MORPHOMETRIC COMPARISONS OF TERM PLACENTAE FROM NORMOTENSIVE AND PRE-ECLAMPTIC PREGNANCIES SUGGEST MALADAPTATIONS OF THE FETAL COMPONENT OF THE PLACENTA IN PRE-ECLAMPSIA

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2011

DECLARATION

I, Jennifer Frances Ducray declare that

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Signed: _____ Date: _____
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Prof Thajasvarie Naicker: Supervisor

DEDICATION

For my husband Peter and my Parents Noel and Patricia Bollen,

without whose constant sacrifice and encouragement

this would not have been possible.

"Never regard study as a duty, but as the enviable opportunity to learn, to know ....for your own personal joy and to profit the community to which your later work belongs." Albert Einstein

"Mother and Child"
by Seshadri Sreenivasan
Available at http://artgallery.com.ua/bigpicture

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- and finally to my God to whom I owe everything.

ABSTRACT

Adequate maternal, intervillous and fetal blood flow are all necessary for fetal wellbeing. Compromise to any part of this exchange would be detrimental to pregnancy outcome. Pre-eclampsia is associated with reduced maternal spiral artery flow, resulting in reduced placental perfusion. This in turn creates an ischemic environment which may pre-dispose morphological changes in placental villi. This pilot study utilized morphometric image analysis to examine some features of the fetal component of the placenta in normotensive (NT) and pre-eclamptic (PE) groups. The features examined included: density of placental villi (expressed as percentage of field area occupied by placental tissue); stem vessel carrying capacity (expressed as percentage of stem villus area occupied by vessel lumina); the thickness of the stem arterial walls relative to artery size (expressed as percentage of artery area occupied by arterial wall) and the extent of fibrosis associated with villi (expressed as percentage of field area occupied by fibrosis). The results were as follows: density of placental villus arrangement NT:51.89±6.19, PE:64.78±6.93 (P<0.001); carrying capacity of stem villi NT:17.20±11.78, PE:8.67±8.51 (P<0.001); relative thickness of stem villi arterial walls NT:74.08±12.92, PE: 86.85±10.55 (P<0.001); and extent of fibrosis NT:0.727±0.310, PE:1.582±0.707 (P<0.001). These significant differences between normotensive and pre-eclamptic placentae suggest possible fetal maladaptations in response to the intervillous ischemia, compounding the existing maternal compromise to materno-fetal exchange.

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LIST OF ABBREVIATIONS

AEDF	Altered end diastolic flow
BMI	Body mass index
BP	Blood pressure
CTB	Cytotrophoblasts
DBP	Diastolic blood pressure
DPX	Dibutyl phthalate with Xylene
HELLP	Hemolysis, Elevated liver enzymes, Low platelet count
HIV	Human Immunodeficiency virus
IUGR	Intra-uterine growth restriction
MAP	Mean arterial pressure
N or NT	Normotensive group
PE	Pre-eclamptic group
p.f.	Post fertilization
SBP	Systolic blood pressure
STB	Syncytiotrophoblast
STBM	Syncytiotrophoblast microparticles
VSM	Vasculo-syncytial membrane

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LIST OF RELATED PUBLICATIONS AND PRESENTATIONS

PEER REVIEWED FULL PUBLICATION

- Ducray, J.F., Naicker, T. and Moodley, J. (2011). Pilot study of comparative placental morphometry in pre-eclamptic and normotensive pregnancies suggests possible maladaptations of the fetal component of the placenta. *European Journal of Obstetrics & Gynaecology and Reproductive Biology* 156 (1): 29-34. (ISBN: 0301-2115; Appendix 7)

PEER REVIEWED ABSTRACTS

- Ducray, J.F., Naicker, T., Moodley, J. (2009). Placental morphometry related to materno-fetal blood flow in pre-eclampsia. *Placenta* 30 (10): A66. (ISBN: 0143-4004).
- Ducray, J.F., Naicker, T., Moodley, J. (2010). Comparative placental morphometry suggests possible maladaptations of fetal component in placenta from pre-eclamptic pregnancies. *MSSA proceedings,* 40:17. (ISBN: 0-620-35056-3).
- Ducray, J.F., Naicker, T., Huppertz, B., Moodley, J. (2011). Pilot investigation: Immunolocalization of activated caspase 8 in cytotrophoblasts of normotensive and pre-eclamptic pregnancies. P*lacenta,* 32 (9): A143. (ISBN: 0143-4004).

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- Ducray, J.F., Naicker, T., Huppertz, B., Moodley, J.(2011) Pilot investigation: Immunolocalization of activated caspase 8 in cytotrophoblasts of normotensive and pre-eclamptic pregnancies. *International federation of placenta associations*, Geilo, Norway.

NATIONAL PRESENTATIONS

- Ducray, J.F., Naicker, T., Moodley, J. (2010) . Placental morphometry related to materno-fetal blood flow in pre-eclampsia. *DUT Faculty of Health Research Day*, Durban, South Africa..

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CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND

Placental growth and function involve a magnificent orchestration of multiple factors in order to ensure the life-giving exchange of pregnancy. This orchestration involves changes in the maternal uterine and systemic circulation, growth and development of the fetal circulation and of course the development of the specialized interface for materno-fetal exchange. Burton *et al.* (2006) states that "the placenta is manifestly the result of maternal-fetal interactions", however, the individual contributions and interrelationship between maternal and fetal circulations in ensuring adequate physiological exchange within the placenta is still an area of much debate and ongoing investigation.

In a brief historical overview, Burton *et al*.(2006) mention several key researchers who contributed to our current understanding of placental structure. In the 1700's two brothers, William and John Hunter did a series of investigations in which they injected molten wax into the circulation of gravid uteri. In this manner, they were able to provide proof that the maternal and fetal circulations were separate. Subsequently, in the 1800's Weber, Dalrymple and Stieve made significant contributions to the understanding of the structure of the fetal villous tree, and German researchers Borell and coworkers used cineradiography to describe the flow of blood through the intervillous space in 1958 (Burton *et al*., 2006) Original articles not available.

Advances in tissue preparation techniques and microscope technology have allowed rapid growth in our understanding and interpretation and as a result, theories of placental development, function and malfunction are constantly evolving.

The birth of a healthy infant at term is undoubtedly dependent upon normal placental development, and disordered placentation is responsible for pregnancy complications ranging from miscarriage to pre-eclampsia (Kingdom, 1998). Development of the villous tree under normal circumstances is governed by several factors, which if perturbed have the capacity to induce adaptive or maladaptive changes (Kingdom *et al*., 2000).

Amongst the many possible contributing factors for pre-eclampsia, it is clear that the placenta is involved (Redman, 1991). The role of the placenta in the aetiology of pre-eclampsia is confirmed by several factors:

- Pre-eclampsia occurs in molar pregnancies, a complication of pregnancy in which there is a placental mass with dysregulated trophoblast proliferation but no fetus (Benirschke and Kaufmann, 2000; Chun et al., 1964; Clarke and Nelson-Piercy, 2008).
- An increased incidence in pregnancies with larger placentas such as twin pregnancies (Basso and Olsen, 2001; Sibai et al., 2000).
- Resolution of the disorder only occurs with delivery of the placenta (Borzychowski *et al*., 2006).

For this reason, research in pathogenesis has predominantly focused on the role of the placenta in this syndrome.

1.2 PLACENTAL DEVELOPMENT

1.2.1 CLEAVAGE

The fertilized ovum undergoes a series of changes as it moves down the fallopian tube and into the uterus (Fig 1.2.1). Initially, cleavage (involving rapid mitoses without cellular growth) results in the formation of the multicellular morula. As the morula enters the uterine cavity fluid enters forming a fluid filled cavity, the blastocyst cavity. The developing zygote is now referred to as a blastocyst, and consists of an inner cell mass (the embryoblast) which will develop into the embryo, umbilical cord and amnion, and an outer cell mass (the trophoblast) which will develop into the fetal membranes and placenta (Benirschke and Kaufmann, 2000; Boyd and Hamilton, 1970; Ross and Romrell, 1989)

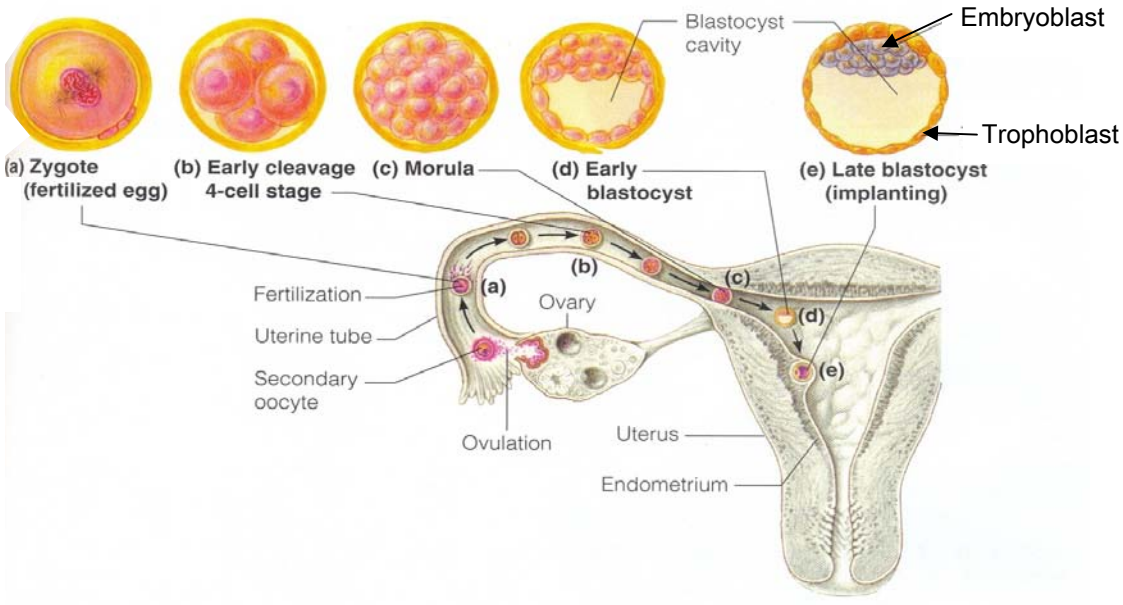

Figure 1.2.1: Cleavage of the zygote, giving rise to the blastocyst Adapted : (Marieb, 2003).

