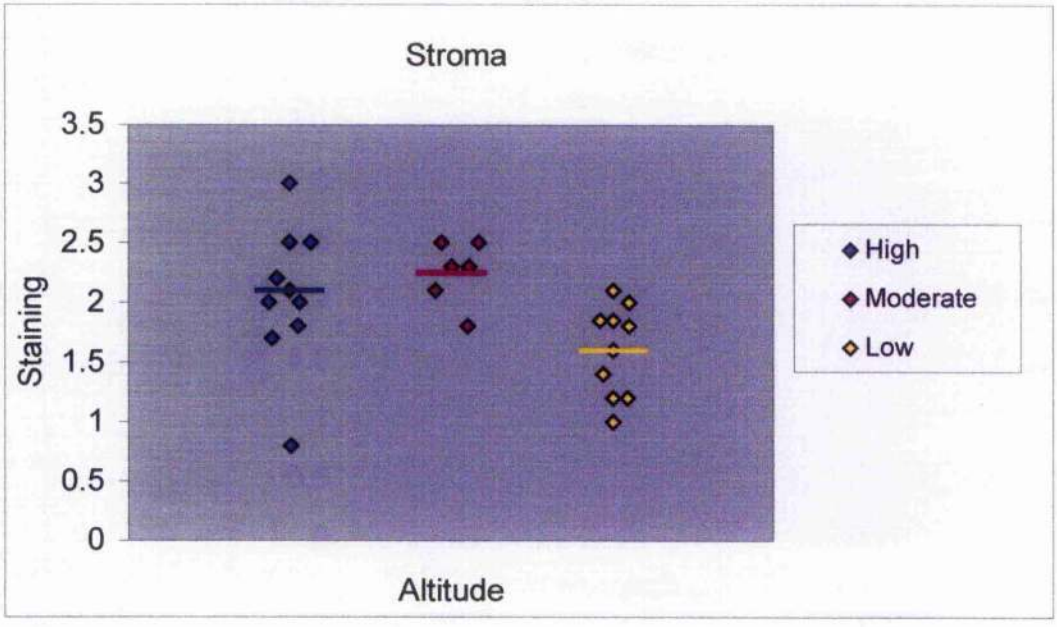
b)



c)


Muscle
3
2.5
2
1.5
1
0.5
0
Staining
Altitude
High
Moderate
Low

d)


Stroma
3.5
3
2.5
2
1.5
1
0.5
0
Staining
Altitude
High
Moderate
Low

The data was tested for normality using Shapiro-Wilk analysis and was found to have a normal distribution. Hence parametric testing was used for the statistical analysis. The statistical analysis is summarized in table 40.

A one-way ANOVA showed no differences in vCTB staining between the two altitudes. A one-way ANOVA for endothelial MMP-9 staining almost reached significance ($p=0.066$). Post-hoc analysis using Fisher's PLSD showed significantly lower staining at high altitude than at low altitude ($p<0.05$). No other differences were found in endothelial staining. ANOVA showed a significant change in stromal MMP-9 staining with increasing altitude ($p<0.05$). Post-hoc analysis using Fisher's PLSD showed significantly higher staining at high altitude than at low altitude ($p<0.05$). Post-hoc analysis using Fisher's PLSD also showed significantly higher staining at moderate altitude than at low altitude ($p<0.05$). There was no significant difference between high and moderate altitudes. No differences were observed in muscle staining between the two altitudes.

Table 40 **Analysis of variation in MMP-9 immunostaining between various altitudes**

Test	Altitudes compared	vCTB	Endothelium	Muscle	Stroma
ANOVA	**All three**	N/S*	N/S	N/S	$p<0.05$
Fisher's PLSD	**Low/ moderate**	N/S	N/S	N/S	$p<0.05$
Fisher's PLSD	**Low/ high**	N/S	$p<0.05$	N/S	$p<0.05$
Fisher's PLSD	**Moderate/ high**	N/S	N/S	N/S	N/S

*N/S = Non-significant ($p>0.05$)

6.2 MMP-2 expression in placental sections from varying altitudes

Fewer studies implicate MMP-2 than MMP-9 in pre-eclampsia and MMP-2 has not been shown to be sensitive to oxygen tension. It was therefore decided to repeat the experiment carried out in section 6.1 with an MMP-2 antibody (section 2.2). Due to availability of samples only high (Leadville) and low (Glasgow) altitudes were compared. There were 6 placental sections in each group. Immunohistochemistry was carried out to detect MMP-2 levels and locations in full thickness placental sections. The procedure was as described previously. Staining was scored with the identity of the tissues concealed to avoid bias, on a 0-4 scale where 0 = no staining and 4 = very strong staining. Control slides where sheep IgG or antibody diluent was substituted for primary antibody showed no staining (section 2.2).

The raw data is shown in table 41 and the descriptive statistics are given in table 42. Staining was present in vCTB, endothelium muscle and stroma at all altitudes. Figure 53 shows a representative section from each altitude (Figures 53a high altitude, 53b low altitude). In each picture staining is present on the vCTB, stroma, endothelium and on the muscle surrounding the endothelium. Figure 54 shows the data in scattergram form for a) vCTB, b) endothelium, c) muscle and d) stroma. The median value for each cell type is illustrated by a bar.

The data was tested using Shapiro-Wilk analysis and was found to have a non-normal distribution. Hence non-parametric tests were used in the statistical analysis. The data was analysed using Mann-Whitney analysis (table 43). No significant differences were found in MMP-2 staining between low and high altitude in any of the cell types.

Table 41 **Semi-quantitative analysis of MMP-2 immunostaining at various altitudes**

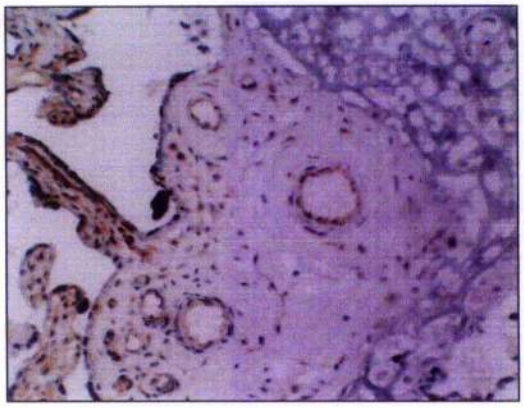
Patient Number	vCTB	Endothelium	Muscle	Stroma
Low Altitude				
1	0.5	2.5	1	1
2	0.5	1.5	0.5	0.5
3	0.5	2.5	0.5	0.5
4	0.5	1	0.5	0.5
5	0.5	1.5	0.5	0.5
6	1	1.5	0.5	0.5
High Altitude				
1	1	1	0.5	0.5
2	1	1	0.5	0.5
3	0.5	0	0.5	0.5
4	0.5	0	0.5	0.5
5	0.5	1.5	1	1
6	0.5	2	0.5	0.5

Table 42 **Descriptive statistics for MMP-2 immunostaining at various altitudes**

Altitude	Cell Type	Max	Min	Range	Mean	Median	SE	SD
High	vCTB	1	0.5	0.5	0.67	0.5	0.11	0.26
Low	vCTB	1	0.5	0.5	0.58	0.5	0.08	0.2
High	Endothelium	2	0	2	0.92	1	0.33	0.80
Low	Endothelium	2.5	1	1.5	1.75	1.5	0.25	0.61
High	Muscle	1	0.5	0.5	0.58	0.5	0.08	0.20
Low	Muscle	1	0.5	0.5	0.6	0.5	0.10	0.22
High	Stroma	1	0.5	0.5	0.58	0.5	0.08	0.2
Low	Stroma	1	0.5	0.5	0.6	0.5	0.1	0.22

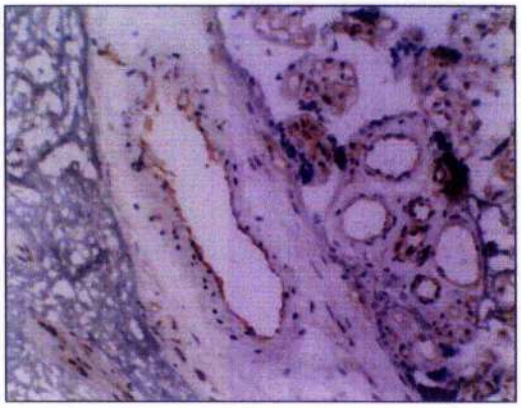
Figure 53 MMP-2 expression in placentae from various altitudes

a) MMP-2 expression at high altitude (Leadville = 3100m)



100μm

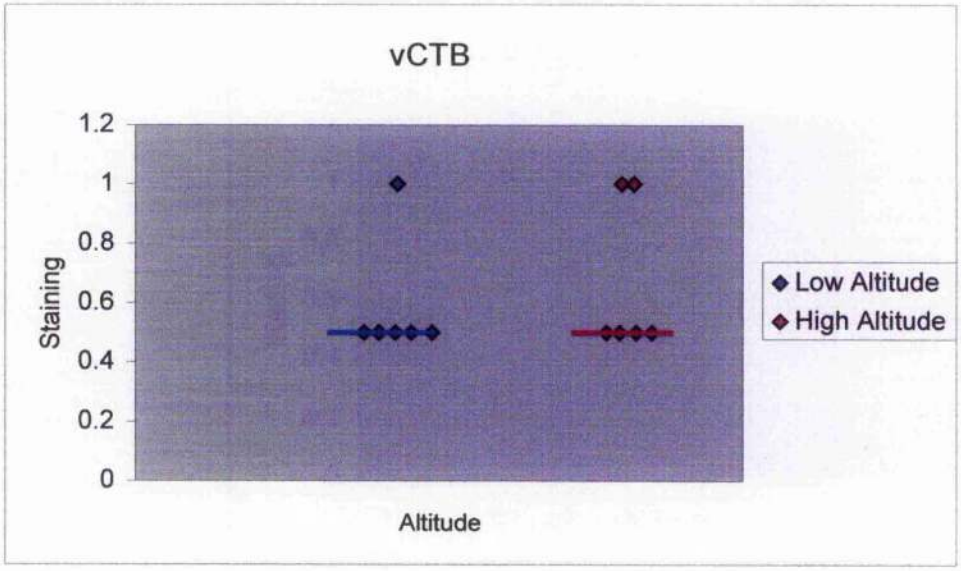
b) MMP-2 expression at low altitude (Glasgow = sea level)



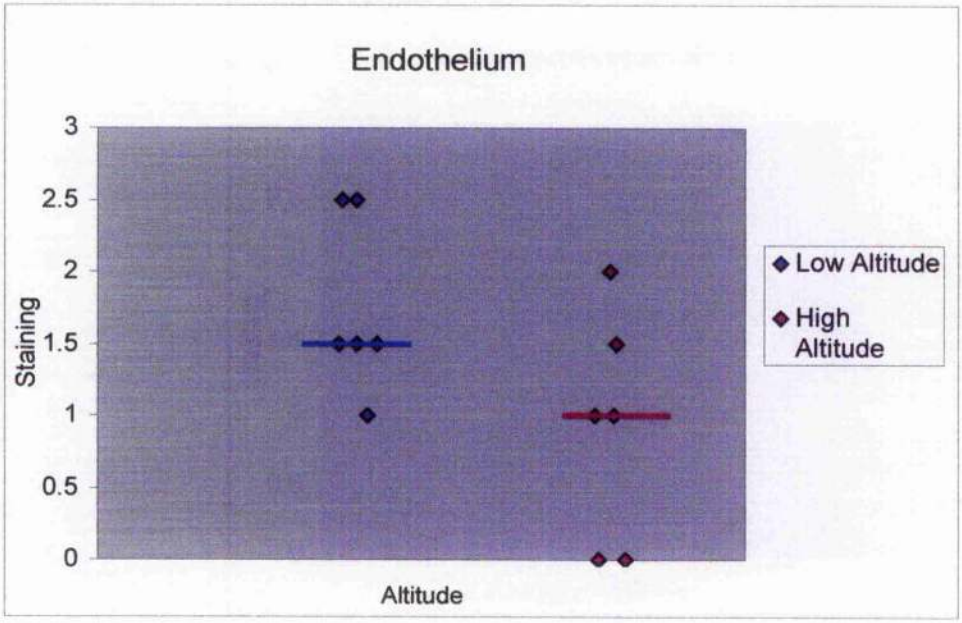
100μm

Figure 54 Scattergrams of MMP-2 expression at various altitudes

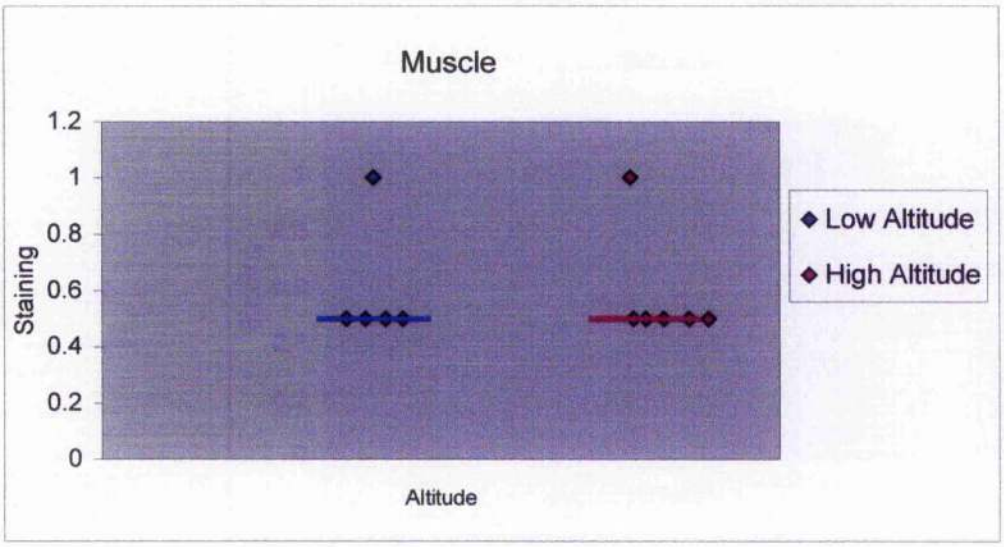
a)



b)



c)


Muscle
1.2
1
0.8
0.6
0.4
0.2
0
Staining
Altitude
Low Altitude
High Altitude

d)

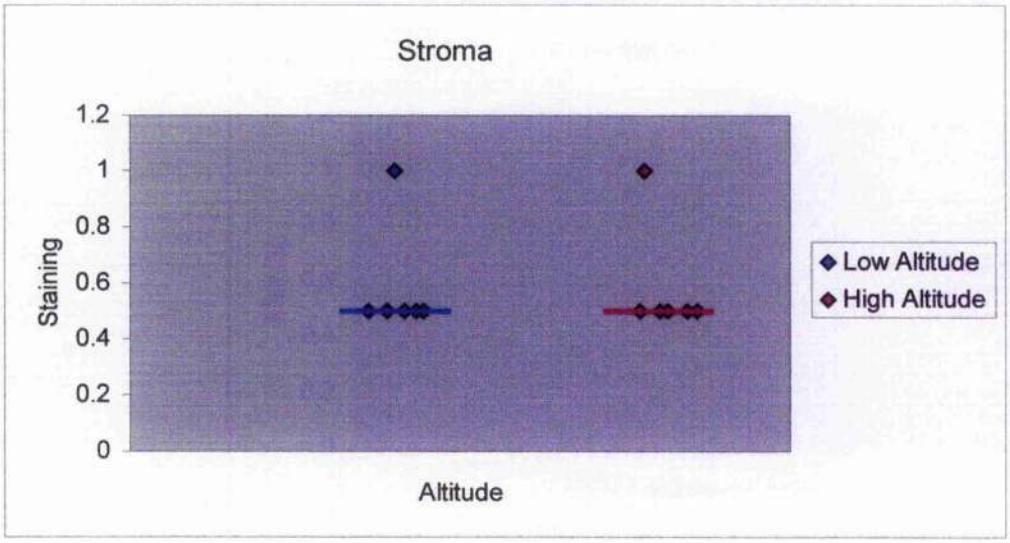

Stroma
1.2
1
0.8
0.6
0.4
0.2
0
Staining
Altitude
Low Altitude
High Altitude

Table 43 **Analysis of variation in MMP-2 immunostaining between various altitudes**

	vCTB	Endothelium	Muscle	Stroma
Mann-Whitney comparison of low/high Altitude	N/S*	N/S*	N/S*	N/S*

*N/S = Non-significant ($p>0.05$)

6.3 Discussion

There are no known published studies on MMP expression in placentae from high altitude pregnancies. It has previously been reported that MMP-9 is reduced in term placental extracts from women with pre-eclampsia (Kolben et al. 1996). A further study reported that term trophoblast cell cultures from normal pregnancies secreted high levels of active MMP-9 while term trophoblast cultures from pregnancies complicated by pre-eclampsia secreted higher levels of inactive pro-MMP-9 (Graham and McCrae, 1996; Lim et al. 1997). Hence it was hypothesised that MMP expression may also be reduced in high altitude placentae. The results of this study showed that MMP-9, but not MMP-2, expression is increased in the villous stroma and reduced in the endothelium in placentae from high altitude pregnancies compared to those at low altitude. None of the studies on pre-eclampsia have reported any differences in MMP expression in the endothelium or stroma.