1.2.2 IMPLANTATION AND VILLOUS DEVELOPMENT

Implantation is the invasion of the blastocyst into the endometrium. It is generally recognized that it begins around 6 days post fertilization (p.f). For implantation to occur, there needs to be apposition between the trophoblast and the endometrial epithelium. In most cases, the attachment of the blastocyst to the endometrium occurs in the region overlying the embryoblast, referred to as the embryoblast pole or the implantation pole (Boyd and Hamilton, 1970).

Implantation begins when the apical plasma membranes of the trophoblast cells, and the apical plasma membranes of the endometrial cells become attached (Fig 1.2.2A). This is most unusual, as the apical plasma membranes of epithelial cells are normally non-adhesive (Denker, 1990). In addition, the blastocyst has not displayed any adhesiveness throughout it's journey in the fallopian tube, or during the pre-implantation time in the uterine cavity. The human blastocyst expresses L-selectin on its surface, and in a fashion akin to that of leucocytes, this receptor interacts with special carbohydrate ligands which results in tethering of the blastocyst to the endometrial surface, (Red-Horse et al., 2004; Norwitz et al., 2001; Vitiello and Patrizio, 2007). Apical adhesiveness of these two cell membranes is only of short duration, a time referred to as the "implantation window" (Psychoyos, 1988; Red-Horse et al., 2004; Achache and Revel, 2006).

The trophoblast cells rapidly proliferate and begin to invade the endometrium. The increased proliferation results in the differentiation of the trophoblast into two distinct layers (Heuser and Streeter, 1941; Benirschke and Kaufmann, 2000). Internally, is a mitotically active cytotrophoblast layer. The ongoing fusion of the proliferating trophoblast cells forms an outer

multinucleate cytoplasmic mass known as the syncytiotrophoblast (Fig 1.2.2B). The syncytiotrophoblast is a non-mitotic invasive layer, displaying fingerlike extensions that invade into the endometrium. The cytotrophoblasts essentially support the growth and regeneration of the syncytiotrophoblast by mitoses and subsequent fusion with the overlying syncytium (Burton et al., 2006; Gauster et al., 2009). Over the following days, the progressive invasion along with cytotrophoblast proliferation and fusion results in the syncytiotrophoblast becoming relatively thick over the implantation pole of the blastocyst.

Around day 8 p.f. small vacuole-like spaces appear in the syncytiotrophoblast. These enlarge and fuse in such a way as to become confluent, forming a system of lacunae. The sections of separating syncytiotrophoblast are referred to as trabeculae (Burton *et al*., 2006). Around day 12p.f.(Fig 1.2.2C) the blastocyst is deeply embedded and the endometrial epithelium closes over the implantation site (Boyd and Hamilton, 1970). At this point, the entire outer surface of the blastocyst consists of syncytiotrophoblast. The cytotrophoblast continues to multiply, and around day 12 p.f. the proliferating cytotrophoblast layer of the chorionic plate begins sending cords of cells into the syncytiotrophoblast trabeculae, forming what are referred to as primary chorionic villi. On day 14 p.f. (Fig 1.2.2D) mesenchymal cells derived from the cyto-trophoblast spread on its inner surface transforming into a loose network, the embryonic mesenchyme (Enders and King, 1988).

Shortly after this, secondary chorionic villi are formed (Fig 1.2.2E) by the embryonic mesenchyme invading and forming a central core of loose connective tissue, and reducing the cytotrophoblasts to a single layer (Boyd and Hamilton, 1970; Wislocki and Streeter, 1938).

These secondary villi become tertiary chorionic villi (Fig 1.2.2F) once fetal blood vessels develop in the connective tissue cores (Demir et al., 1989; Burton et al., 2006; Kingdom et al., 2000). As the tertiary villi are forming around day 15, some cytotrophoblast cells in reaching the peripheral ends of the trabeculae, meet up with cytotrophoblasts from neighbouring villi and grow laterally to form an outer boundary called the trophoblastic shell (Boyd and Hamilton, 1970).

The longer villi which remain attached to the trophoblastic shell form the anchoring villi. Proliferative activities together with branching result in the development of the primitive villous trees. The cytotrophoblastic cells at the foot of the anchoring villi are called cell columns, and their proliferation allows for longitudinal growth of the villi. These cells also give rise to what is referred to as the extravillous trophoblast population (see section 1.2.3). As the invasive syncytiotrophoblast encounters maternal vessels, it erodes their walls, allowing maternal blood to fill the interlinking lacunar system.

The difference in pressure between the maternal arterial and venous elements linking into the lacunae allow for directional flow through the arteries and back through the veins, thus beginning to establish uteroplacental circulation. Initially sluggish, the flow increases as the invasion deepens to include larger vessels (Boyd and Hamilton, 1970; Burton et al., 2006). As the lacunae enlarge, they link up forming a large collective space surrounding the developing villi. By definition this is now the intervillous space, and is filled with maternal blood in contact with the syncytiotrophoblast surface of the villous trees (Kingdom *et al*., 2000).

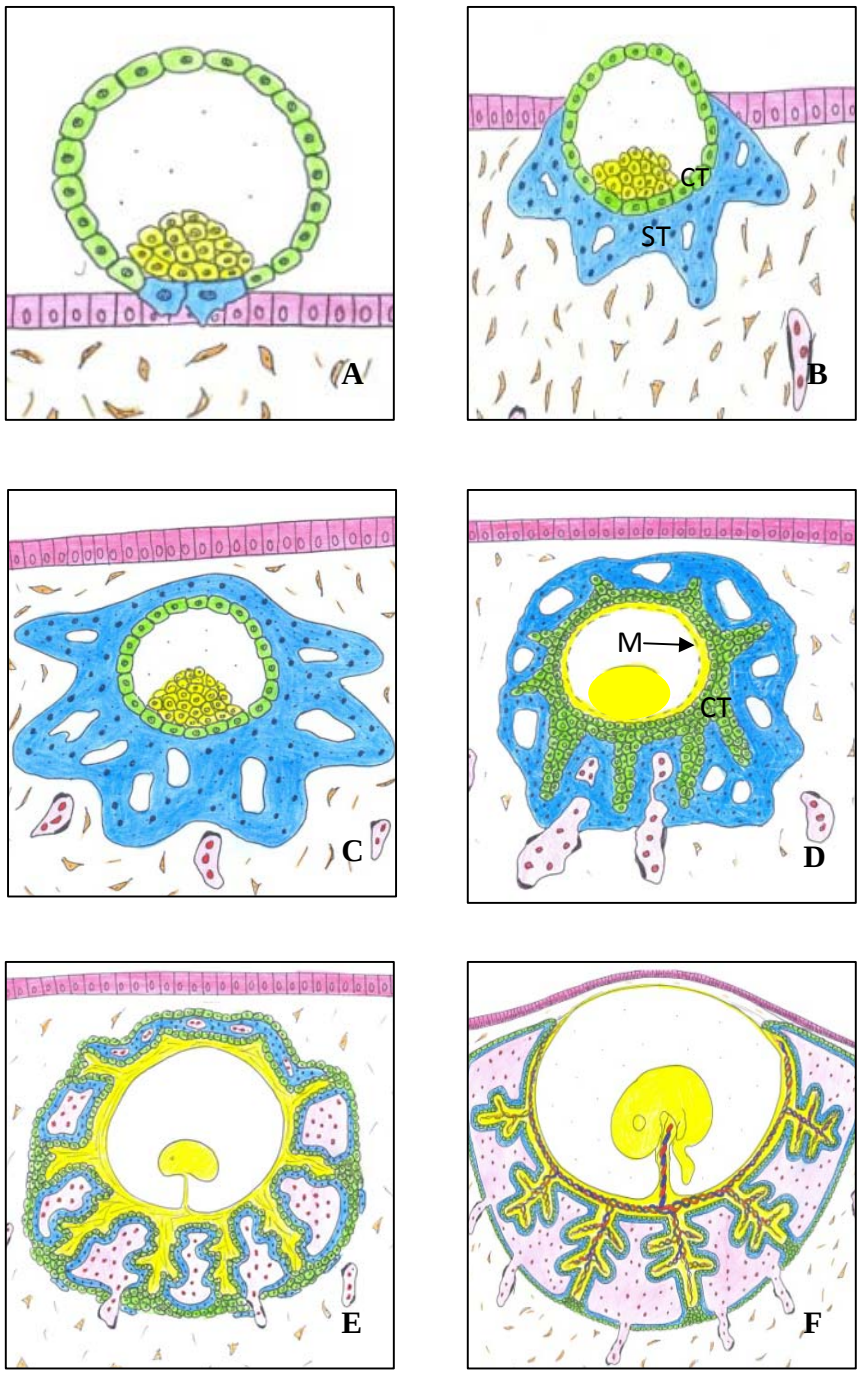

Figure 1.2.2: Implantation of the blastocyst and villous development. **A:**Apposition between the blastocyst trophoblast and endometrial epithelium. **B:** Invasion of the syncytiotrophoblast (S) which is formed by mitoses and fusion of the cytotrophoblast cells (CT). **C:**Repair of endometrial epithelium and formation of lacunar system. **D:**Cords of cytotrophoblast (CT) cells invading the syncytiotrophoblast, and formation of embryonic mesenchyme (M) internally. **E:**Invasion of embryonic mesenchyme to form secondary chorionic villi. **F:**Development of fetal blood vessels in the connective tissue cores, transforming them into tertiary villi. Adapted from Ross and Romrell (1989) and Benirschke and Kaufmann (2000).