Gallery et al reported significantly reduced levels of MMP-1 but not MMP-9 secreted by cultured decidual microvascular endothelial cells from pre-eclamptic patients (Gallery et al. 1999). The authors suggested that this may be associated with the relative failure of trophoblasts to invade maternal decidual blood vessels in pre-eclamptic pregnancy. If MMP expression in the endothelium is associated with vascular remodeling during the later stages of gestation, reduced MMP expression at high altitude may represent a failure of physiological adaptation to reduced oxygen levels found in such environments. Since maternal blood has lower oxygen saturation it follows that the endothelial lining of blood vessels would also be exposed to lower O_2 levels. Reduced MMP-9 expression in CTB has been linked to low oxygen in several studies (Caniggia et al. 1999; Caniggia et al. 2000a). It is thought that the low oxygen environment present in the intervillous space during the early weeks of gestation may in fact prevent MMP expression and therefore trophoblast invasion. Hence it is not inconceivable that a similar mechanism may operate in the endothelium although there are no published studies on HIF-1α expression in the placenta. In the current study the levels of endothelial MMP-9 expression at moderate altitude were not significantly different from high altitude or low altitude and therefore moderate altitude may be a partial intermediate between the two extremes.

The increase in stromal staining with increasing altitude was even more marked than the difference in endothelial staining in the current study. Staining

increased between low and moderate and between low and high but not between moderate and high altitudes. Staining tended to be fairly general throughout the stroma. The role of MMP-9 in the stromal villi during pregnancy is unclear but it is possible that in the third trimester it may have a role in structural remodelling. As described in section 1.6, pregnancy at high altitude is associated with increased markers of placental hypoxia such as formation of syncytial knots, persistence of vCTB at term and hypercapillarisation (Ali, 1997). To adjust to the hypoxia it has been suggested that diffusion distances across the intravascular barrier might decrease, particularly across the trophoblast layer which may be sparser (Jackson et al. 1988). A decrease in perivascular cells around many of the villous capillaries has also been noted (Zhang et al. 2002) perhaps adding to the thinning of the maternal fetal interface. Alterations in villous capillary diameter have also been found at high altitude (reviewed in Mayhew, 2003). Above 3600m villous surface area is reduced and to compensate, there is an enlargement of the intervillous space. Under hypoxic conditions, increased branching of fetal capillaries is thought to occur (Kaufmann et al. 1985; Kaufmann, Luckhardt and Leiser, 1988). It is possible that changes in MMP-9 expression may contribute to some of these changes in villous configuration.

It would have been informative to study placentae from earlier in pregnancy in order to establish whether EVT expression of MMP-9 was reduced at high altitude as this would further establish the suitability of high altitude as a model for pre-eclampsia. Logistically this was not possible in the current study but would be of interest for future work.

Fewer studies have associated MMP-2 expression in cultured CTB with oxygen tension, trophoblast invasion and in the development of pre-eclampsia. It was found in this study that MMP-2 expression did not differ significantly between high and low altitude in any of the cell types examined. Again it would be interesting to study expression in EVT in early pregnancy to determine if there were any differences between the two altitudes. While MMP-2 is constitutively expressed in many human tissues, the regulation of its gene expression is likely to be sensitive to numerous agents through the upstream binding site for various transcription factors. While high altitude pregnancy may be a suitable model for some aspects of pre-eclampsia, care must be taken when evaluating it as a potential model. This is discussed further in chapter 7.

Chapter 7: Serum markers of pre-eclampsia in serum from high and moderate altitude pregnancies

7.1 Maternal concentrations of circulating VCAM-1 at high and moderate altitude

Maternal circulating concentrations of the cell adhesion molecule VCAM-1, a marker of endothelial activation, are increased in pre-eclampsia (section 1.7.5). The hypothesis was tested that pregnant women living at high altitude, although the pregnancy outcomes are normal, may show similarities to women with pre-eclampsia regarding endothelial cell activation.

Serum samples were obtained from pregnant women living at high (HA) and moderate altitudes (MA) as described in section 2.4.2.3. The demographic details of the overall sample group are shown in table 44a. For each assay (VCAM-1, E-Selectin and ICAM-1) the majority of the samples were used. The samples were initially assayed blind and hence in a few cases two samples were obtained from the same patient in a particular trimester were included in the assay and analysis. However the data was reanalyzed without these samples and the means did not differ substantially and there were no differences in statistical test results. Hence the initial results are presented herein. The precise numbers for each are shown in table 44b.

Concentrations of VCAM-1 were assayed by ELISA (R&D Systems, UK) in early (8-14 weeks), mid (15-28 weeks) and late (29 weeks–term) pregnancy and post partum (PP) (n = 5, 21, 15 and 13 at MA and 10, 17, 19 and 11 at HA respectively) as described in section 2.10.3.

The gestations of the samples were analyzed using Shapiro-Wilk analysis and the data was found to have a non-normal distribution. Thus the median gestation for each trimester was calculated and is shown in table 45. When the gestations of the samples were compared using a Mann-Whitney U test there were no significant differences between the gestations of high and moderate samples in first, second or third trimesters. All post-partum samples were collected at three months post-partum.

Table 44a Demographic details of sample populations in altitude study

Maternal Characteristics, means ± SD

	1600 m n=15	3100 m n=16
Age (years)	28 ± 4	27 ± 7
Height (cm)	167 ± 6	165 ± 8
Pre-pregnant weight (kg)	62 ± 6	61 ± 6
BMI (kg/m^2)	22 ± 2	22 ± 2
Weight gain with pregnancy (kg)	14 ± 4	16 ± 5
Maternal Education (years)	17 ± 3	14 ± 3**
Paternal Education (years)	16 ± 3	16 ± 3
Birth weight (grams)	3332±346	3076±390*
Gestational Age (weeks)	39.2 ± 1.2	39.8 ± 1.7
Baby Length (cm)	50.2 ± 2.6	50.3 ±3.1
Placental weight (grams)	517 ±109	596 ± 85**

*p<0.05, one tailed test (p=0.06 two tailed test)

** P<0.05 two tailed test

Table 44b Number of samples used in each assay

Assay	VCAM-1	VCAM-1	E-Selectin	E-Selectin	ICAM-1	ICAM-1
Trimester	**Moderate Altitude**	**High Altitude**	**Moderate Altitude**	**High Altitude**	**Moderate Altitude**	**High Altitude**
1	5	10	5	10	5	10
2	21	17	15	17	21	16
3	15	19	11	16	15	18
Post-Partum	13	11	7	11	13	11

Table 45 **Statistical comparison of the gestations of serum samples from high and moderate altitude used in VCAM-1 assay**

	Moderate Altitude Median (Range)	High Altitude Median (Range)	p-value
1st trimester	12 (12-14)	12 (11-14)	N/S*
2nd trimester	25 (15-27)	25 (15-28)	N/S
3rd trimester	37 (32-39)	37 (31-40)	N/S

* N/S = Non-Significant ($p>0.05$)

After the assay was performed, a standard curve was drawn using the standard samples provided in the assay kit. The standard curve is shown in figure 55. The calculated values for the samples in the assay using the standard curve are shown in table 46 and the descriptive statistics of the data are shown in table 47. The data was then plotted on a scattergram (figure 56). The mean + SE values (ng/ml) were as follows: 1st trimester 563.82±108.18 at MA and 468.3 ±27.87 at HA, 2nd trimester 529.3±40.1 at MA and 456.3±24.4 at HA, 3rd trimester 639.39±62.0 at MA and 545.21±38.5 at HA and PP 585.77±61.05 at MA and 450.5±30.35 at HA.

The data was initially analyzed at the two altitudes separately to determine if there were any variations in VCAM-1 concentrations between the individual trimesters. The data was first tested to determine if it approached a normal distribution using Shapiro-Wilk analysis. Since it did, an ANOVA/ Fisher's PLSD test was used and the results are shown in table 48 (moderate altitude) and table 49 (high altitude). No variation in VCAM-1 concentration with trimester was found at moderate altitude but at high altitude VCAM-1 was significantly higher in the third trimester than in the second trimester ($p<0.05$).

The data was subsequently analyzed using a t-test to determine if there were any significant differences in circulating VCAM-1 concentrations at high compared with moderate altitude firstly in the groups as a whole and then in each of the three trimesters or post-partum. The results of the statistical analysis are shown in table 50. Although the initial t-test reached significance ($p<0.01$), further analysis showed no significant differences between the two altitudes in the 1st, 2nd or 3rd trimesters or post-partum samples.

Finally, to test if there was any correlation between gestation and VCAM-1 concentration a Pearson's correlation analysis was carried out. The results are shown in table 51. There was no correlation between VCAM-1 and gestation in either the HA or MA groups individually but when the groups were combined there was a positive correlation of VCAM-1 with gestation.

Figure 55 Standard curve for VCAM-1 assay

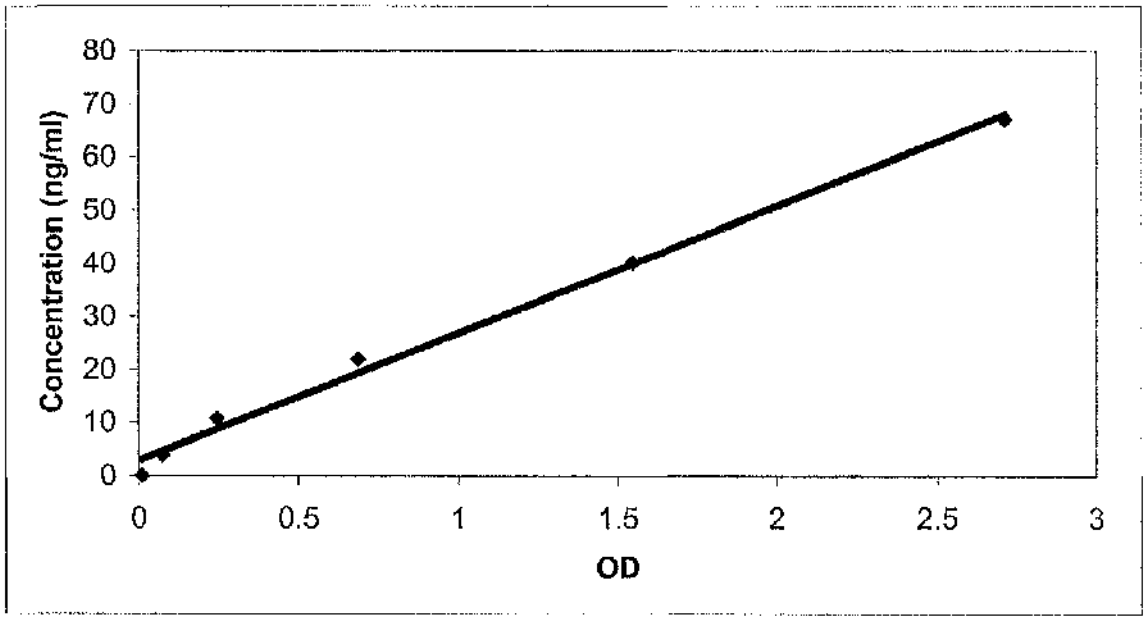


Table 46 **VCAM-1 concentrations in maternal serum throughout pregnancy – raw data**

Patient Number	Sample Gestation (weeks)	VCAM-1 ng/ml
Moderate Altitude		
1 Denver (D)	22	560.5
	27	678.5
	39	572
	PP	400.34
2D	27	497.4
	37	580
	PP	321.1
3D	12	479.45
	27	404
	32	417.15
	PP	520.37
4D	14	256.29
	26	400.9
	34	394.3
5D	12	497.4
	24	464.3
	34	458.15
	PP	613
6D	12	900
	24	518
	34	1128
	PP	766
7D	14	686
	27	1096
	38	689
	PP	891.5
8D	27	626
	36	852
	PP	453.15
9D	17	681
	26	756.5
	37	943.5

	PP	1053.5
10D	15	451.95
	26	621
	39	880
	PP	458.15
11D	15	645.5
	25	347.15
	37	854.5
	PP	562.38
12D	17	405.14
	26	618.8
	39	834.86
	PP	758
13D	24	289.9
	36	389.53
	PP	391.93
14D	16	363.12
	26	303.11
	36	390.73
	PP	425.54
15D	16	485.56
	38	507.14
High Altitude		
1 Leadville (L)	25	349.9
	38	482.5
	PP	317.51
2L	11	394.3
	24	397.6
	37	394.3
	PP	523.5
3L	12	442.6
	27	404.15
	31	962.1
	40	592.39

5L	11	339.3
	25	339.3
	36	360.4
6L	14	433.15
	26	374.15
	37	479.45
	PP	448.85
8L	12	563.5
	25	538
	35	485.5
	PP	591
10L	12	574.5
	27	746.5
	37	251
	PP	566.5
11L	15	549.5
	26	470.4
	37	535
	PP	563.5
12L	24	500.5
	37	610
	PP	417.14
13L	12	461.2
	24	526.5
	37	479.45
	PP	410.7
14L	25	439.45
	37	580
	PP	454.35
15L	26	480.76
	39	481.96
	PP	355.92
16L	24	417.15
	32	736.5
	38	711.5
	PP	306.71

17L	13	607.5
	28	342.85
	38	585.5
18L	13	476.45
	24	410.7
	35	778
25L	12	390.5
	25	470
	36	506
	38	347.52

Table 47 **VCAM-1 concentrations in maternal serum throughout pregnancy – descriptive statistics**