1.2.3 EXTRAVILLOUS TROPHOBLAST INVASION

The extravillous trophoblast cells invade the endometrium along two pathways: *Interstitial invasion* takes place into the endometrial tissue, whilst *endovascular invasion* takes place into the vessel lumina of spiral arteries (Fig 1.2.3). This invasion of the extravillous trophoblast populations transforms the previously narrow and vasoreactive spiral arteries into large sinusoidal like vessels (Pijnenborg et al., 2006; Brosens et al., 1967). This is achieved by the invading trophoblasts degrading and replacing the endothelium, smooth muscle and internal elastic lamina, thus reducing vascular reactivity (Brosens *et al*., 1967). These low-pressure high-flow vessels ensure adequate blood flow into the intervillous space to meet the oxygen and nutrient demands of the fetus (Kaufmann *et al*., 2003).

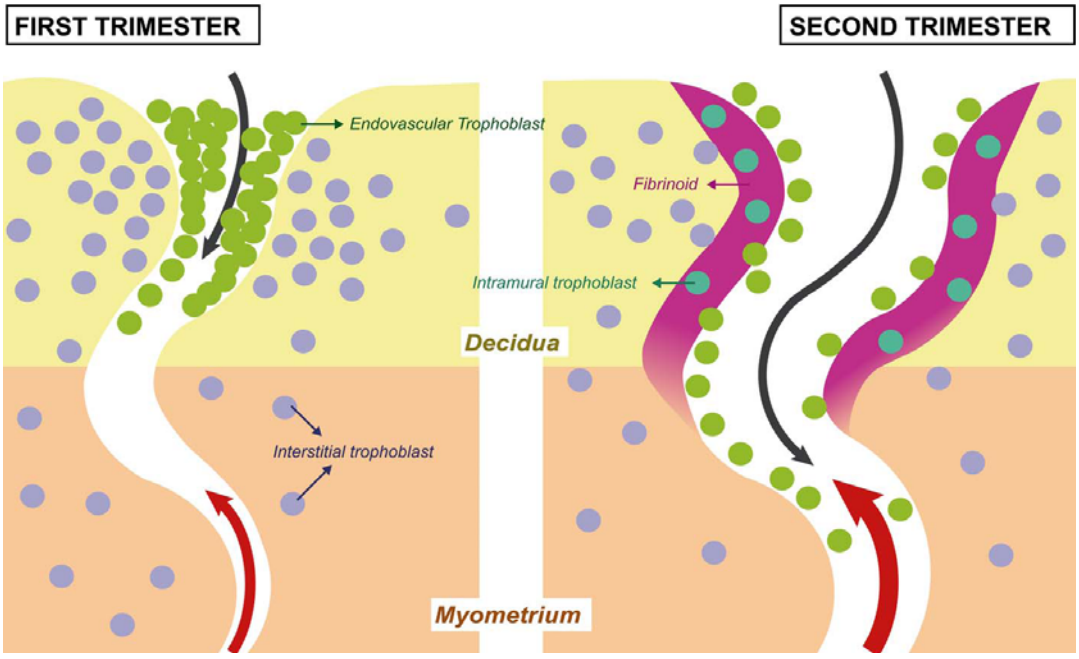

Figure 1.2.3: Diagram illustrating the concept of two waves of trophoblastic invasion into the spiral artery. On the left hand side one can see the endovascular tophoblast migration into the decidual segments of the spiral arteries which occurs during the first trimester. On the right hand side which is illustrating a second semester spiral artery, this endovascular migration is seen extending into the myometrial segments the of spiral arteries. In addition, the intramural trophoblast invasion which transforms the vessel walls and alters vasoreactivity is illustrated. Red arrow: direction of blood flow; black arrow: direction of endovascular trophoblast migration. Adapted from Pijnenborg *et al.* 2006.

1.3 PLACENTAL STRUCTURE

1.3.1 GENERAL OVERVIEW

The human placenta is disc-shaped thickening of the membranous sac of the fetus, in which the exchange area, the intervillous space, is located between the chorionic plate and the basal plate (Fig.1.3.1). Towards the placental margin, there is a narrowing and eventual obliteration of the intervillous space, until the placenta ends with the fusion of the basal plate and chorionic plate to form the chorion laeve. Overlying the chorion laeve is the amnion, and underlying the basal plate (consisting of stroma of the maternal decidua) lies the myometrium (Benirschke and Kaufmann, 2000).

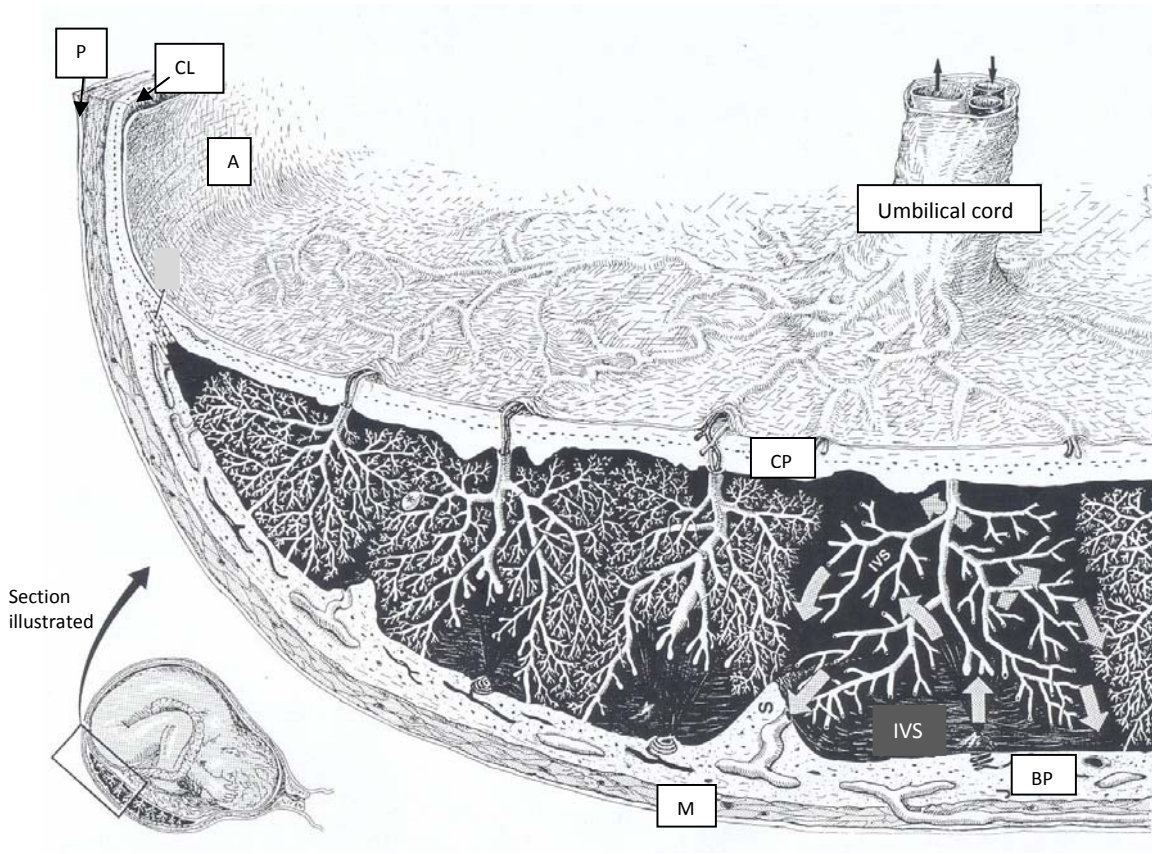

Figure 1.3.1: A section through the human placenta showing the intervillous space (IVS) between the chorionic plate (CP) and basal plate (BP) fusing peripherally to form the chorion laeve (CL) The amnion (A) is seen overlying the chorion laeve, and under the basal plate lie the myometrium (M) and perimetrium (P). (Adapted from Bernirschke and Kaufmann 2000).

1.3.2 THE VILLOUS TREE

The placental design is classified as "villous" (Boyd and Hamilton, 1970; Burton *et al*., 2006). In villous placentas, numerous branching tree-like villi project out of the chorionic plate into the intervillous space. These villi constitute the fetal side of the placenta. They have a core of connective tissue or mesenchymal stroma in which the fetal vessels are located. Surrounding this is a covering of trophoblast. The fetal vessels of the villi are involved in the exchange of nutrients and oxygen with the maternal blood, and are connected to the actual fetal circulation via the larger vessels of the chorionic plate, as well as the umbilical vessels (Benirschke and Kaufman, 2000).

The trophoblast layers separate the maternal blood of the intervillous space from the interior of the villi (Fig. 1.3.2). The outer multinucleated syncytiotrophoblast is in contact with the maternal blood. Between the basement membrane of the syncytiotrophoblast lie the cytotrophoblast cells. In early fetal life they are seen as a continuous layer, but as pregnancy progresses, the number of cytotrophoblasts relative to syncytiotrophoblast area diminishes and they become discontinuous, only being seen as isolated cells or clumps of cells. However, although the number in a given area is decreasing, studies have shown that the actual number of cytotrophoblasts increases steadily until term (Simpson *et al*., 1992).

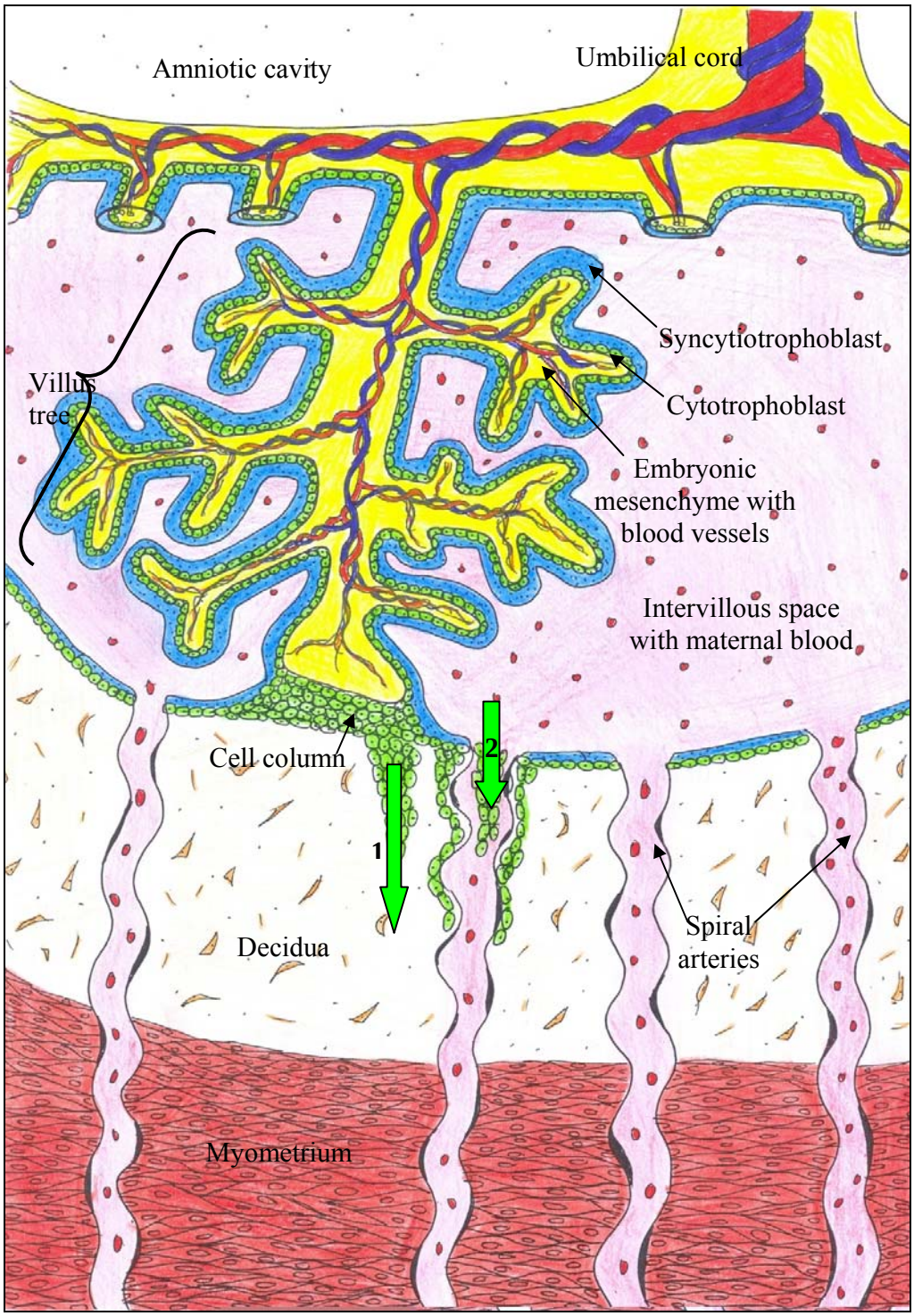

Figure 1.3.2: Single anchoring tertiary villus with trophoblastic cell column. Waves of invading extratrophoblast cells 1: interstitial invasion and 2: endovascular invasion.

The villous branches in differing positions in the villous tree display different features in relation to their function. Five villous types (Fig.1.3.3) have been described (Benirschke and Kaufmann, 2000; Burton et al., 2006; Kaufmann et al., 1979; Sen et al., 1979)

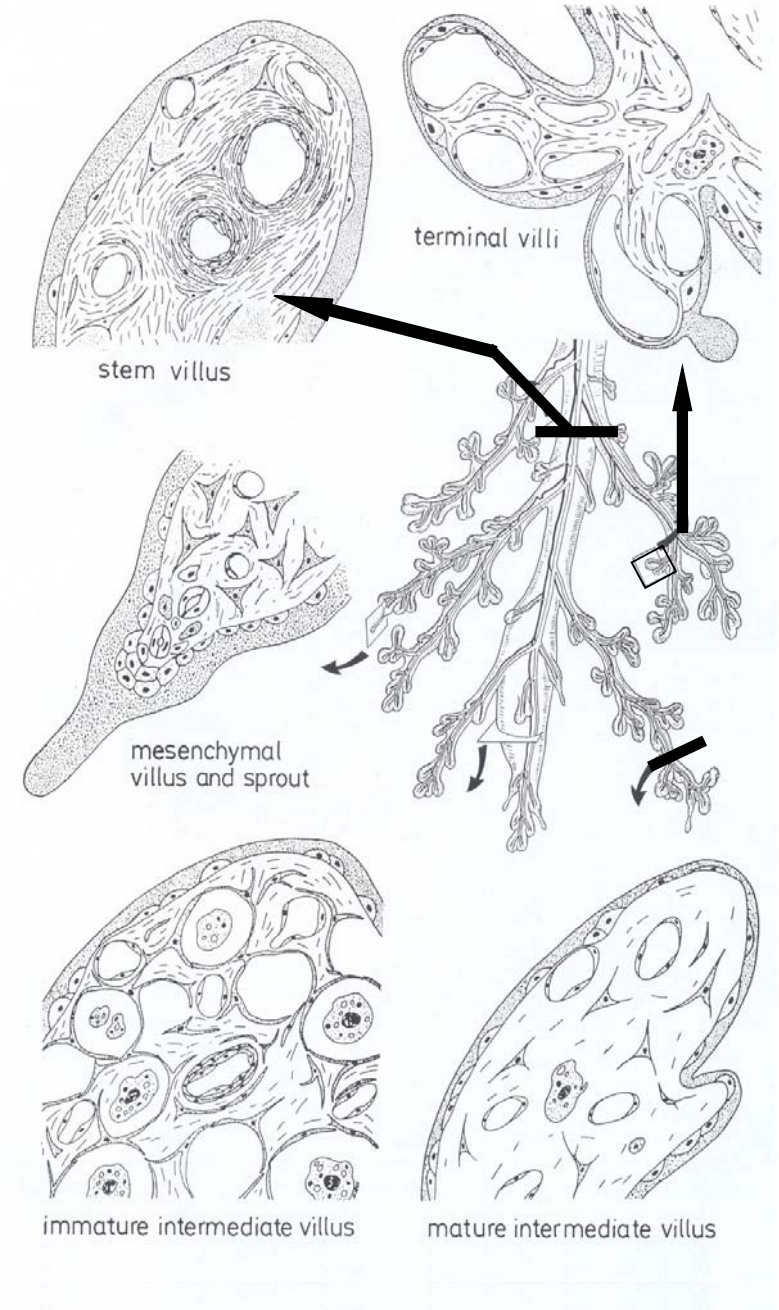

Figure 1.3.3: Diagrammatic representation of a single villous tree together with typical cross sections of the different villous types (Adapted from Benirschke and Kaufmann, 2000)

1.3.2.1 Stem Villi

Stem villi include: the main central trunks connecting the villus trees to the chorionic plate; several generations of branching from these trunks; and the anchoring villi. They comprise about 20-25% of the total villous volume in the normal mature placenta. Stem villi are characterised by a condensed fibrous stroma made up of bundles of collagen and occasional fibroblasts. Whilst the peripheral connective tissue contains non-contractile fibroblasts, the more centrally located connective tissue cells (those located closer to the vessels) are myofibroblasts (Demir *et al*., 1997). The central myofibroblasts have been referred to as the perivascular sheath (Graf *et al*., 1995). The trophoblastic cover often degenerates towards term, becoming replaced by perivillous fibrinoid (Benirschke and Kaufmann, 2000), Fig.1.3.4. One or more arteries/veins (or arterioles and venules in smaller calibre villi) are present, with a clearly visible media or adventitia. These are generally accompanied by paravascular capillaries representing vasa vasora (Kingdom *et al*., 2000).

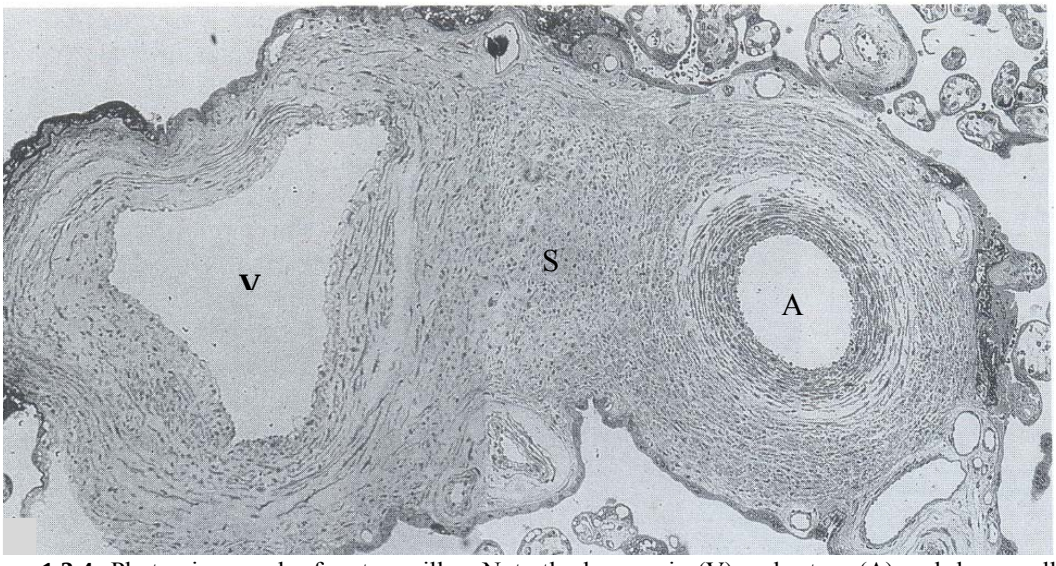

Figure 1.3.4: Photomicrograph of a stem villus. Note the large vein (V) and artery (A) and dense collagenous stroma (S) Initial mag 115X. (Benirschke and Kaufmann, 2000).