(ng/ml)	Mean	Median	SD	SE	Min	Max	Range between min and max
HA 1st trimester	468. 3	451.9	88.12	27.87	339.3	607.5	268.2
HA 2nd trimester	456.3	439.5	100.7	24.4	339.3	746.5	407.2
HA 3rd trimester	545.21	506	167.6	38.5	251	962.1	711.1
HA Post Partum	450.5	448.85	100.66	30.35	306.7	591.0	284.29
MA 1st trimester	563.82	497.4	241.89	108.18	256.29	900	643.71
MA 2nd trimester	529.3	497.4	183.74	40.1	289.9	1096	806.1
MA 3rd trimester	659.39	580.0	240.15	62.0	389.53	1128.0	738.47
MA Post Partum	585.77	520.37	220.13	61.05	321.1	1053.5	732.4

Figure 56 **Scattergraph of maternal concentrations of circulating VCAM-1 at high and moderate altitude**

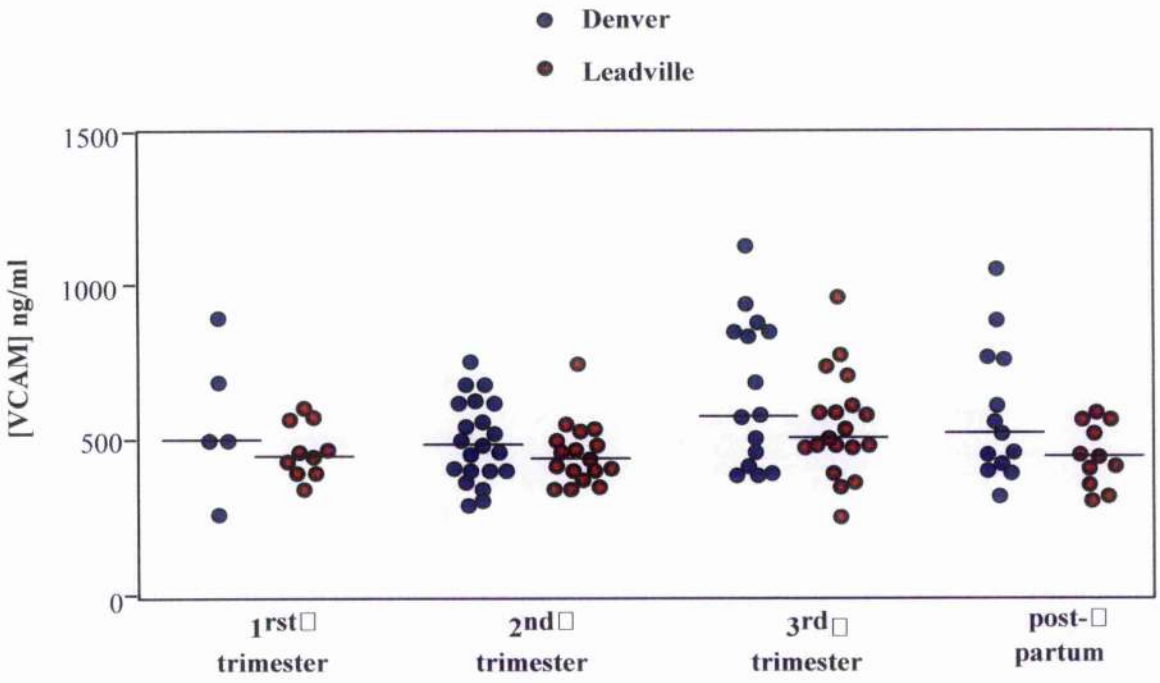


Table 48 **Statistical analysis of VCAM-1 concentration variation at moderate altitude**

Test	Trimesters analysed	p-value
ANOVA	All three trimesters + PP	N/S*
Fisher's PLSD	$1^{st}/2^{nd}$	N/S
Fisher's PLSD	$1^{st}/3^{rd}$	N/S
Fisher's PLSD	$2^{nd}/3^{rd}$	N/S
Fisher's PLSD	1^{st}/PP	N/S
Fisher's PLSD	2^{nd}/PP	N/S
Fisher's PLSD	3^{rd}/PP	N/S

* N/S = Non-Significant ($p>0.05$)

Table 49 **Statistical analysis of VCAM-1 concentration variation at high altitude**

Test	Trimesters analysed	p-value
ANOVA	All three trimesters + PP	N/S*
Fisher's PLSD	$1^{st}/2^{nd}$	N/S
Fisher's PLSD	$1^{st}/3^{rd}$	N/S
Fisher's PLSD	$2^{nd}/3^{rd}$	$p<0.05$
Fisher's PLSD	1^{st}/PP	N/S
Fisher's PLSD	2^{nd}/PP	N/S
Fisher's PLSD	3^{rd}/PP	N/S

* N/S = Non-Significant ($p>0.05$)

Table 50 **Statistical comparison of VCAM-1 concentrations at moderate and high altitude**

Test	Trimesters analysed	p-value
t-test	All three trimesters + PP	$p<0.01$
t-test	1st	N/S*
t-test	2nd	N/S
t-test	3rd	N/S
t-test	Post-Partum	N/S

* N/S = Non-Significant ($p>0.05$)

Table 51 **Correlation analysis of VCAM-1 concentration with gestation at moderate and high altitude**

Altitudes	p-value
Moderate	N/S*
High	N/S
Moderate and High	0.04 R=0.22

* N/S = Non-Significant (p>0.05)

7.2 Maternal concentrations of circulating E-Selectin at high and moderate altitude

Maternal circulating concentrations of the cell adhesion molecule E-Selectin, another marker of endothelial activation, are also increased in pre-eclampsia (section 1.7.5). Therefore in the next experiment concentrations of E-Selectin were assayed in the maternal circulation from women living at high and moderate altitude. E-Selectin was assayed by ELISA (R&D systems) in early (8-14 weeks), mid (15-28 weeks) and late (29 weeks–term) pregnancy and post partum (n = 5, 15, 11 and 7 at MA and 10, 17, 16 and 11 at HA respectively). The gestations of the samples were analyzed using Shapiro-Wilk analysis and the data was found to have a non-normal distribution. Thus the median gestation for each trimester was calculated and is shown in table 52. When the gestations of the samples were compared using a Mann-Whitney U test there were no significant differences between high and moderate samples in first, second or third trimesters.

Table 52 **Statistical comparison of the gestations of serum samples from high and moderate altitude used in E-Selectin assay**

	Moderate Altitude Median (Range)	High Altitude Median (Range)	p-value
1st trimester	12 (12-14)	12 (11-14)	N/S*
2nd trimester	26 (15-27)	25 (15-28)	N/S
3rd trimester	37 (32-39)	37 (32-39)	N/S

* N/S = Non-Significant ($p>0.05$)

After the assay was performed a standard curve was drawn using the standard samples provided in the assay kit. The standard curve is shown in figure 57. The raw data for the samples in the assay is shown in table 53 and the descriptive statistics of the data are shown in table 54. The data was then plotted on a scattergram (figure 58). The mean + SE (ng/ml) values were as follows: 1st trimester 32.49±5.32 at MA and 38.87±4.77 at HA, 2nd trimester 39.8±2.95 at MA and 37.05±2.92 at HA, 3rd trimester 36.6±4.63 at MA and 37.09±4.27 at HA and PP 37.1±4.03 at MA and 43.3±5.88 at HA.

Initially the data was analyzed at the two altitudes separately to determine if there were any variations in E-Selectin concentrations between the individual trimesters at either altitude using an ANOVA/ Fisher's PLSD test. No variation in E-Selectin concentration with trimester was found at either of the altitudes ($p>0.05$).

The data was subsequently analyzed using t-tests to determine if there were any significant differences in circulating E-Selectin concentrations at high compared with moderate altitude initially between the two groups as a whole and subsequently in any of the three trimesters or post-partum. There were no significant differences in E-Selectin concentration between moderate and high altitudes in 1st, 2nd or 3rd trimester or post-partum.

Finally, to test if there was any correlation between gestation and E-Selectin concentration a Pearson's correlation analysis was carried out. There was no correlation between E-Selectin and gestation in either the HA or MA groups or in both groups combined.

Figure 57 Standard curve for E-Selectin assay

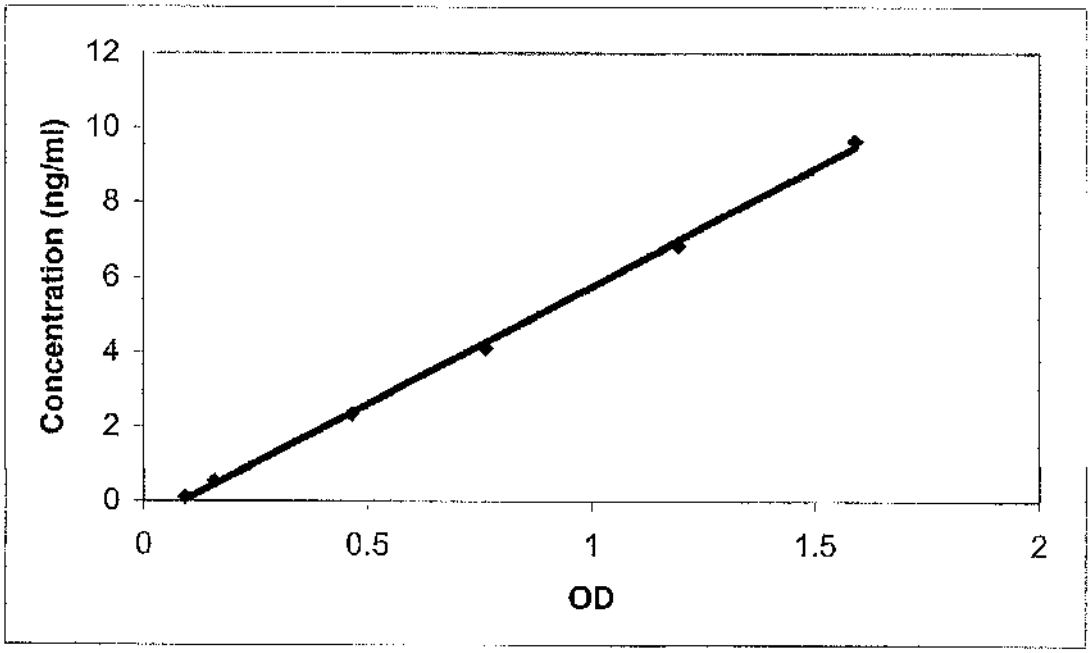


Table 53 **E-Selectin concentrations in maternal serum throughout pregnancy – raw data**

Patient Number	Sample Gestation (weeks)	E-Selectin ng/ml
Moderate Altitude		
1 Denver (D)	22	47.28
	27	57.48
	39	39.98
	PP	52.06
2D	27	47.78
	37	48.28
3D	12	31.16
	27	36.2
	32	31.41
	PP	33.55
4D	14	28.51
	26	29.77
	34	29.9
5D	12	52.69
	24	62.64
	34	73.59
6D	12	29.02
	24	37.33
	34	45.26
	PP	32.29
7D	14	21.09
	27	30.78
	38	24.74
	PP	27.26
8D	27	28.9
	36	19.83
	PP	52.44
9D	17	35.94
	26	28.89
	37	22.47
	PP	33.68

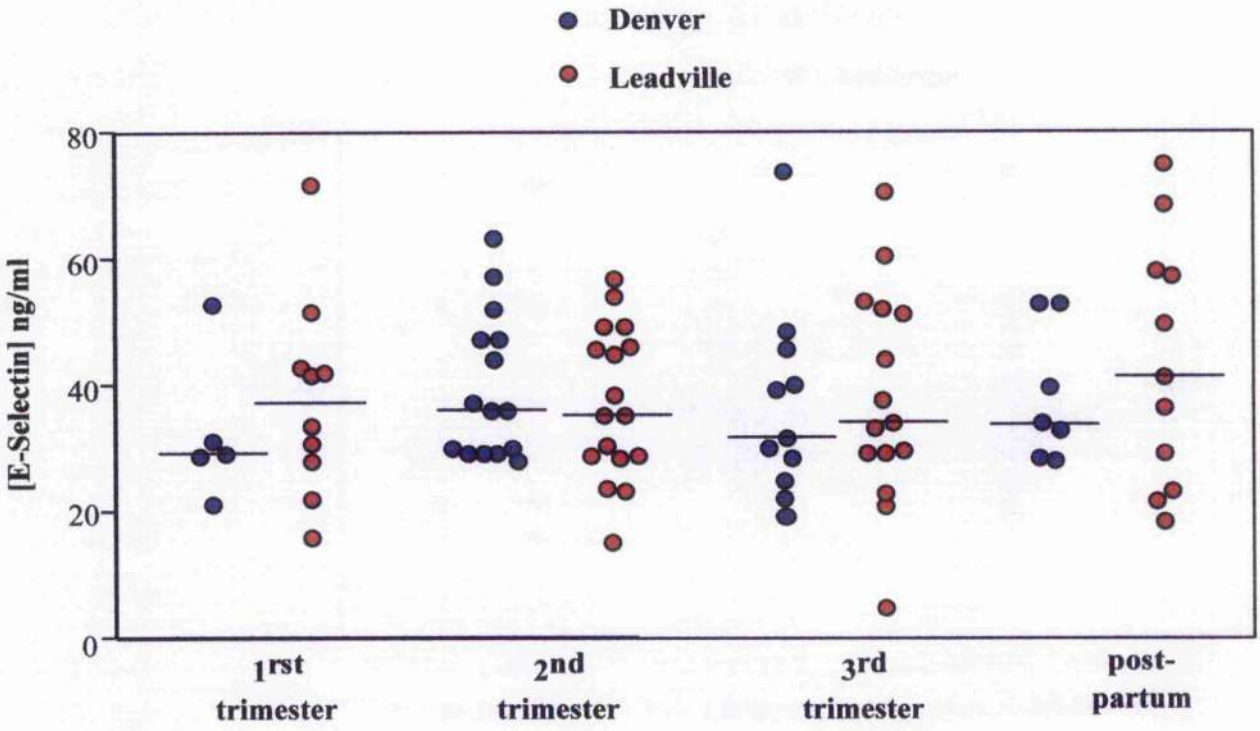
10D	15	28.14
	26	29.52
	39	28.14
	PP	28.14
11D	15	52.44
	25	44.51
	37	38.97
High Altitude		
1 Leadville (L)	25	53.95
	38	32.92
	PP	40.98
2L	11	32.42
	24	30.03
	37	21.97
	PP	36.32
3L	12	42.49
	27	49.17
	PP	74.48
5L	11	33.3
	25	22.97
	36	20.58
6L	14	51.31
	26	56.6
	37	52.82
	PP	57.1
8L	12	27.76
	25	28.64
	35	29.52
	PP	29.14
10L	12	15.8
	27	14.92
	37	4.59
	PP	18.19
11L	15	28.26
	26	34.81

	37	22.72
	PP	22.97
12L	24	23.35
	37	43.63
	PP	49.29
13L	12	30.78
	24	34.94
	37	28.89
	PP	21.59
14L	25	45.89
	37	33.93
	PP	57.98
15L	26	38.08
	39	37.58
16L	24	49.17
	32	70.32
	38	60.37
	PP	68.31
17L	13	71.58
	28	44.76
	38	53.07
18L	13	41.36
	24	28.77
	35	29.27
25L	12	41.86
	25	45.51
	36	51.18

Table 54 **E-Selectin concentrations in maternal serum throughout pregnancy – descriptive statistics**

(ng/ml)	Mean	Median	SD	SE	Min	Max	Range between min and max
HA 1st trimester	38.87	37.3	15.1	4.77	15.8	71.58	55.78
HA 2nd trimester	37.05	34.94	12.05	2.92	14.92	56.6	41.68
HA 3rd trimester	37.09	33.43	17.06	4.27	4.59	70.32	65.73
HA Post Partum	43.3	40.98	19.52	5.88	18.19	74.48	56.29
MA 1st trimester	32.49	29.02	11.91	5.32	21.09	52.69	31.6
MA 2nd trimester	39.8	36.2	11.4	2.95	28.14	62.64	34.5
MA 3rd trimester	36.6	31.4	15.37	4.63	19.83	73.59	53.76
MA Post Partum	37.1	33.6	10.7	4.03	27.26	52.44	25.18

Figure 58 **Scattergraph of maternal concentrations of circulating E-Selectin at high and moderate altitude**



7.3 Maternal concentrations of circulating ICAM-1 at high and moderate altitude

Maternal circulating concentrations of the cell adhesion molecule ICAM-1, another marker of endothelial activation, are not increased in pre-eclampsia (section 1.7.5). Therefore in the next experiment concentrations of ICAM-1 were assayed in the maternal circulation from women living at high and moderate altitude. ICAM-1 concentrations were assayed by ELISA (R&D systems) in early (8-14 weeks), mid (15-28 weeks) and late (29 weeks – term) pregnancy and post partum (n = 5, 21, 15 and 13 at MA and 10, 16, 18 and 11 at HA respectively). The gestations of the samples were analyzed using Shapiro-Wilk analysis and the data was found to have a non-normal distribution. Thus the median gestation for each trimester was calculated and is shown in table 55. When the gestations of the samples were compared using a Mann-Whitney U test there were no significant differences between high and moderate samples in first, second or third trimesters.