Functionally, stem villi serve as a mechanical support to the villus tree, as well as carrying vessels conducting the fetal blood to and from the peripheral exchange areas. Taking into consideration the lack of fetal capillaries as well as the degeneration observed in the trophoblast layer, it is generally assumed that their role in materno-fetal exchange can be considered negligible. However, the presence of large fetal vessels with thick muscular walls with myofibroblasts suggests a role in auto-regulation of the fetal aspects of placental circulation (Benirschke and Kaufmann, 2000). In addition, because stem villi extend far into the villous space and, in the case of anchoring villi, actually connect the chorionic plate and the basal plate, contraction of longitudinally oriented muscle in the perivascular sheath of these villi could conceivably effect a decrease in the intervillous volume, thus increasing uteroplacental flow impedance (Fig.1.3.5). In this way, the fetus could gain some control over flow of the maternal blood through the intervillous space by increasing or decreasing impedance (Demir *et al*., 1997).

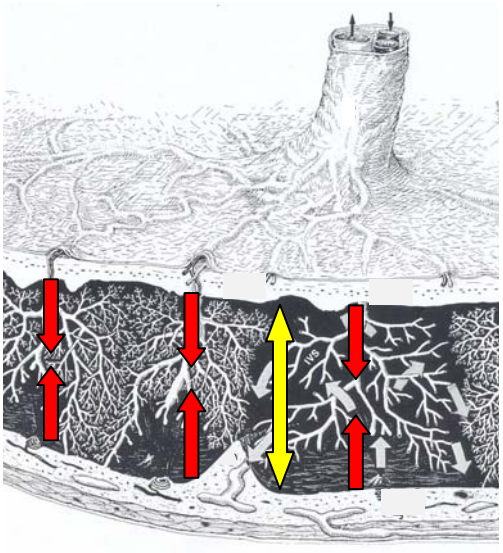

Figure 1.3.5: Diagram illustrating how the anchoring villi, which span from the chorionic plate (CP) attaching onto the basal plate (BP), have the capacity to reduce the intervillous depth (Yellow arrow) thereby increasing impedance in the intervillous space (Benirschke and Kaufmann, 2000).

1.3.2.2 Mature Intermediate Villi

These are the long slender branches of stem villi from which the terminal villi arise. They are typically zigzag in appearance due to this branching (Fig.1.3.6). About a quarter of villous volume of the mature placenta is comprised of mature intermediate villi. They lack the dense collagenous stroma of the stem villi and have rather loose bundles of connective tissue fibers and fixed connective tissue cells (Benirschke and Kaufmann, 2000).

The vessels are slender elongated fetal capillaries, as well as small terminal arterioles and collecting venules (although these arterioles and venules lack a media or adventitia identifiable by light microscopy) (Kaufmann, 1982; Kingdom et al., 2000). In cross section, they are very similar in appearance to terminal villi, but can be differentiated on the basis of a lower percentage of the villous volume being occupied by vessel lumina-21% as opposed to the 45% of terminal villi (Benirschke and Kaufmann, 2000).

Functionally, the mature intermediate villi give rise to the terminal villi. During development, the capillary growth exceeds that of the villi themselves, causing lateral bulges in the capillaries, which push against the trophoblast, bringing fetal blood close to the intervillous space and forming the terminal villi. The high degree of vascularisation along with the large contribution to the surface area (31%) shown in intermediate villi indicate their importance in materno-fetal exchange. Together with the terminal villi they form the exchange surface area of the villous tree (Benirschke and Kaufmann, 2000; Kaufmann et al., 1985).

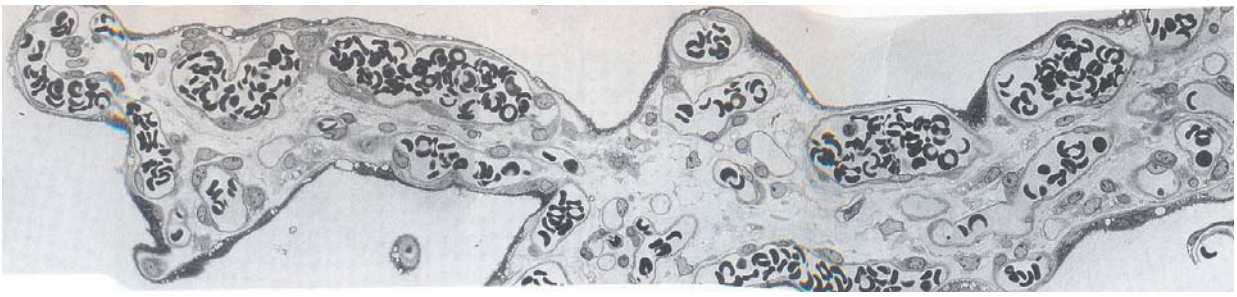

Figure 1.3.6: Photomicrograph of a longitudinal section of an intermediate villus. Note the zig-zag appearance, the loose connective tissue stroma and small calibre vessels located relatively close to the trophoblastic cover. Initial mag 430X (Benirschke and Kaufmann, 2000).

1.3.2.3 Terminal Villi

The final grapelike ramifications of the mature intermediate villi generally branch from a constricted neck region of the mature intermediate villi on the convex side (Fig.1.3.7). Terminal villi comprise 40% of villous volume, and 50% of villous surface area. They are characterised by a high degree of capillarization many of which are sinusoidally dilated. These capillaries occupy more than 50% of stromal volume and more than 35% of villous volume (Benirschke and Kaufmann, 2000; Kaufmann et al., 1985).

The dilated sinusoidal capillaries often bulge against the thin trophoblastic layer, attentuating it into an extremely thin vasculosyncytial membrane (VSM) devoid of nuclei and other large organelles. The result is that the diffusion barrier is extremely thin in these areas (Burton *et al.*, 2006). It has also been noted that there is an inverse relationship between the incidence of villous VSM's and fetal hypoxia (Benirschke and Kaufmann, 2000).

The high degree of vascularisation of terminal villi, as well as the small mean materno-fetal diffusion distance are features suited to diffusional exchange. Together with the mature intermediate villi, the terminal villi represent the main sites of materno-fetal exchange in the third trimester placenta (Kaufmann *et al*., 1985).

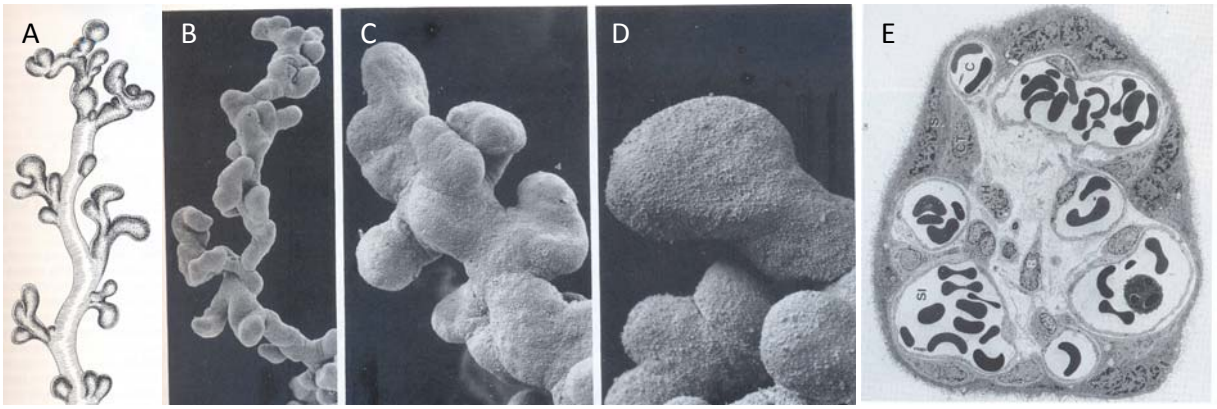

Figure 1.3.7: Diagram (A) and scanning electron-micrographs (B-D), showing appearance of an intermediate villus with the grape-like terminal villi branching off the the convex sides. Initial mags: B 180X, C 470X, D500X. Transmission electron micrograph of a cross section through a terminal villus (E). Initial mag 2000X (Benirschke and Kaufmann, 2000).

1.3.2.4 Immature Intermediate Villi

These are distal bulbous continuations of stem villi, which whilst not yet mature, will ultimately mature into stem villi (Fig.1.3.8). They are numerous in early pregnancy and persist in small groups in the mature placenta. They are covered in a thick trophoblastic layer.

The large amounts of collagen characteristic of the mature villus is absent, and instead they have a reticular net like arrangement of processes from the connective tissue cells. Numerous fluid filled channels are located between the fibres. There are capillaries, arterioles and venules, but the larger arteries and veins are absent.

Functionally, the immature intermediate villi are the growth centres of the villous trees (Benirschke and Kaufmann, 2000).

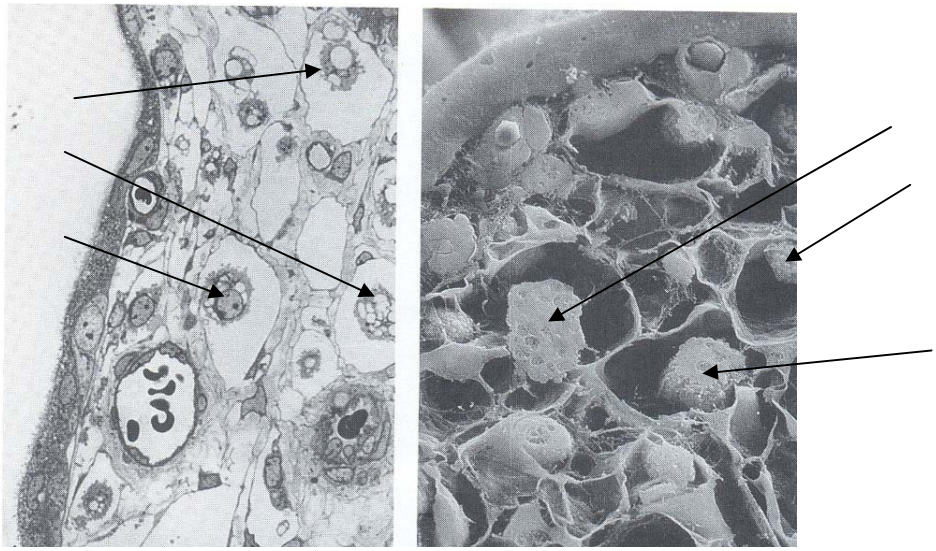

Figure 1.3.8: Transmission (A) and scanning (B) electron micrographs of the typical reticular appearance of the internal stroma in an immature intermediate villus. Vacuolated macrophages (Hofbauer cells) can be seen in the stromal channels (see arrows). Initial mag 520X. (Benirschke and Kaufmann, 2000).