Table 55 **Statistical comparison of the gestations of serum samples from high and moderate altitude used in ICAM-1 assay**

	Moderate Altitude Median (Range)	High Altitude Median (Range)	p-value
1st trimester	12 (12-14)	12 (11-14)	N/S*
2nd trimester	25 (15-27)	25 (15-28)	N/S
3rd trimester	37 (32-39)	37 (31-40)	N/S

* N/S = Non-Significant (p>0.05)

After the assay was carried out a standard curve was drawn using the standard samples provided in the assay kit. The standard curve is shown in figure 59. The raw data for the samples in the assay is shown in table 56 and the descriptive statistics of the data are shown in table 57. The data was then plotted on a scattergram (figure 60). As seen in table 57, the mean ICAM-1 concentrations (ng/ml) were as follows: 1st trimester: 270.65±31.8 at MA vs 255.29±20.09 at HA, 2nd trimester: 279.16±13.79 at MA vs 246.7±14.29 at HA, 3rd trimester: 279.89±28.15 at MA vs 272.87±24.0 at HA and for PP 331.7±39.3 at MA vs 316.02±24.88 at HA.

Initially the data was analyzed at the two altitudes separately to determine if there were any variations in ICAM-1 concentrations between the individual trimesters at either altitude using an ANOVA/ Fisher's PLSD test. At high altitude ICAM-1 was significantly lower ($p<0.05$) in the second trimester than post partum. No other significant differences were found.

The data was subsequently analyzed using t-tests to determine if there were any significant differences in circulating ICAM-1 concentrations at high compared with moderate altitude firstly in the groups as a whole and then in each of the three trimesters or post-partum. There were no significant differences between high and moderate ICAM-1 concentrations in any trimester or post partum.

To test if there was any correlation between gestation and ICAM-1 concentration a Pearson's correlation was carried out. There was no correlation between ICAM-1 and gestation at moderate or high altitude or in both groups combined.

Figure 59 Standard curve for ICAM-1 assay

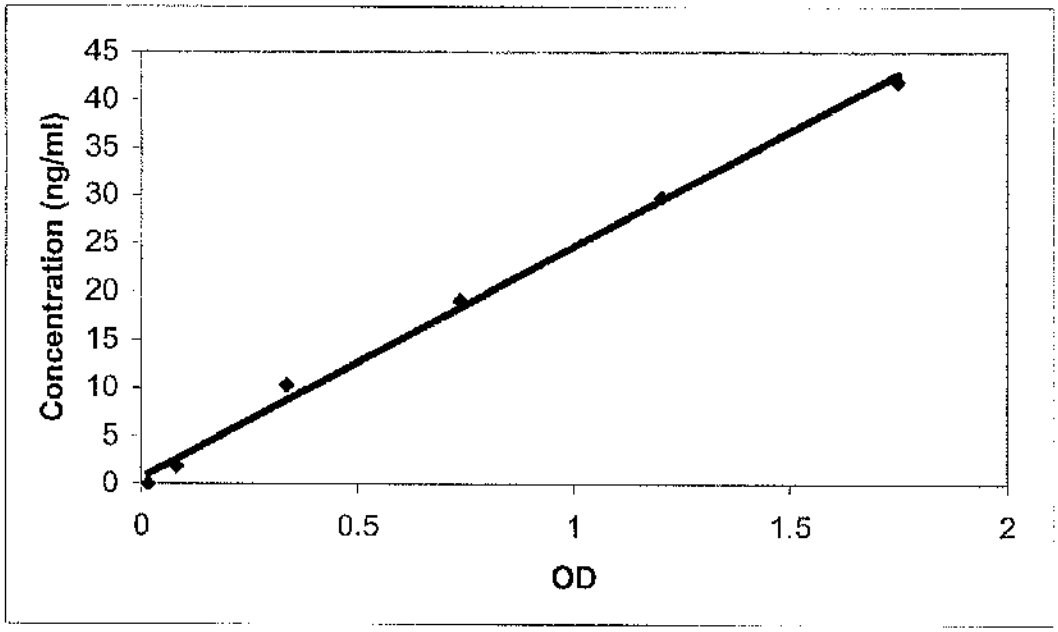


Table 56 **ICAM-1 concentrations in maternal serum throughout pregnancy – raw data**

Patient Number	Sample gestation (weeks)	ICAM ng/ml
Moderate Altitude		
1 Denver (D)	22	263.65
	27	209.11
	39	233.59
	PP	142.87
2D	27	255.67
	37	247.03
	PP	401.11
3D	12	378.56
	27	288.79
	32	296.47
	PP	211.51
4D	14	277.27
	26	298.39
	34	85.26
5D	12	191.35
	24	192.79
	34	252.31
	PP	310.39
6D	12	282.07
	24	183.19
	34	266.71
	PP	222.07
7D	14	223.99
	27	231.67
	38	175.03
	PP	284.47
8D	27	260.47
	36	233.59
	PP	253.27
9D	17	292.63
	26	256.15
	37	283.51

	PP	179.83
10D	15	223.51
	26	239.35
	39	190.87
	PP	282.07
11D	15	287.83
	25	284.47
	37	296.95
	PP	444.8
12D	17	369.43
	26	422.71
	39	571.04
	PP	464
13D	24	387.2
	36	367.51
	PP	499.5
14D	16	239.83
	26	331.99
	36	331.99
	PP	616.17
15D	16	343.51
	38	366.55
High Altitude		
1 Leadville (L)	25	248.95
	38	213.43
	PP	282.55
2L	11	233.11
	24	128.94
	37	189.91
	PP	370.39
3L	12	397.76
	27	331.03
	31	449.6
	40	507.2
	PP	445.76

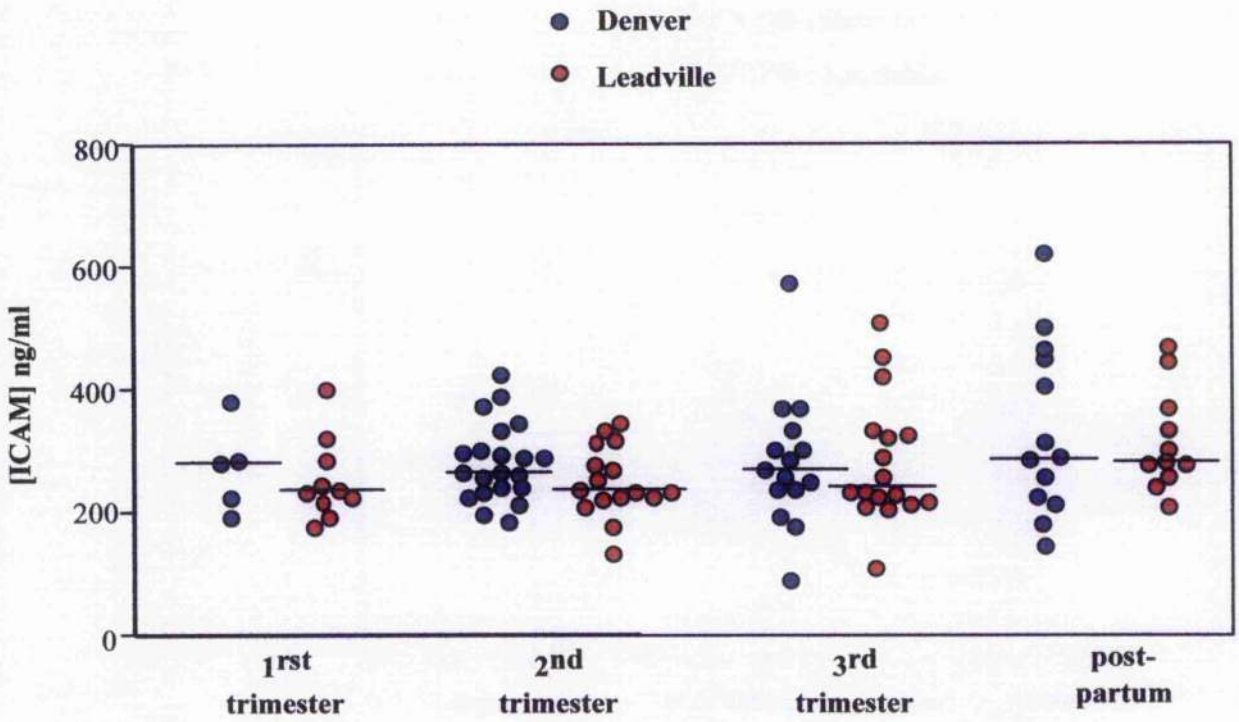
5L	11	213.9
	25	223.51
	36	228.79
6L	14	319.51
	26	313.27
	37	285.43
	PP	305.11
8L	12	230.23
	25	230.71
	35	224.47
	PP	278.71
10L	12	243.67
	27	175.99
	37	106.39
	PP	243.67
11L	15	304.63
12L	24	343.03
	37	419.36
	PP	470.71
13L	12	233.59
	24	275.83
	37	255.67
	PP	256.63
14L	25	267.67
	37	220.63
	PP	334.39
15L	26	221.59
	39	228.79
	PP	210.07
16L	24	235.99
	32	329.59
	38	320.47
	PP	278.23
17L	13	283.99
	28	205.27
	38	201.91

18L	13	223.03
	24	217.75
	35	316.15
25L	12	174.07
	25	222.55
	36	205.27
	38	208.63

Table 57 **ICAM-1 concentrations in maternal serum throughout pregnancy – descriptive statistics**

(ng/ml)	Mean	Median	SD	SE	Min	Max	Range between min and max
HA 1st trimester	255.29	233.35	63.53	20.09	174.07	397.76	223.69
HA 2nd trimester	246.7	233.35	57.16	14.29	339.3	746.5	214.09
HA 3rd trimester	272.87	228.79	101.82	24.0	106.39	507.2	400.81
HA Post Partum	316.02	282.55	82.52	24.88	210.07	470.71	260.64
MA 1st trimester	270.65	277.27	71.2	31.8	191.35	378.56	643.71
MA 2nd trimester	279.16	263.65	63.18	13.79	183.19	422.71	239.52
MA 3rd trimester	279.89	266.71	109.0	28.15	85.26	571.04	485.78
MA Post Partum	331.7	284.5	141.6	39.3	142.87	616.17	473.3

Figure 60 **Scattergraph of maternal concentrations of circulating ICAM-1 at high and moderate altitude**



7.4 Correlation of VCAM-1, ICAM-1 and E-Selectin throughout pregnancy

VCAM-1, ICAM-1 and E-Selectin concentrations were analysed to determine if there was any correlation between the concentrations of each at either or both altitudes. When high altitude was analysed alone there was a positive correlation between ICAM-1 and VCAM-1 concentrations ($p<0.01$ $R=0.4$) i.e. when a woman had high concentrations of ICAM-1 she also tended to have high concentrations of VCAM-1. There were no correlations when moderate altitude samples were analysed alone. When both altitudes were included in the analysis there was a positive correlation between ICAM-1 and E-Selectin concentrations ($p<0.01$ $R=0.33$) i.e. when a woman had high concentrations of ICAM-1 she also tended to have high concentrations of E-Selectin.

7.5 Discussion

A central pathological characteristic of pre-eclampsia is endothelial dysfunction. Women with the disease show increases in markers of endothelial dysfunction and blood vessels of these women also show altered endothelial function ex-vivo. Markers of endothelial activation include increased cytokines such as interleukin (IL)-6 (Greer, 1995) and cell adhesion molecules such as E-Selectin and VCAM-1 (Lyall et al. 1994, Lyall et al. 1995).

Women residing at high altitude with normal pregnancy outcomes have physiological changes and placental vascular remodelling intermediate between that observed in normal pregnancy and pre-eclampsia (Palmer et al. 1999, Zamudio et al. 1993). The model of chronic hypoxia in pregnancy offered by high altitude residence is unique in allowing the opportunity to distinguish between specific circulating and molecular markers of pre-eclampsia from those which may be due to hypoxia alone, without pathological consequences.

The aim of this study was to measure maternal circulating concentrations of the cell adhesion molecules VCAM-1, E-Selectin and ICAM-1 throughout pregnancy and post-partum in pregnant women residing at high altitude who had normal pregnancy outcomes. The hypothesis tested was that circulating cell adhesion molecule concentrations would be increased as a result of chronic hypoxia even in the absence of any symptoms of pre-eclampsia. No variation in VCAM-1 concentration between the trimesters was found at moderate altitude but at high altitude VCAM-1 was significantly higher in the third trimester than in the second trimester ($p<0.05$). It is possible that this increase is related to chronic exposure to hypoxia. VCAM-1 concentrations were lower overall in the high altitude group than in the moderate altitude group. However no differences in VCAM-1 were found between the moderate altitude and high altitude group between any of the individual trimesters. No other major changes were observed with either E-Selectin or ICAM-1. These data suggest that chronic hypoxia per se is not responsible for increased cell adhesion molecules in pre-eclampsia and is likely to be a secondary response to other events.

Acute high altitude exposure leads to an increase in IL-6 and other indicators of an immune system inflammatory response (Mazzeo et al. 2001, Bailey et al. 2004,

Kubo et al. 1998). Maternal circulating concentrations of the pro-inflammatory cytokines IL-6, TNF-alpha, and IL-8 are all elevated late in pregnancy in women residing at high altitude, but did not differ even marginally in the non-pregnant state. The same subjects failed to increase their levels of anti-inflammatory (Th-2) IL-10 during pregnancy, causing a marked reduction in circulating concentrations relative to low altitude control subjects that was, again, most pronounced in the third trimester when pregnancy complications develop (Coussons-Read et al. 2002). It may be that pregnant women at high altitude do not make a complete switch from Th1 to Th2-type immune responsiveness as women residing at lower altitude (Coussons-Read et al. 2002) or that the overall profile of cytokine production during pregnancy at high altitude is altered by sympathoadrenal activation secondary to the interaction of hypoxia and pregnancy and therefore favours the development of complications at the extremes of the normal range of variation. Alternatively, altered cytokine production or degradation may be a reflection of underlying mechanisms that contribute both to the observed alterations in circulating concentrations and the development of complications without one necessarily causing the other.