1.3.2.5 Mesenchymal Villi

As the most primitive villi, they predominate during the first stages of pregnancy. They are basically the tertiary villi of early placentation, and all other villi types are derived from mesenchymal villi. Later in pregnancy, this type of villus is not readily seen, although can be found on the tips of immature intermediate villi where they represent zones of proliferation and branching (Benirschke and Kaufmann, 2000). Table 1.1 summerizes the main features of the three main villus types.

Table 1.1: Table summarising main features of the three main villi types of the mature placenta. Constructed from data in Benirschke and Kaufmann, 2000.

Villus type	% of total villus volume in the mature placenta	% contribution to the total surface area of the mature placenta	% of villlus occupied by fetal vessel lumina	µm
Stem	22.7	12.4	26	80-3000µm
Mature intermediate	27.8	31	21	60-150µm
Terminal	38.7	46.3	45.2	30-80µm

1.3.3 FACTORS AFFECTING MATERNO-FETAL EXCHANGE

1.3.3.1 Circulation Through The Placenta

Amongst the mammals of the animal kingdom one encounters various anatomical arrangements of maternal and fetal blood flow in the exchange areas (Fig 1.3.9). The more efficient an exchange system is, the higher the feto:placental weight ratio at term (Dantzer *et al*., 1988).

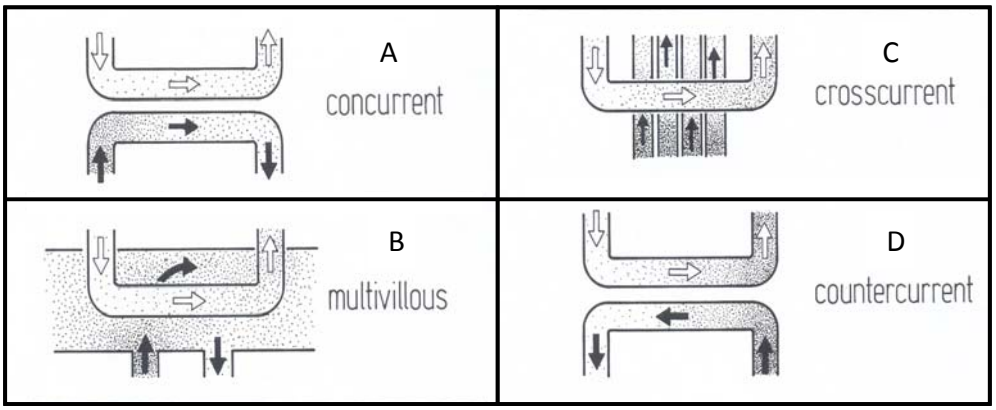

Figure 1.3.9: Diagrammatic representation of various materno-fetal exchange systems. Maternal blood represented by black arrows and fetal blood by white arrows. Density of dots gives indication of efficiency of exchange. In the concurrent system (A), maternal vessels run parallel to fetal vessels with matching direction of flow. This is the least efficient system. In the countercurrent system (D), maternal and fetal vessels run parallel with opposing directions of flow creating a very efficient arrangement for maximum exchange. A crosscurrent system (C) would be less efficient. The human placenta has a multivillous system (B). (Adapted from Benirshke and Kaufmann 2000).

The human placenta has a "multivillous" exchange system which is not as efficient as the countercurrent mechanism which is the most effective vascular arrangement when placental transfer relies on passive diffusion (as is the case for water, oxygen and carbon dioxide). A multivillous exchange system therefore depends more heavily on good maternal oxygen loading as well as good maternal, intervillous and fetal flow.

1.3.3.2 Materno-Fetal Barrier

According to the Grosser classification of materno-fetal barriers (Benirschke and Kaufmann, 2000) the human placenta is a "hemochorial barrier". In this kind of placental barrier, the invading fetal trophoblast cells end up in direct contact with the maternal blood. Initially, the barrier is a hemo-dichorial barrier in which there is both syncytiotrophoblast and cytotrophoblast cell layers. As pregnancy progresses, however, the population of cytotrophoblasts diminishes as stated previously, forming what is classified as a hemo-monochorial barrier (Fig 1.3.10). Fetal vessels may even abutt directly onto the syncytiotrophoblast, or even protrude into the syncytiotrophoblast, reducing this layer to a negligible barrier, and forming what is referred to as a vasculo-syncytial membrane (Burton *et al*., 2006). According to Fick's law of diffusion, any reduction in the diffusion distance between maternal and fetal blood is going to impact greatly on the materno-fetal diffusional exchange. This is the case for all passively exchanged substances (which include oxygen, carbon dioxide and water) (Benirschke and Kaufmann, 2000).

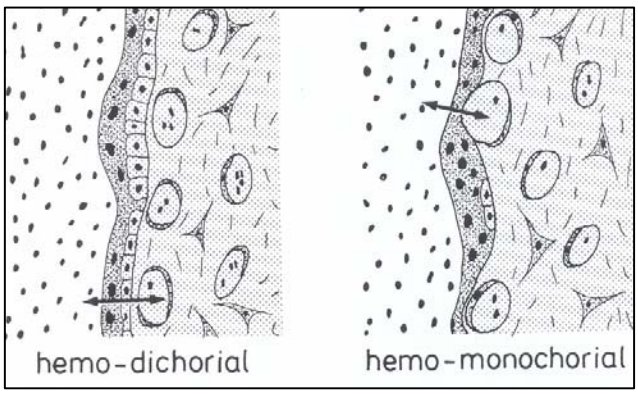

Figure 1.3.10: Diagrammatic representations of two different classifications of the materno-fetal diffusion barrier. Both these are seen in the human placenta at different stages of pregnancy (Adapted from Benirschke and Kaufmann 2000).

1.3.3.3 Surface Area

Other forms of transport across the materno-fetal barrier include facilitated diffusion (eg glucose); active transport (ions and amino acids); and vesicular transport (proteins and lipids). In the case of these forms of transport, the limiting factor is not diffusion distance. The limiting factors here are the available surface area of the syncytiotrophoblast, as well as the activity of the transport mechanisms (carrier molecules, enzymes and receptors) (Benirschke and Kaufmann, 2000). Syncytial surface area can be increased by an increase in villous growth as well as by microvillous folding of the apical plasma membrane (Fig. 1.3.11).

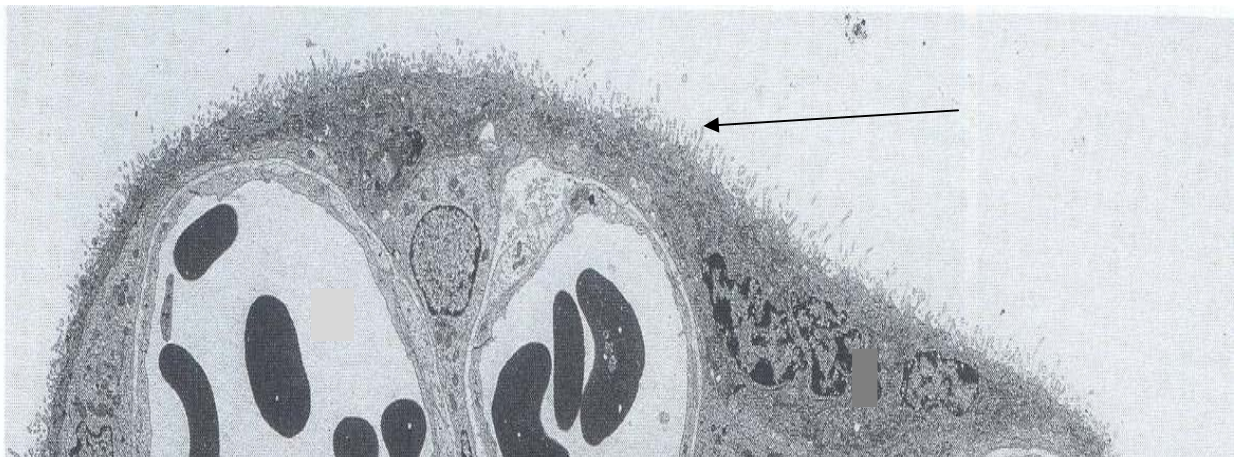

Figure 1.3.11: Electron micrograph of a section of terminal villus showing the microvilli on the syncyotrophoblast which protrude into the intervillous space and increase surface area for materno-fetal exchange Initial mag 2000X . (Adapted from Benirschke and Kaufmann 2000).

1.4 PRE-ECLAMPSIA

1.4.1 THE INCIDENCE OF PRE-ECLAMPSIA

Pre-eclampsia is a condition unique to pregnancy characterised by abrupt onset of hypertension and proteinuria. It is associated with an increase in maternal morbidity and mortality, and with a five-fold increase in perinatal mortality (Baker and Kingdom, 2004; Moodley, 2008; Sahin, 2003). Worldwide, pre-eclampsia and eclampsia account for over 50 000 deaths annually, occurring in about 2-8% of pregnancies in developed countries, but being up to three fold higher in developing countries (Sahin, 2003; Clarke and Nelson-Piercy, 2008). In South Africa, hypertensive disorders of pregnancy (HDP) account for 19 % of all maternal deaths, and of these, 83 % are due to pre-eclampsia and eclampsia (Moodley, 2008).