Vascular endothelial growth factor (VEGF) is an important survival factor for endothelium so systemic inhibition would be expected to cause generalised endothelial dysfunction. Circulating concentrations of free VEGF are reduced in the maternal circulation in pre-eclampsia and it was predicted that this may be related to alterations in receptors for VEGF (Lyall et al. 1997) e.g. the soluble receptor for vascular endothelial growth factor known as sFlt-1. sFlt-1 was first detected in 1993 (Kendall and Thomas, 1993) who confirmed (Kendall et al. 1996) that it could inhibit the actions of VEGF. Clinical trials of a neutralising monoclonal antibody to VEGF for the treatment of metastatic colorectal or renal cancer have shown that hypertension and proteinuria are the commonest side effects (Kabbinavar et al. 2003, Yang et al. 2003). Serum soluble flt-1 is increased in pre-eclampsia (Maynard et al. 2003). Because it is complexed to VEGF, its high levels in pre-eclampsia can explain the variable reports of changes of plasma VEGF in this condition. The findings may explain the results of some studies which have reported that total VEGF, rather than unbound free VEGF is increased in pre-eclampsia (Baker et al. 1995). The origin of the circulating sflt-1 is presumed to be the placenta although this has not yet been directly demonstrated. The most compelling evidence is its rapid decline in

concentration after delivery (Maynard et al. 2003). In agreement the highest concentrations of free VEGF are found in non-pregnant women (Lyall et al. 1997). VEGF is increased by hypoxia and in a similar population to the present study total (bound and free) VEGF was increased in pregnant women at 3100m throughout pregnancy compared with pregnant women living at 1600m (Wheeler et al. 2002). That the VEGF may of placental origin was supported by the observation that no differences were found post-partum. VEGF was inversely associated with birth-weight at 3100m but not at 1600m. Measurements of free VEGF were not performed. The observation that not all of the endothelial factors such as cell adhesion molecules are elevated in maternal plasma at high altitude suggest that chronic hypoxia is only responsible for some aspects of maternal activation in pre-eclampsia.

Chapter 8: Villous explant culture as a model for trophoblast invasion

8.1 Explant Culture: Determination of oxygen tension in culture medium incubated in a low oxygen incubator

CTB proliferation and differentiation has been reported to be regulated by oxygen tension (section 1.5). The development of *in vitro* models to determine the effects of different oxygen concentrations has been important in beginning to understand this process (Genbacev et al. 1996; Genbacev et al. 1997; Watson et al. 1998; Caniggia et al. 2000a; Huppertz et al. 2003). A key issue in the studies of this type is the actual oxygen levels which cultured cells are exposed to during incubation in different oxygen conditions. In some studies, specific methods to determine the oxygen concentration in the culture medium is reported (Genbacev et al. 1996; Fitzpatrick and Graham, 1998; Watson et al. 1998; Huppertz et al. 2003) while in others only the measurement of oxygen in the incubator is mentioned (Caniggia et al. 2000a; Kudo et al. 2003; Nelson et al. 2003). The aim of this study was to determine the oxygen environments encountered in the cell culture system which would subsequently be used to culture placental villous explants.

As described in section 2.5, studies were carried out using two Forma Scientific water-jacketed incubators, one set at 5% CO_2 (18% O_2) in air for standard culture conditions and one set at 5% CO_2/ 93% N_2 (2% O_2) for low oxygen conditions. Oxygen levels in each incubator were measured using a Fyrite gas analyser and were 18% and 2% in the standard and low oxygen incubators respectively.

There are many commercially available oxygen probes and meters for measuring dissolved oxygen in a liquid environment but various difficulties exist in measuring oxygen in cell culture systems such as those used in trophoblast and explant cultures. A Jenway portable dissolved Oxygen/^{o}C meter and electrode was chosen because of its suitability, in particular the integral temperature compensation. The oxygen meter was calibrated as described in the manufacturers instructions in 21% oxygen in water saturated air and to 0% using the zero salts supplied. All experiments were performed in 125ml of M199 supplemented with 10% FBS and 1% antibiotic/antimycotic solution) in a T75 vented-lid culture flask to allow stirring and so that the depth of culture medium was sufficient to cover the electrode. The flask was placed flat in the incubators to allow maximum surface area for gaseous exchange.

The surface area/ volume was 75 cm^2/ 125ml compared to 8 cm^2/ 4 ml in 35 mm culture dishes, 1.9 cm^2/ 0.4 – 1 ml in 24 well plates and 0.6 cm^2/ 200 µl in the Millicell inserts used in many trophoblast and explant culture experiments. Three experiments were performed.

a) Culture medium was warmed to 37°C in standard culture conditions. Dissolved oxygen levels were measured and the flask was then transferred to low oxygen conditions at 37°C for up to 24hr. Dissolved oxygen levels in the culture medium were measured at intervals during the incubation period.

The dissolved oxygen concentration in culture medium in standard culture conditions measured approximately 18%. When culture medium was transferred to the low oxygen incubator, oxygen levels decreased gradually to 6-8% after 4hr and 2-3% after 24hr of incubation (figure 61).

b) Nitrogen gas was bubbled through the culture medium (2-3 psi) for 30min to eliminate dissolved oxygen in the culture medium. The opening to the flask was sealed with parafilm in which there was an inlet for the pipette delivering the nitrogen gas and an outlet to allow gas to escape. The flask was immediately transferred to a 2% oxygen environment at 37°C. Dissolved oxygen levels in the culture medium were measured after 1hr and following an overnight (16hr) incubation.

When nitrogen gas was bubbled through the culture medium, dissolved oxygen levels fell from 18-20% to approximately 1.5% after 15min and to 0% after 30min. When pre-gassed culture medium (0%) was placed directly into the incubator with the 2% oxygen environment, oxygen levels in the culture medium equilibrated to approximately 2% after 1hr and remained at 2-3% following overnight incubation.

c) Nitrogen gas was bubbled through culture medium for 30min to eliminate dissolved oxygen in the culture medium, as described above. The culture medium was then dispensed into smaller volumes (5 x 25ml) to mimic culture medium changes (total dispensing time 4min) and then returned to the flask. Dissolved oxygen in the culture medium was measured immediately and the flask placed in the low oxygen incubator at 37°C. Dissolved oxygen levels were measured at time-points up to 16hr.

When culture medium pre-gassed with nitrogen to eliminate oxygen (0%) had been dispensed in air, dissolved oxygen levels increased to 8.5%. Further incubation of the cell culture medium in a hypoxic environment of 2% oxygen for approximately 16hr was required for dissolved oxygen levels in the culture medium to decrease to 2%.

8.1.2 Discussion

There are many factors that must be given consideration when measuring dissolved oxygen levels in a cell culture system. Many oxygen electrodes and meters are not suitable for use with cell cultures. Many are too large for the direct measurement of oxygen in cell cultures maintained in, for example, 35 mm dishes, 24-well plates or Millicell inserts. Smaller probes are available but often at much greater expense. Temperature is of vital importance as it affects the concentration of dissolved oxygen in a liquid e.g. the MI-730 electrode (Microelectrodes, Inc, USA) is quoted by the manufacturers as having a change in probe response of 2.2% per degree change in temperature. Thus, unless an electrode has an integral temperature compensation function care must be taken to ensure that calibration and all measurements are carried out at the same temperature, which may be difficult. Many manufacturers recommend calibration in ambient air, while pre-gassing liquids results in changes in liquid temperature that are difficult to control. Dissolved oxygen measurements cannot accurately be carried out in a static system such as that used in explant culture due to oxygen starvation at the membrane of the electrode. This would result in false low oxygen readings, while agitation of cultures could dislodge cells or explants. Some authors have reported using a blood gas analyser to measure dissolved oxygen in culture medium sampled from cell cultures (Huppertz et al., 2003). Initially a Bayer 288 blood gas analyser was used in these experiments. However, this was not suitable as the analyser could only accurately measure oxygen levels in whole blood and gave false readings with culture medium.

In order to characterise oxygen levels within the oxygen controlled cell culture environment in a way that best compensated for the factors discussed above, an oxygen electrode with automatic temperature compensation was chosen. Dissolved oxygen levels were measured in test culture medium maintained within the same environments as are our cultured cells.

Dissolved oxygen levels in culture medium incubated in a hypoxic environment of 2% oxygen following prior exposure to atmospheric oxygen or standard culture conditions decreased gradually to 2% over an incubation period of 24hr. During this time cultured cells would be exposed to varying oxygen concentrations and degrees of hypoxia depending on the length of time in culture before the desired oxygen concentration was reached. These experiments were carried out using a large volume of culture medium due to the requirements of the oxygen electrode in use. With the much smaller volumes of culture medium used in the actual cell culture experiments, the rate of gaseous exchange between the culture medium and the environment would presumably be greater and hypoxic conditions would be established more quickly. However, problems associated with culturing cells in a static environment, which may limit the rate of gaseous exchange, still remain.

Pre-gassing the culture medium, either with nitrogen to eliminate oxygen from the medium or with a gas of the desired mix and oxygen level has been suggested as a means of avoiding the situation described above. Experiment b) showed that after pre-gassing with nitrogen to 0% oxygen, dissolved oxygen levels equilibriated to 2% and remain at that level when maintained within a 2% oxygen environment. However, manipulation and microscopic monitoring of cultured cells is often carried out in air, thus exposing the culture medium and cells to atmospheric oxygen.

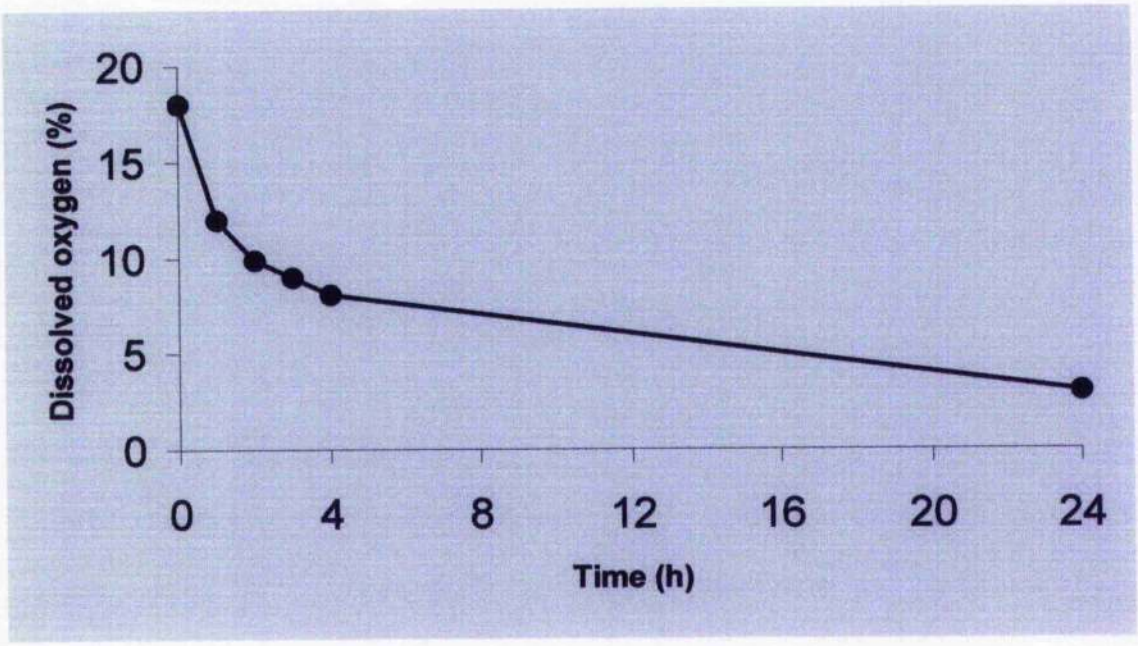
As shown in experiment c) culture medium that has been pre-gassed with nitrogen quickly absorbs atmospheric oxygen on exposure to air. Further incubation in a 2% oxygen environment for 16hr was required for dissolved oxygen levels in the culture medium to equilibrate back to 2%. Gassing cultures directly to avoid uptake of atmospheric oxygen also has difficulties particularly when millicell dishes are used e.g. dislodging the cells or explants during gassing, the cooling effect of the gas on the culture medium and the time involved in gassing individual cultures in an experiment.

Cultures maintained in a low oxygen environment would be exposed to increased oxygen levels during handling and during the associated time delay before the cell culture environment equilibrated to appropriate levels on return to the incubator, thus disrupting the exposure of the cells to the required oxygen concentration. The only way to avoid uptake of atmospheric oxygen would be to use a chamber flushed with the appropriate gas mix to allow manipulation of cultures in the same oxygen environment as the incubator atmosphere (Genbacev et al. 1996) and

then to be able to transfer the dishes to the incubator without exposure to atmospheric air.

The experiments described in this section highlight the importance of, and difficulties associated with, the validation of oxygen levels *in vitro* cell culture studies investigating the effects of oxygen on placental development and function. The issues highlighted are particularly important with regard to reproducibility and the comparison of data between different research laboratories.

Figure 61 **Oxygen concentration in culture medium incubated in a 2% O_2 incubator after various time-points**



8.2 Proliferation/invasion at high and low oxygen tension

The initial aim of this experiment was to set up an explant culture system similar to the one described in section 1.3. Villous explant cultures were prepared using a method based on that of Genbacev et al. (1992) as described in section 2.5.2. The collection of all tissue is described in section 2.4.

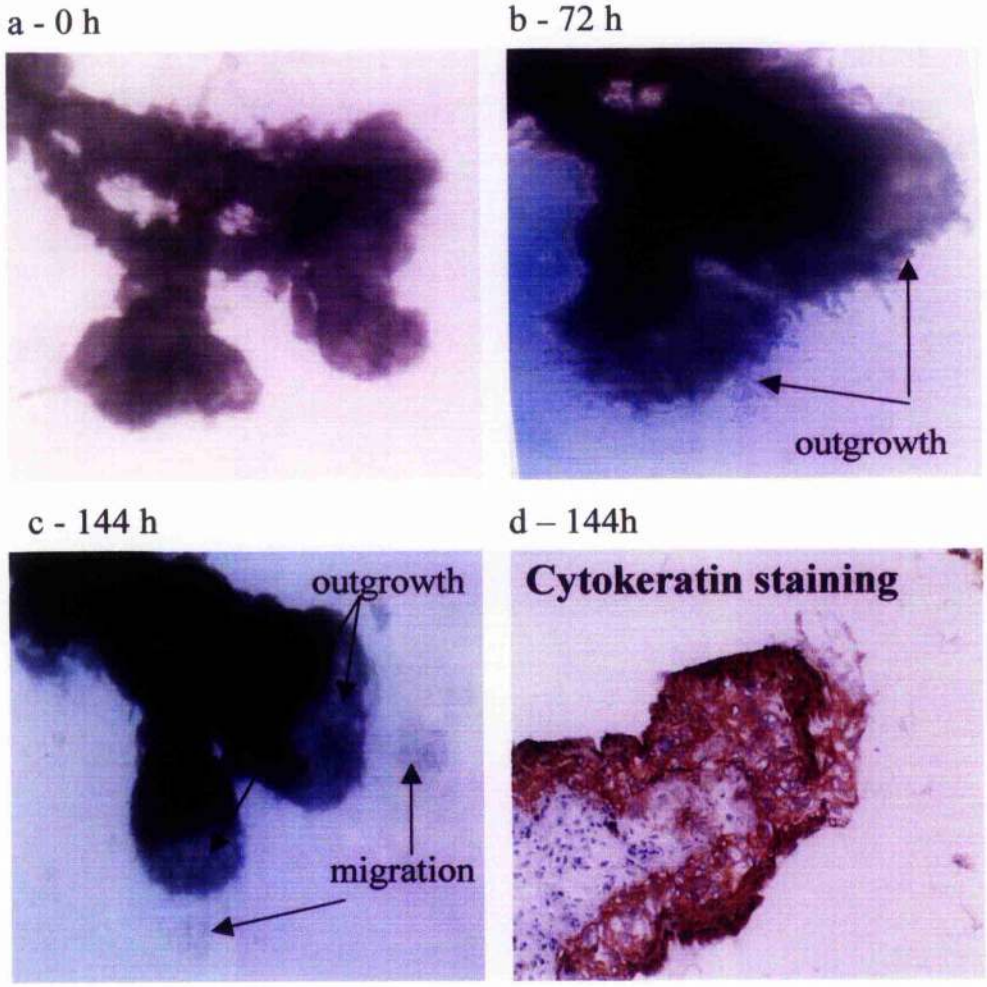
Briefly, villous tissue was isolated from TOPs, washed several times in sterile PBS and dissected to isolate small fragments of placental villi. Villous tissue (5-15mg wet weight) was arranged on the surface of matrigel-coated inserts and cultures placed in a 37°C incubator (5% CO_2 in air) for 2hr after which culture medium was added to the lower chamber of the culture dishes. Cultures were maintained at 37°C in standard culture conditions for a further 24hr and then 200µl of supplemented culture medium was carefully added to the upper chamber of the culture dish containing the explant (0hr). A set of 6 explants were maintained at 37°C in standard culture conditions (18% O_2) for 144hr. Six separate placentae were studied and from each placenta six explants (i.e. 36 explants) were cultured under standard conditions.