1.4.2 THE CLINICAL PICTURE OF PRE-ECLAMPSIA

It is generally accepted that a woman is pre-eclamptic if she develops new onset hypertension during pregnancy in which her blood pressure is ≥140/90mmHg on two separate occasions in addition to proteinuria (Clarke and Nelson-Piercy, 2008; Davey and MacGillivray, 1988; Baker and Kingdom, 2004). Hypertension and proteinuria are, however, only two features of a very complex clinical syndrome involving many body systems. Severe pre-eclampsia can involve the cardiovascular, pulmonary, renal, digestive and nervous systems as follows:

- Endothelial damage with activation of the coagulation cascade and altered permeability.
- Low cardiac output and increased peripheral resistance.
- Pulmonary edema or cyanosis.
- Impaired liver function with increased release of enzymes such as transaminases and transferases secondary to parenchymal necrosis.
- Epigastric tenderness due to stretching of Gilson's capsule with possible hepatic rupture.
- Headache, dizziness, blurred vision, tinnitus and altered conciousness.
- Decreased renal function with oliguria (<400-500mℓ in 24hr).
- Thrombocytopenia (<100 000/mm^3) and haemolysis.

Collectively, the hemolysis, elevated liver enzymes and low platelet count are referred to as the HELLP syndrome of pre-eclampsia (Hayman and Myers, 2004).

1.4.3 THE AETIOLOGY OF PRE-ECLAMPSIA

There are various theories as to how pre-eclampsia occurrs. As far back as 1939, researchers had associated the development of pre-eclampsia with reduced placental perfusion (Page, 1939). In later years, examination of the placental bed in women with pre-eclampsia demonstrated shallow trophoblast invasion with associated failure of the vascular remodelling that is characteristic of normal pregnancy (Brosens *et al*., 1972). This finding has since been corroborated by numerous studies demonstrating that in pregnancies complicated by intrauterine growth retardation and pre-eclampsia, extravillous trophoblast invasion only

extends into the decidual portions of the spiral arteries. These non-invaded and thus unmodified vessels retain a narrow lumen and an intact muscular wall, hindering a normal blood supply to the placenta which in turn leads to decreased perfusion of the intervillous space (Kaufmann et al., 2003; Meekins et al., 1994; Moodley and Ramsaroop, 1989; Naicker et al., 2003; Sheppard and Bonnar, 1981; Vitiello and Patrizio, 2007). Doppler waveflow studies of the uterine arteries done at 20 and 24 weeks of pregnancy in which there is a raised pulsatility index, have been linked with an increased risk of developing pre-eclampsia and/or intra-uterine growth restriction (Clarke and Nelson-Piercy, 2008).

Redman and colleagues (1991) suggest that pre-eclampsia should be considered a two-stage disorder with the first stage being a reduction in placental perfusion, which leads to the second stage or maternal syndrome (Fig.1.4.1), (Borzychowski et al., 2006; Redman, 1991).

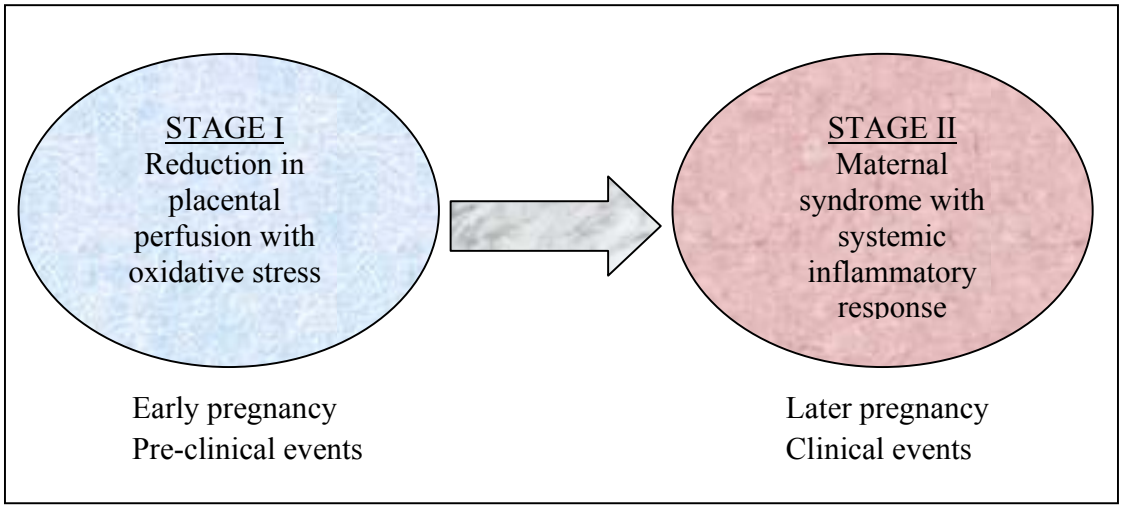

Figure 1.4.1: The two stage theory of pre-eclampsia. Adapted from Roberts and Hubel (2009)

The first stage of the two-stage model is pre-clinical, occurring in early pregnancy. It is essentially a localized condition of placental oxidative stress, caused by an inadequate utero-placental blood supply (Pijnenborg *et al*., 2006). The second stage is characterized by a systemic inflammatory response which involves all the maternal inflammatory mechanisms including the endothelium (Borzychowski *et al*., 2006).

The challenges have been to identify the factors that could predispose pregnant women to inadequate trophoblast invasion, and then to define the precise mechanisms which could link stage 1 and stage 2.

Multiple predisposing factors have been identified. Primiparity almost doubles a woman's risk of developing pre-eclampsia, with 75% of pre-eclamptic women being nulliparous (Chelsey, 1984). This could possibly have its roots in immune tolerance towards paternal genes, as a change of partner also increases risk (Esplin et al., 2001; Lie et al., 1998). Age seems to play a role, with women aged more than 35 years having an increased risk of developing pre-eclampsia (Saftlas *et al*., 1990). Pre-existing disease including chronic hypertension, renal disease and diabetes are risk factors (Garner et al., 1990; Samadi et al., 2001). Obesity, which interestingly is also a risk factor in diabetes, cardiovascular disease and hypertension, has been linked to an increased risk of a woman developing pre-eclampsia (Baker and Kingdom, 2004). Genetic factors play a role, such as family history of pre-eclampsia and ethnicity. Also amongst the possible contributors, it is believed that angiotensin-II and the renin–angiotensin pathways could be mediators of suboptimal implantation and placentation (Baker and Kingdom, 2004; Clarke and Nelson-Piercy, 2008).

In identifying possible factors which could link stages 1 and 2, substances being released from the villi into the maternal circulation have been investigated. This release would possibly occur as a result of reduced oxygen delivery. Factors shown to increase in pre-eclampsia include cytokines, antiangiogenic factors, and syncytiotrophoblast microparticles (STBM). If released into the maternal circulation, these elements could potentially cause cell injury, endothelial dysfunction, vasoconstriction and activation of the coagulation cascade with the resulting systemic maternal syndrome (Fig.1.4.2), (Clarke and Nelson-Piercy, 2008; Roberts and Hubel, 2009). Another possibility presented by Roberts and Hubel is that given the reduced placental perfusion and subsequent reduced delivery of nutrients, the fetal-placental unit may release materials intended to provide the needed stimulation in order to modify maternal

metabolism with a view to increasing nutrient availability. The large percentage (70%) of normal size-for-age infants who are born of pre-eclamptic mothers would support this theory. The two stage theory has been modified over time to include maternal genetics, constitutional and behavioural factors, as well as environmental factors, with the linkage of the two stages likely being multifactorial (Roberts and Hubel, 2009).