To compare explants cultured in low oxygen with those cultured in standard conditions, a subset of explants from each placenta were transferred to a low oxygen incubator at 0hr (following incubation of explants in standard conditions and addition of culture medium to the upper chamber of the insert). As for those cultured in normal conditions, six explants from each of six separate placentae were cultured for 144 h.

Attachment of villous explants was observed following incubation at 37°C in standard culture conditions (5% CO_2 in air) overnight. There was little change in the overall morphology of explants from plating down of the tissue to the addition of culture medium to the upper culture chamber and the start of the culture period (0hr) (figure 62a). Areas of outgrowth of cells were observed extending from the tips of some but not all villi after 72 and 144hr in culture (figure 62b and 62c). Outgrowth was observed during the first 24hr of culture in some of the explants studied but most outgrowth occurred between 24hr and 72hr. In some cases, the number of villous tips exhibiting outgrowth increased from 72hr to 144hr and in many cases the area of existing regions of outgrowth increased between 72hr and 144hr. Immunohistochemistry showed that cells at regions of outgrowth were cytokeratin-positive CTB cells (figure 62d).

Migration of individual cells and groups of cells out into the matrigel and down through the matrigel was observed in most explants at 72 (figure 62b) and 144hr (figure 62c). Migration of cells into the matrigel became apparent mainly between 24hr and 72hr of culture. Cells were also visible emerging from the tips of villi where regions of cell proliferation and outgrowth were observed (figure 62b and 62c). Migrating cells in the matrigel were easier to visualise and quantify when viewed directly under the microscope than following photography and image reproduction.

Figure 62 **Explant of first trimester villous tissue (8 weeks of gestation) cultured at 37°C in standard conditions**



8.3 Morphology and semi-quantitative analysis of explant outgrowth and migration

8.3.1 Standard culture conditions

Explant morphology was examined at 0, 72 and 144hr by two independent observers using an Olympus CK2 inverted phase contrast microscope to assess cell outgrowth and migration. Cultures were photographed at 0, 72 and 144hr using an Olympus 5ZH microscope and Olympus camera attachment and detailed sketches of each explant were drawn to record any changes over the culture time period. The number of sites of outgrowth of cytotrophoblast cells in each explant were recorded and migration of cells into or through the matrigel was scored on a scale of 0 – 5 (0 – no migration, 1 – one or two sites of localised migration, 2 – several sites of localised migration, 3 – moderate migration, 4 - moderate to extensive migration, 5 - extensive migration from several sites around the explant).

The number of villous tips exhibiting outgrowth and the area of outgrowth varied between explants from the same placenta as well as from explants from different placentae. The number of villous tips exhibiting outgrowth at 0, 72 and 144hr in six explants from the same placenta cultured under standard conditions are shown graphically in Figure 63a. Figure 64a illustrates the mean number of villous tips giving rise to outgrowth in explants cultured under standard conditions, derived from analysis of the complete data set, which included six explants for each condition from six different placentae (36 explants).

Migration scores at 0, 72 and 144hr of six explants from one placenta cultured under standard conditions are shown in figure 63b. Mean (±SE) migration scores derived from the complete set of explants cultured under standard conditions are presented in figure 64b. Migration scores representing the number of sites where migration originated from and the extent of the migration into the matrigel tended to increase from 0 – 72hr ($p<0.05$) and from 72 – 144hr ($p<0.05$) as determined by a Spearman's correlation.

8.3.2 Low oxygen conditions

Outgrowth and migration of cells in explants cultured under low oxygen conditions following an overnight pre-incubation in standard culture conditions was also examined. The morphological changes were found to be similar to those described above for explants cultured in standard conditions. Villous outgrowth and migration of cells into the matrigel was present in some but not all cultures and where present tended to increase during the culture period. The number of sites of outgrowth (figure 63c) and the migration scores (figure 63d) of six explants from one placenta cultured in low oxygen conditions is shown in figure 63. The number of villous tips exhibiting outgrowth, the area of villous outgrowth, the extent of migration into the matrigel and the number of sites from which migrating cells originated varied considerably between explants from the same placenta and between different placentae in low oxygen as with explants cultured under standard conditions. The mean number of villous tips that produced outgrowth and the mean migration scores of explants cultured under both standard and hypoxic culture conditions from the six placentae studied are presented in figure 64.

Figure 63 Time course of outgrowth and migration of cells in first trimester villous explant cultured in either standard or hypoxic conditions

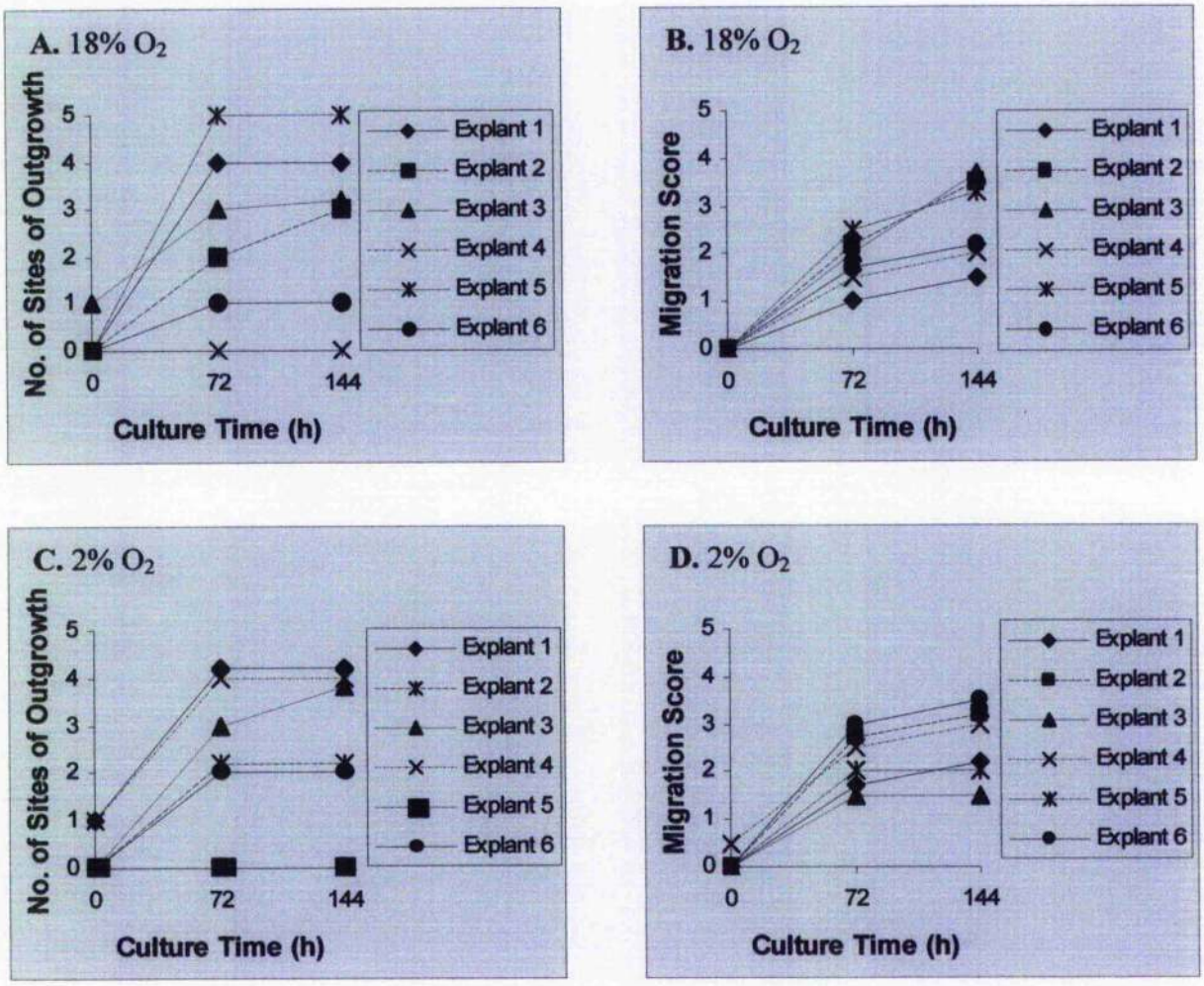


Figure 63: Time course of outgrowth and migration of cells in explants from one first trimester placenta (8 weeks of gestation) cultured at 37°C in either standard (18% O_2) or low oxygen conditions (2% O_2, with 24hr pre-incubation in standard conditions) for up to 144hr. These graphs illustrate the variation in the number of sites of outgrowth and migration scores between explants from one placenta cultured simultaneously in either standard or low oxygen conditions.

Figure 64 Cytotrophoblast outgrowth and migration in villous explants cultured in either standard or hypoxic conditions

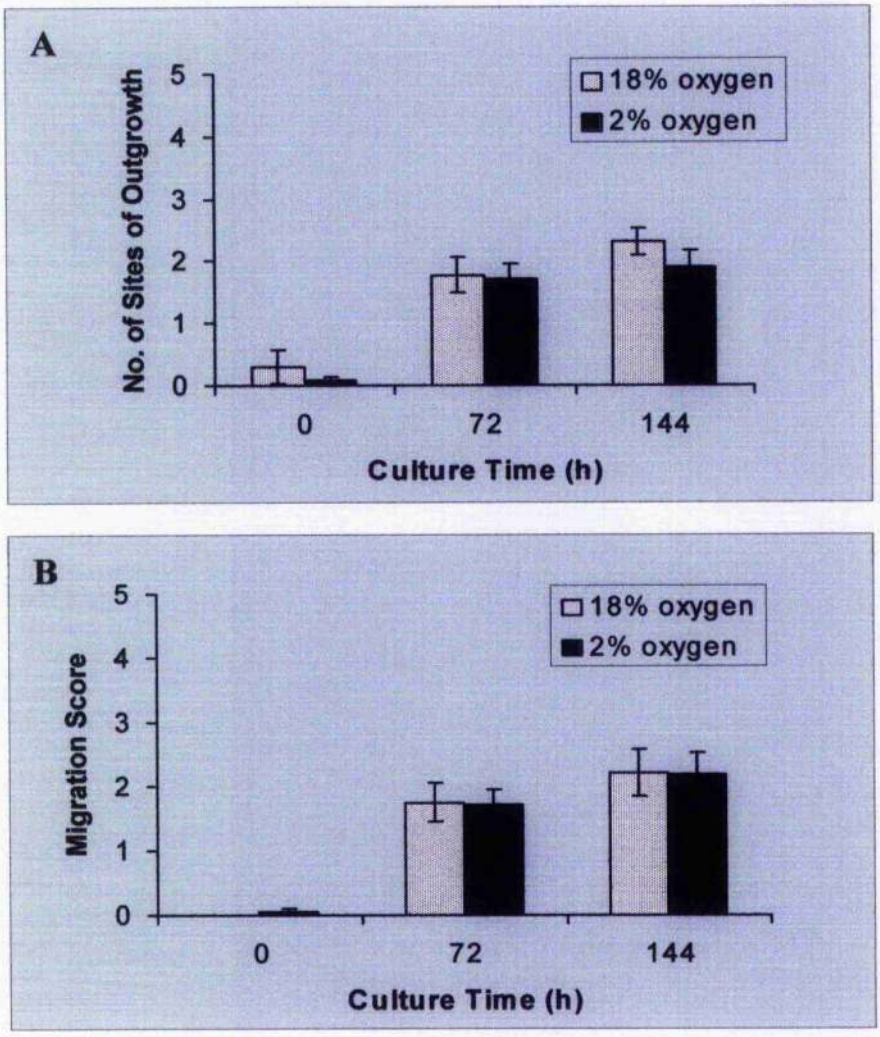


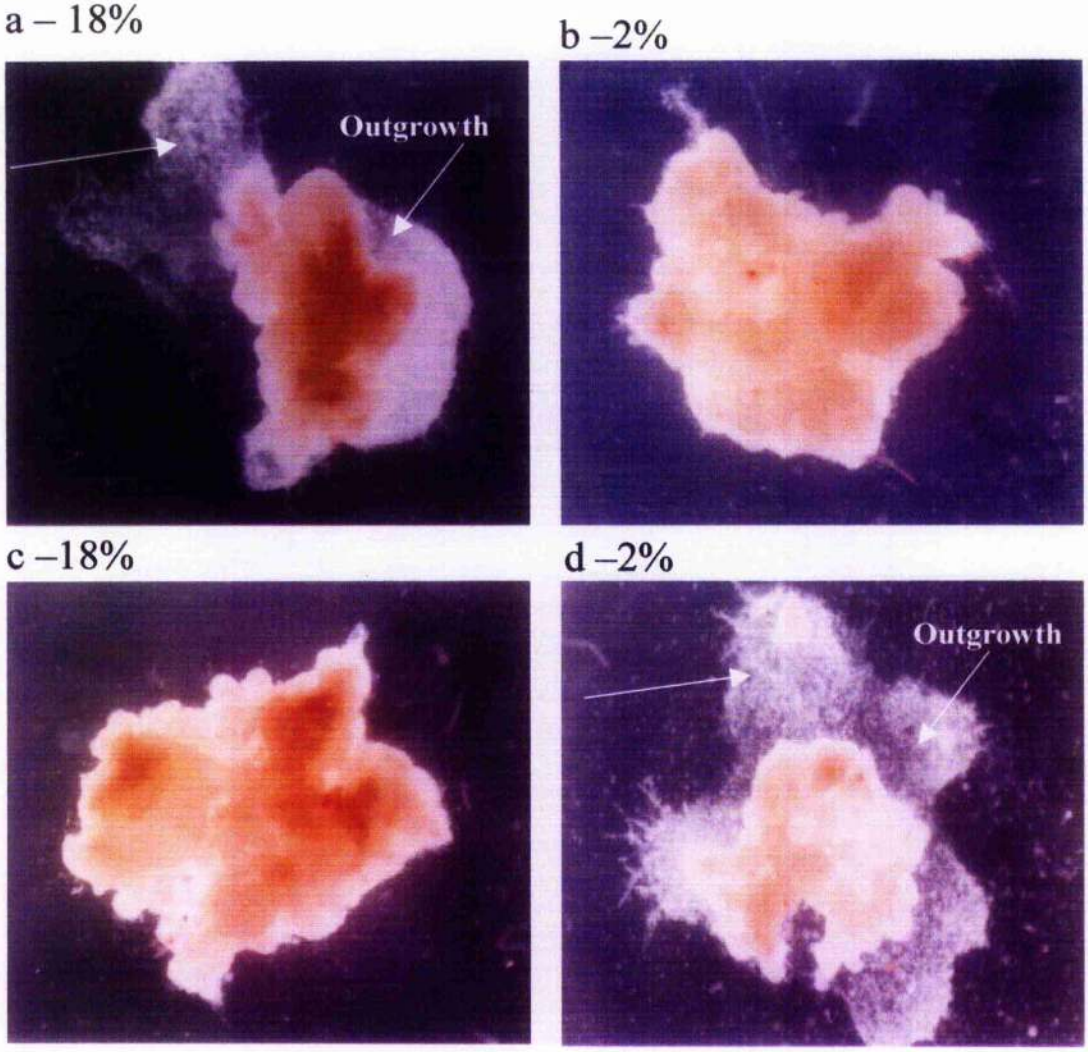
Figure 64: Cytotrophoblast outgrowth (A) and migration (B) in first trimester (8 – 9 weeks of gestation) villous explants cultured at 37°C in standard (18% O_2) or low oxygen (2% O_2) conditions for up to 144hr. Data is expressed as the mean ± SE of observations from explants cultured from six different placentae (six explants per treatment from each placenta).

8.3.3 Comparison of explants cultured under standard and low oxygen conditions

In comparing the morphology of the explants cultured under standard and hypoxic conditions, it would be possible to identify subsets of explants that appeared to show greater outgrowth and migration under standard conditions than in low oxygen conditions and vice versa. For example, figure 65a shows an explant cultured in standard conditions for 144hr. There are four clear sites of outgrowth extending from villous tips and cells can be seen migrating into the matrigel. On the other hand, in Figure 65b, an explant cultured for the same time in a low oxygen environment shows no evidence of outgrowth or migration. This may lead to the interpretation that explants cultured in standard conditions produce more outgrowth and migration than those cultured in a low oxygen environment. In contrast figure 65b shows an explant cultured in standard conditions that shows little evidence of outgrowth or migration, while in figure 65d both outgrowth and migration are clearly visible in an explant cultured in low oxygen conditions. Thus the opposing conclusion, that greater outgrowth and migration occurs in a low oxygen environment compared to standard conditions, could be assumed.

Analysis of the complete data set of 36 explants cultured in standard conditions and 36 explants cultured in low oxygen conditions showed no significant differences in the number of sites of outgrowth or in the migration scores from explants cultured in a low oxygen environment compared to those maintained in standard culture conditions ($p>0.05$) as determined by Wilcoxon analysis.

Figure 65 **Villous explants from first trimester placentae cultured at 37°C in either standard or hypoxic conditions for 144 hr**



8.4 Analysis of explants exposed directly to low oxygen without preincubation in standard culture conditions

To address the possibility that the trigger that determines whether or not explants form outgrowths or migrate into the matrigel may occur during the 24hr pre-incubation in standard conditions prior to exposure to a reduced oxygen environment, additional explant cultures (six explants per culture condition from two placentae) were set up and were placed directly into the low oxygen incubator without prior incubation (used to facilitate attachment) under standard conditions. Explants from the same placentae cultured as described previously with pre-incubation in standard conditions before culture under either standard or low oxygen conditions were also set up as controls.

Analysis of explant behaviour with regard to the establishment of sites of outgrowth and migration of cells into the matrigel revealed similar morphological changes in explants placed directly into a low oxygen environment compared to those that were exposed to standard culture conditions prior to incubation in the 2% oxygen environment (figure 66).

Figure 66 **Time course of outgrowth and migration of cells in first trimester villous explants cultured in either standard or hypoxic conditions without preincubation in standard culture conditions**

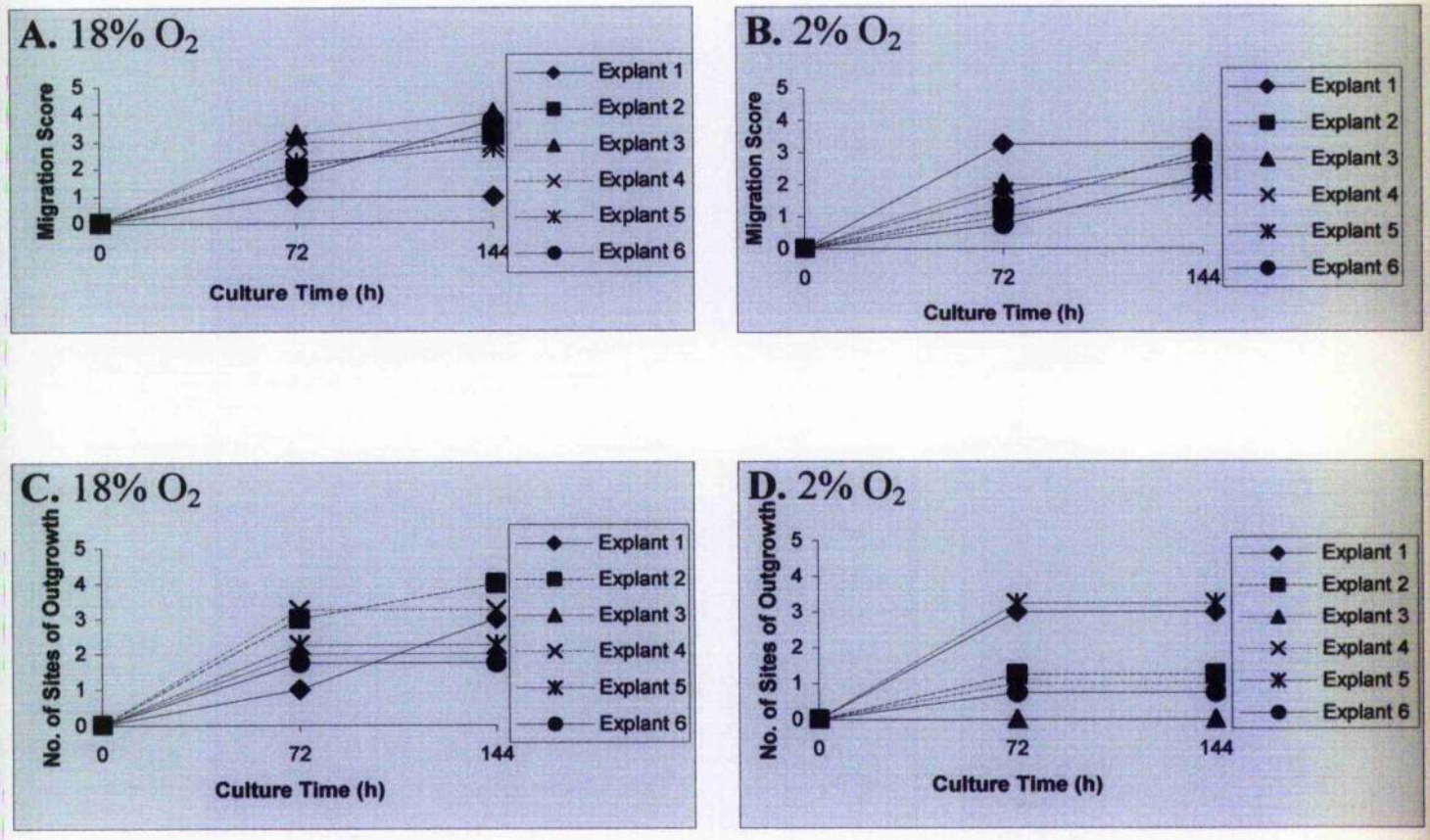


Figure 66: Time course of outgrowth and migration of cells in explants from one first trimester placenta (6 weeks of gestation) cultured at 37°C in either standard (18% O_2) or low oxygen conditions (2% O_2, without preincubation in standard conditions) for up to 144hr. Migration of cells into the matrigel tended to increase throughout the culture period both standard (A) and low oxygen (B) conditions. The number of sites of outgrowth originating from the tips of villi, visible mainly after 72hr in culture, ranged from none to 4 in explants cultured in standard (C) and low oxygen (D) conditions. Changes in explant morphology observed in explants placed directly into a low oxygen environment were similar to those observed in explants exposed to standard conditions prior to incubation in 2% oxygen.

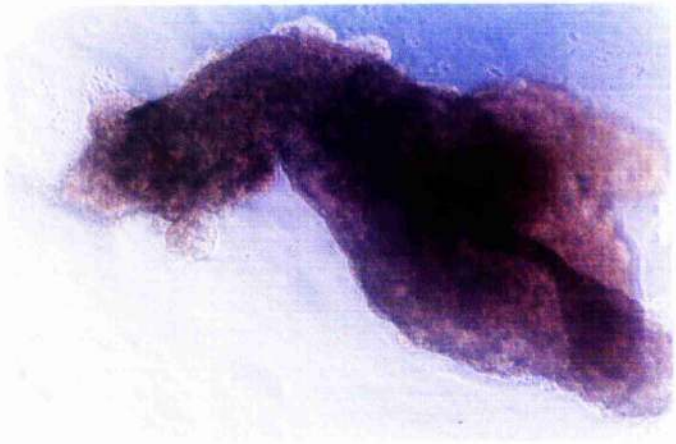
8.5 Attempt to culture chorionic villous sample (CVS) explants

Since placental villous tissue from 6-10 weeks of gestation was successfully cultured on matrigel, it was decided to attempt to culture CVS on the same matrix. It was hoped that it would be possible to correlate invasive potential of the CVS explant with pregnancy outcome in terms of the development of pre-eclampsia or IUGR.

CVS from 10-14 weeks gestation are routinely processed in the department in which the study was undertaken for pre-natal diagnosis of chromosome abnormalities. Hence small pieces of leftover tissue were readily available. No tissue from earlier than 10 weeks of gestation was available since CVS is not generally performed before this gestation. When pieces of villous tissue from 10 CVS were cultured on matrigel, no proliferation or migration of cells was seen in any of the explants in either 2% or 18% oxygen. Around three explants were obtained from each sample and the tissue pieces were considerably smaller (2mg) than those obtained from termination samples (5mg-15mg). Frozen explants were sectioned and subsequent slides were stained with LP34 and Ki67 antibodies as trophoblast and proliferation markers respectively. No signs of proliferation or cell migration were observed in any of the explants. Photographs are shown below of a representative CVS (figure 67) cultured in normal (a) and low (b) oxygen tension for 144hr.

Figure 67 **Villous explants from CVS cultured at 37°C in either standard or hypoxic conditions**

a)



10 weeks of gestation CVS 144hr culture period (standard O_2)

b)

10 weeks of gestation CVS 144hr culture period (low O_2)

8.6 Discussion

8.6.1 Morphology of villous explants cultured in standard culture conditions

Culture of villous explants from first trimester placentae on matrigel resulted in outgrowth of CTB at the tips of placental villi and migration of individual cells and groups of cells into the matrigel. Outgrowth of CTB cells at villous tips was visible in 92% of cultures maintained in standard culture conditions and migration in 94% of cultures. Villous outgrowth and migration tended to increase during the culture period. These changes described are comparable with other studies that have demonstrated that culture of first trimester villous explants on an ECM resulted in CTB proliferation and differentiation and migration of cells into the surrounding matrix (Genbacev et al. 1993a; Genbacev et al. 1993b; Genbacev et al. 1993c; Caniggia et al. 1997; Aplin et al. 1999).

8.6.2 Comparison of villous explants cultured in normal and low oxygen conditions

One objective of this study was to evaluate and characterise explant cultures by semi-quantitative analysis of these morphological changes to allow statistical comparisons between explants cultured under different conditions to be made. Explants cultured in a low oxygen environment (2% O_2) showed similar changes to explants cultured in standard conditions. Villous outgrowth and migration was observed in 86% and 88% respectively of explants of explants cultured in 2% oxygen. Statistical analysis showed no differences in the number of sites of outgrowth or in the migration scores in explants cultured in low oxygen condition compared to those cultured in standard conditions.

These results contrast with some previous studies that have demonstrated a more defined role for oxygen tension in the regulation of trophoblast differentiation and proliferation *in vitro*. Genbacev et al. (1997) reported that explants cultured from first trimester placentae in an environment of 2% oxygen showed more prominent outgrowth than in explants cultured in 20% oxygen due to increased proliferation of EVT. Invasion of CTB into the matrigel was inhibited by exposure to hypoxia.

Caniggia et al. (2000a) reported that explants cultured in standard conditions showed little outgrowth and migration while those cultured in 3% oxygen showed extensive outgrowth but no evidence of migration of cells into the matrigel. The increased outgrowth in low oxygen conditions was reported to be due to increased proliferation rather than migration of cells. Increased proliferation in first trimester explants in a 2% oxygen environment was also reported by Huppertz et al. (2003). Genbacev et al. (1996) reported that a low oxygen environment induced proliferation and inhibited differentiation of CTB. These *in vitro* studies suggest that low oxygen tension maintains CTB cells in a highly proliferative, non-invasive state.

The reasons for the lack of effect of low oxygen tension on explant outgrowth and migration in this current study are not clear. However a number of issues may have an impact in the comparison of data between laboratories. Most notably, there was considerable variation with regard to the extent of outgrowth and migration observed in explants cultured in similar environments. Variability in the size of villous outgrowth in explants cultured in standard conditions was also described by Aplin et al. (1999). Consistent with the present study, they showed that the area of outgrowth from an explant varied greatly, mainly depending on the size and number of sites of origin. The size of outgrowth also varied widely between cultures and was apparent in different cultures from the same or different placentae. This variability may be due to a number of factors that are difficult to control e.g. size and quality of the tissue used for individual explants, the region of the placenta from which the villi are sampled and whether or not the selected tissue contains or has begun to form anchoring villi. Aplin also pointed out that CTB proliferation, migration, and colonization of decidua occur *in vivo* both before and after the oxygen transition (Aplin et al. 2000; Pijnenborg et al. 1980; Kaufmann et al. 1997). The findings raise the questions of how morphological changes should be assessed, the appropriate number of explants required per condition and how many placentae should be studied for accurate and reproducible data on explant behaviour. Another key issue in *in vitro* culture studies is the validation of the oxygen levels to which the cultures are exposed as discussed in section 8.1.

Processing of villous tissue for explant culture, culture medium changes and daily microscopic monitoring of cultures tissues is usually carried out in air, so from the time of delivery of the placenta to establishment of cultures in a reduced oxygen environment tissues are exposed to atmospheric oxygen. Key events that control CTB

differentiation may therefore be affected by exposure to high levels of oxygen before the culture conditions are established. Studies by Burton and colleagues suggested that first trimester villous tissue are exposed to relatively higher levels of oxygen and oxidative stress than in the *in vivo* environment as a result of the collection procedure in theatre (Burton et al. 2003). They also showed that the level of exposure to oxygen and oxidative stress also depends on which area of the placenta the villous tissue was taken from which cannot be determined in termination material. Physiological adaptations to oxidative stress may pre-dispose explants to a particular pathway of proliferation or differentiation. In the present study, explants were initially incubated in standard culture conditions overnight prior to transfer of explants to a low oxygen environment. Further experiments revealed that the morphological changes seen in explants placed directly in a low oxygen environment were similar to those seen in explants pre-incubated in standard conditions prior to exposure to low oxygen levels.

8.6.3 Culture of CVS explants

With regard to the attempt to culture CVS explants, it was hoped that it would be possible to study the pregnancies from which these samples were obtained through gestation and correlate the outgrowth seen in explant culture with pregnancy outcome. Further to this, analysis could be undertaken of MMP secretion into the culture medium from explants cultured in standard and low oxygen conditions.

However almost no outgrowth was observed in any of the 10 separate CVS studied, from which explants were cultured both in normal and in low oxygen tension. The CVS obtained were from 10-13 weeks of gestation while the TOPs used for explant culture were from 7-9 weeks of gestation. No CVS from earlier in pregnancy were available. When a villous explant from a TOP of the same stage was cultured considerably less outgrowth was observed than in the earlier TOPs, suggesting that less outgrowth and migration may occur in these later stages of the first trimester than between 7-10 weeks of gestation. In addition to the difficulties with the gestations, the amount of villous tissue available from the CVS was very small compared with the amount available from the TOP samples. The sampling technique employed in obtaining CVS may also damage the tissue. It thus seems likely that explant culture with CVS may not be viable in the current situation.

8.6.4 Conclusions

The development of suitable culture models has and will continue to provide tools to further enhance our knowledge of placentation. Although this study does not support the findings of some previous studies on the effects of oxygen on trophoblast differentiation in first trimester explant cultures, a role for oxygen in the regulation of trophoblast differentiation and invasion in normal and pathological pregnancies is not excluded. The current study highlights some of the difficulties that need to be addressed, in particular, the importance of developing protocols to allow comparison of data generated in different laboratories and some of the issues in interpreting *in vitro* data.

Chapter 9: Final Conclusions

Deficiencies in placentation, in particular in trophoblast invasion and remodelling of the maternal spiral arteries, can result in various complications of pregnancy. Pre-eclampsia is a common major complication of human pregnancy and has been studied extensively for over 100 years yet despite this, the fundamental causes of the disorder remain unknown. The generalised maternal endothelial damage characteristic of the disease is thought to be secondary to defective placentation early in pregnancy, specifically defective trophoblast invasion and transformation of the maternal spiral arteries. Accumulating evidence supports the hypothesis that pre-eclampsia is a complex two-stage process in which defective placentation results in decreased placental perfusion, hypoxia/reperfusion injury to the placenta and the secretion of factors into the maternal circulation which result in the clinical presentation of the disease. Further complex feedback interactions cause increasing damage to the maternal endothelium.

As discussed in section 1.4, successful placentation and trophoblast invasion require regulated secretion of proteinases, which degrade the ECM and allow invasion to proceed. Several studies have described the importance of MMPs in this process, in particular the gelatinases MMP-2 and MMP-9. Coupled with the expression of MMPs is the expression of their regulatory molecules TIMPs which inhibit the action of the former and the transcription factor HIF which may be responsible for the regulation of MMP expression by oxygen tension in the in–utero environment. Despite the evidence for the role of MMPs and their regulators in placentation, the precise temporal and spatial expression of these molecules is controversial and no reports have investigated MMP, TIMP and HIF expression in the same study. One of the aims of this thesis therefore was to study MMP, TIMP and HIF expression in placentae throughout gestation using whatever methods were most appropriate.

In section 1.5 the evidence for the role of oxygen in regulating trophoblast invasion is presented. For many years investigators have sought to develop reliable models in which to study the effects of oxygen tension on the parameters of trophoblast behaviour and invasion. Several *in vitro* models of trophoblast invasion are discussed in section 1.3 and the focus of some of the work done in this thesis was the villous explant culture model developed by Genbacev et al. and widely used by many others in recent years. Three aspects of this model were investigated in the course of the work presented herein; the determination of the actual oxygen tension encountered by cells at the culture interface, the comparison of trophoblast behaviour

in standard compared with low oxygen conditions and the culture of villous explants from CVS.

In vivo studies of normal pregnancies in residents at high altitude have also allowed study of the effects of chronic hypoxia on various parameters of placentation and maternal response (section 1.6). Trophoblast remodelling of the maternal spiral arteries has been reported to be abnormal in high altitude pregnancy and this together with the increased incidence of pre-eclampsia and low birth weight at high altitude has led to the speculation that pregnancies at high altitude may be intermediate in several aspects between normal pregnancy and pre-eclampsia. The current study aimed to compare the concentration of CAMs in the maternal circulation as markers of endothelial activation in high and moderate altitude pregnancy. Several CAMs are elevated in pre-eclampsia compared to normal pregnancy and other studies have shown increased concentrations of other markers of endothelial activation in both pre-eclampsia and high altitude pregnancy. Further to this, immunohistochemistry was used to investigate MMP-9 and MMP-2 expression in term placentae from high, moderate and low altitude pregnancy in order to determine whether differential MMP expression may be a feature of placentation in hypoxic conditions.

9.1 MMPs TIMPs and HIF-1α

Although a number of studies have investigated MMP expression at various stages in pregnancy, few have compared TIMP and MMP expression in the same study. Furthermore, while the transcription factor HIF-1α had been postulated as a regulator of trophoblast invasion and specifically of MMP-9 expression, few have investigated HIF and MMP expression concurrently. Hence this study attempted to provide a comprehensive analysis of the expression and activity of MMPs and their regulators throughout pregnancy.

The results in chapter 3 showed that gelatin zymography was the optimal method for determining the activity of MMPs in placental homogenates. Immunohistochemistry was performed to determine in which cells MMP-2, MMP-9, TIMP-1, TIMP-2 and HIF-1α were expressed and to semi-quantitatively analyse expression of these proteins from 7-19 weeks of gestation and at term. The results are summarised in table 58.

Table 58 Summary of MMP, TIMP and HIF analysis

	1st trimester	2nd trimester	Term	Zymographical Analysis
MMP-9	EVT	↓ EVT	Endothelium (Muscle/Stroma)	Zymography showed negative correlation of activity with gestation through 1st trimester only
MMP-2	vCTB, EVT, (Stroma)	↓ vCTB EVT Endothelium	Endothelium (Muscle/Stroma)	Zymography showed negative correlation of activity with gestation through pregnancy and activity decreased from 1st/2nd and from 1st/3rd trimester
TIMP-1	EVT, Stroma	↑ Muscle, Stroma ↓ EVT	↑ Muscle, Stroma	
TIMP-2	vCTB, EVT, (Endothelium, Stroma)	↓ vCTB EVT Endothelium	(vCTB), Endothelium, Muscle, Stroma	
HIF-1α	vCTB, EVT, (Endothelium)	↓ vCTB EVT Endothelium	(vCTB), Endothelium, Stroma	

Note: Where a cell type is indicated in brackets, only limited staining was present.

Both immunohistochemistry and zymography results showed a greater decrease in MMP-2 activity with gestation than MMP-9.Immunohistochemical analysis showed that MMP-2 was expressed at much higher levels than MMP-9 in the vCTB. vCTB expression of MMP-2 decreased with gestation, as did EVT expression of both proteinases. The expression of both increased on endothelium and to some extent on the villous stroma and muscle surrounding the blood vessels possibly indicating a role for MMPs in vascular remodelling and development in the later stages of pregnancy. Expression of both TIMP molecules on EVT decreased from the first to the third trimesters. TIMP-1 increased in muscle and stroma, while TIMP-2 showed a high degree of co-distribution with MMP-2, declining on vCTB through pregnancy and increasing on the endothelium, muscle and stroma. It is possible that the secretion of MMP-2 by vCTB in the first trimester is important in the initial stages of placentation and is regulated in an autocrine manner by TIMP-2. HIF-1α expression was particularly high in the vCTB in the first trimester when no MMP-9 was present in these cell types which adds weight to reports by the Caniggia group that MMP-9 expression is down-regulated by the expression of HIF-1α in response to hypoxia early in pregnancy.

The work presented herein is comparable with several published studies as discussed previously. Furthermore it proposes a more significant role for MMP-2 in placentation than has been suggested in some other studies. Regulation of expression of MMPs and TIMPs in the EVT and decidua during trophoblast invasion requires further investigation in placental bed specimens.

The work herein shows particular similarities to that published by Niu et al. (2000) and further to the conclusions presented therein highlights the need to investigate the role of the villous environment in influencing trophoblast behaviour *in vitro*. It would be interesting to determine the secretion profiles of each of these molecules from cultured trophoblast and from villous explants throughout pregnancy to allow comparison between the different culture environments on the secretion of these molecules.

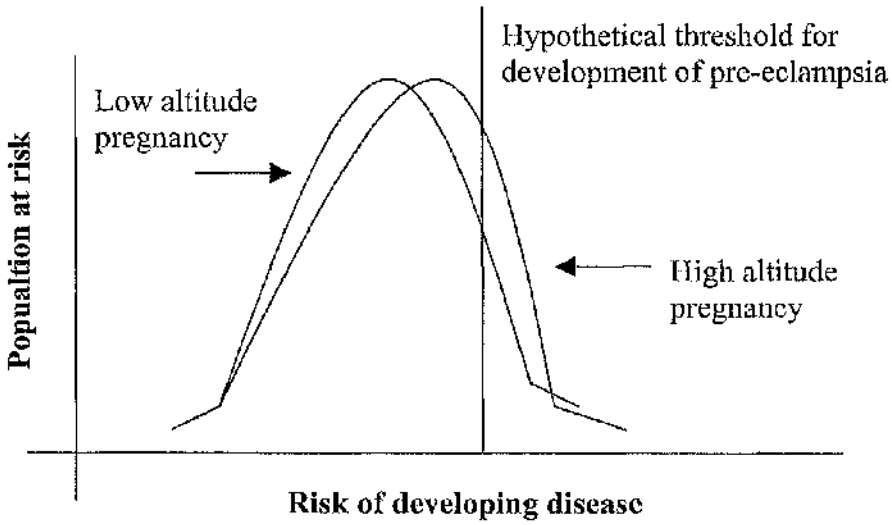
The study of each of these molecules in pre-eclamptic pregnancy both *in vitro* and in placental sections may also be informative. Pregnancies which end in miscarriage may also have defects in trophoblast invasion and immunohistochemical study is underway of MMP expression in these pregnancies.

9.2 High Altitude Pregnancy

As discussed in section 1.6, high altitude pregnancy has been postulated as a model for the development of pre-eclampsia for several years. Placentas at high altitude are subjected to chronic pre-placental hypoxia as opposed to utero-placental or post-placental hypoxia, a factor which must be considered when evaluating studies of this type. Various serum markers of endothelial activation which are increased in the disease are also elevated in normal pregnancy at high altitude. Hence the hypothesis that CAMs may also be elevated was tested. The hypothesis was not supported and it is likely that the increase in CAM concentration in the maternal circulation in pre-eclampsia is not due to hypoxia per se but rather to a more complex interaction of factors resulting from the oxidative stress in placental tissue in these circumstances. It is important to note that hypoxia is not the same as oxidative stress and it may be that the hypoxia experienced at high altitude shifts the normal distribution of women further to the right so that a greater proportion are pre-disposed to the development of pre-eclampsia (figure 68). This may result from reduced trophoblast invasion and transformation of the maternal spiral arteries.

It would be of interest to determine non-pregnant values of circulating CAMs at both altitudes to determine if the reduction in VCAM-1 seen in this study at high altitude would also pertain to non-pregnant subjects. Further work is also underway to study other markers of endothelial activation in the same samples in order to determine if any are altered as a consequence of hypoxia.

Figure 68 **Proposed model of increased risk of pre-eclampsia in high altitude pregnancy**



The study of MMP expression in placenta from high altitude provides evidence to support the hypothesis that MMP-9 may be differentially regulated *in vivo* in response to hypoxia, a finding which supports previously reported *in vitro* data on MMP-9 regulation by oxygen tension. Particularly interesting was the observation that MMP-9 was decreased in the endothelium at high altitude - this may occur in response to the lower oxygen in the maternal blood. It is interesting to speculate that a similar mechanism may also be responsible for the decreased trophoblast invasion seen in normal pregnancies at these altitudes. MMP-2 expression was not altered and MMP-9 expression in the stroma was increased suggesting that a variety of interactive factors may be responsible for the final MMP expression and activity present in any cell type. Further study to determine MMP expression patterns early in pregnancy at high altitude would be of value.

In conclusion therefore, high altitude pregnancy is providing a novel means of separating the effects of chronic hypoxia from other more complex issues involved in

the development of pre-eclampsia and determining some of the factors which may lead to an increased risk of the condition.

9.3 Villous explant culture

The development of *in vitro* models to determine the effects of different oxygen concentrations has been important in beginning to understand the processes involved in CTB proliferation and differentiation. This aim of the last section of this thesis was to investigate the widely used villous explant model. Firstly the oxygen concentration encountered by the trophoblast cells at the cell culture interface was investigated. The findings suggested that the oxygen environment in the incubator is only found in the cell culture medium after a time-delay, which is presumably dependent on other conditions such as surface to volume ratio. This highlights the need for standardisation between different laboratories as to incubation times and measurement of oxygen concentration in the culture medium. Bubbling of the culture medium with nitrogen gas did reduce oxygen to zero but this reduction was almost completely lost during dispensing of the medium.

Secondly the difference in proliferation and migration between villous explants cultured in standard and low oxygen conditions was studied. Previous studies have tended to show clear-cut differences between the two conditions. However the current study shows a need for caution in interpretation of results. Many variable factors may be responsible for determining the degree of proliferation/migration in any one villous explant. Standardisations between laboratories and investigators may help to negate some of these differences and allow more reliable comparison of data.

An attempt to culture CVS villous explants was unsuccessful, possibly due to a combination of gestation and size of samples obtained. If larger amounts of villous tissue could be obtained from continuing pregnancies earlier in gestation, it would be interesting to correlate the behaviour of these explants in various culture conditions with the outcome of pregnancy.

9.4 Final conclusions

The work in this thesis suggests that the expression of MMPs and their regulators throughout pregnancy require more study before conclusions about the relative importance of each can be made. The role of MMP-2 in particular needs to be re-evaluated. Furthermore the role of autocrine regulation of these proteinases may be important. Accumulating evidence suggests that HIF-1α may be a fundamental regulator of proteinases expression in placental development and this study adds further weight to that hypothesis. Elucidating the role of each of these individual contributors to the complex processes in normal placentation may allow new insights to be made into the pathological processes which occur in pre-eclampsia. Only by appreciating the normal can we begin to understand the abnormal.

Models of placentation and trophoblast invasion have a vital role to play in understanding the complexities of placentation. This thesis has highlighted the care required in interpreting results obtained using the villous explant model currently favoured by many investigators. Increased standardisation may be required if results between laboratories and between individual experiments are to be comparable. High altitude pregnancy as a model for hypoxia in pregnancy and potentially as a model for aspects of the development of pre-eclampsia is an area which requires further validation but whose impact could be significant.

Insights gained from studies of placental protein expression throughout pregnancy and from models of placentation and trophoblast invasion will contribute to a comprehensive understanding that may ultimately result in improved therapeutic interventions in conditions such as pre-eclampsia.

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