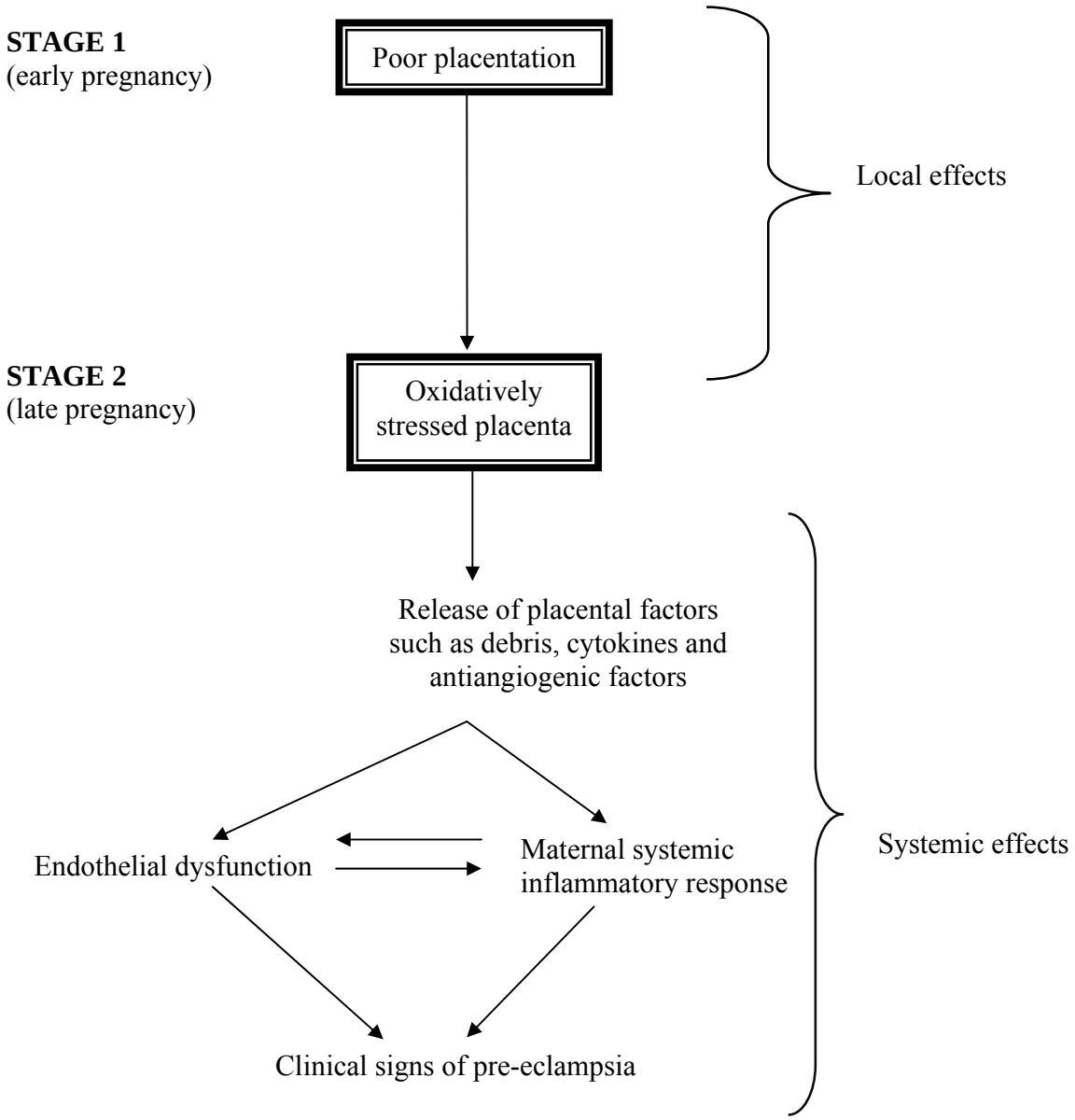

Figure 1.4.2: Flow diagram illustrating the "two stage model" of pathogenesis of pre-eclampsia as proposed by Redman *et al.*(1991). Stage 1 occurring in early pregnancy when insufficient trophoblast invasion leads to poor placentation resulting in placental hypoxia. Stage 2 reflecting the systemic effects of factors released from the oxidatively stressed placenta into the maternal circulation (Adapted from Borzychowski *et al.* 2006).

1.4.4 MORPHOLOGICAL CHANGES OF THE PRE-ECLAMPTIC PLACENTA

Clarity in the relationship between placental function and morphology on the one hand, and pre-eclampsia on the other is still lacking. Kingdom and Kaufmann (1997) suggested that placental changes will depend on intraplacental oxygen status, which in turn depends on both maternal supply as well as fetal extraction (Kingdom and Kaufmann, 1997). Multiple maternal, placental and fetal factors would need to be taken into account when considering the total picture of materno-fetal exchange.

Extending their discussion, these investigators outlined three different scenarios which could result in the fetus being hypoxic. These included uteroplacental hypoxia (where the hypoxia begins at the level of supply into the intervillous space); pre-placental hypoxia (which is caused by a factor such as maternal anaemia, or pregnancy at high altitude); and post-placental hypoxia in which the intervillous space is well oxygenated, but fetal extraction is inadequate (Fig. 1.4.3).

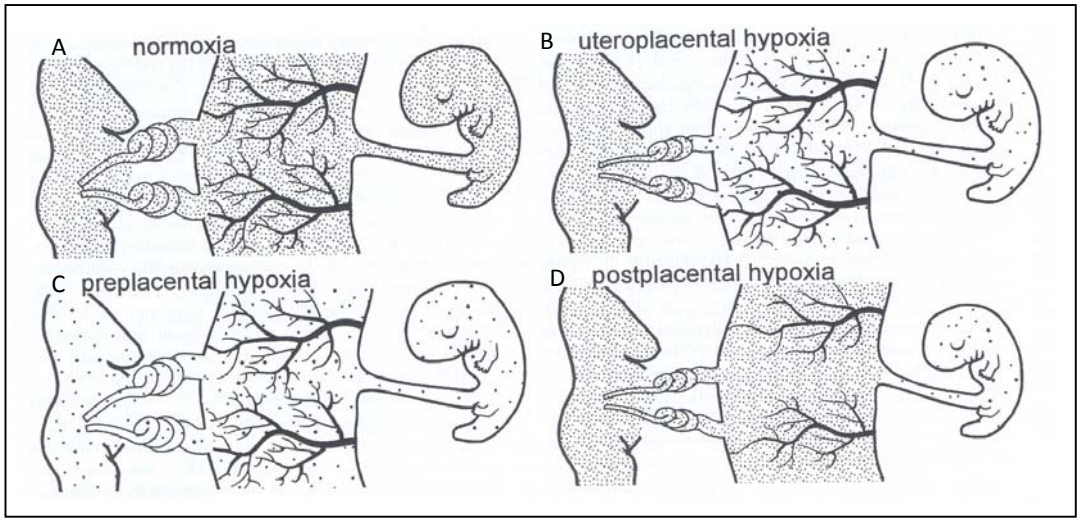

Figure 1.4.3: Diagrammatic representations of normoxia (A) and different forms of materno-fetal hypoxia. In uteroplacental hypoxia (B), there is adequate oxygenated blood in the mother, but inadequate perfision of the placenta. In preplacental hypoxia (C), there is a problem with the mother having adequate oxygenated blood, and in post-placental hypoxia (D), fetal extraction is inadequate in spite of a well perfused plcenta (Adapted from Kingdom and Kaufmann, 1997).

Kingdom and Kaufmann then proceeded to collate data from numerous authors, outlining placental morphological changes related to these different classifications of hypoxia. Conditions falling into the classification of *"preplacental hypoxia"* demonstrated an increase in branching angiogenesis, an increased capillary volume fraction, with the placenta maintaining oxygen transfer by a thinning of the placental barrier. In addition, these placentas demonstrated an increased proliferation of villous cytotrophoblasts, increased syncytial nuclear aggregation, decreased density of stromal matrix and increased numbers of macrophages (Kingdom and Kaufmann, 1997).

In contrast to this, compromise to flow in the umbilical vessels or "*post-placental hypoxia"* in which there is altered end diastolic flow (AEDF) in the umbilical arteries, resulted in a different pattern of villous alterations. There was a reduced proliferation of villous cytotrophoblast together with an increased deposition of villous stroma and decreased number of macrophages (Kingdom et al., 2000; Kingdom and Kaufmann, 1997).

Pre-eclampsia is generally recognized as being characterized by *"uteroplacental hypoxia"*. Whilst the maternal blood entering the intervillous space is oxygenated, the amount of blood entering is inadequate. In the resulting ischaemic environment of the pre-eclamptic placenta, one would expect structural compensations of the villi such as increased surface area or decreased diffusion distance. Alternatively, there could be increased damage such as fibrotic deposition or necrotic areas which would adversely affect placental efficiency.

Most of the structural findings reported by Kingdom and Kaufmann corresponded to those described in the preplacental hypoxia group, including increased capillary volume fraction

increased amount of villous cytotrophoblast proliferation, increased syncytial knotting and an increased number of villous macrophages. Some believe that the entry of maternal blood into the intervillous space is restricted focally, and that as a result placental hypoxia will also be focal and hence any resulting alterations to villous development would be variable rather than homogeneous (Meekins *et al*., 1994). It has also been postulated that in the case of *late* onset pre-eclampsia we may in fact be dealing with an example of "*post-placental"* hypoxia in which fetal elements of the placenta fail to transfer blood to the fetus itself in spite of oxygenated blood entering the villous space. In the latter case, the intervillous space could in fact be hyperoxic as opposed to hypoxic (Kingdom and Kaufmann, 1997).

Studies on the comparative morphology of pre-eclamptic versus normotensive placentas have demonstrated conflicting results. In 1996, Mayhew utilized stereological techniques to compare placentas from pregnancies associated with hypoxic stress with those from normal pregnancies. The latter study related villous surface area to volume, and found that villous surface area altered disproportionately to volume in pregnancies associated with certain hypoxic conditions. This was also true of pre-eclampsia (Mayhew, 1996). In three subsequent studies, working with other investigators, Mayhew found that intrauterine growth restriction (IUGR) was associated with changes in placental morphology such as decreased surface area, impoverished growth of peripheral villi and decreased fetal angiogenesis. However, this was not the case for pre-eclampsia in the absence of IUGR (Mayhew et al., 2007; Mayhew et al., 2003; Mayhew et al., 2004). The latter finding was corroborated by Egbor *et al*. (2006) when they examined features of intermediate and terminal villi (Egbor et al., 2006b; Egbor et al., 2006a).

However, other researchers have demonstrated altered placental morphology associated with pre-eclampsia. Utilising confocal laser scanning microscopy to create three dimensional images, Resta *et al*., (2006) found significant hyper-ramification of the capillary loops in terminal villi of pre-eclamptic placentas together with irregular vessel profiles and narrow vessel lumina (Resta *et al*., 2006). The prevalence of inflammation, infarction, ischaemia, hemorrhage, and syncytial knots was found to be increased in both pre-eclampsia and IUGR by Jain *et al*., (Jain *et al*., 2007). Correa *et al*., found an increased number of terminal villi vessels associated with pre-eclampsia (Correa *et al*., 2008), and it has long been established that the placentas from pre-eclamptic pregnancies display increased syncytial knotting (Haezell et al., 2007; Tenney and Parker, 1940).

To date, many of the studies looking at placental villous morphometry have focused on the *peripheral* (intermediate and terminal) villi, as these villi form the surface for actual materno-fetal exchange. Although stem villi play a critical role in fetal circulation, studies examining them are limited. In conducting fetal blood to the peripheral villi, stem vessels will affect hemodynamics at the exchange sites, including fetal extraction of oxygen. In conducting the oxygenated blood back to the umbilical veins, they will affect umbilical hemodynamics (blood transfer back to the fetus).

1.4.5 THIS STUDY

Taking the view of Kingdom and Kaufman (1997) that pre-eclampsia is an example of *"uteroplacental hypoxia"* this study accepts the reported increase in the capillary volume fraction and the increased amount of villous cytotrophoblast proliferation. These factors essentially focus on exchange surface areas of the peripheral villi, and are expected forms of compensation in the ischaemic environment. However, whilst the alterations in the peripheral or exchange villi are well documented and clear, there is a paucity of data on the conducting pathways within the villi?

The main research questions of this dissertation are the following:

- Whether the increased growth of peripheral villi affects **placental density** (and therefore intervillous flow), and whether the ischaemic environment could have increased **fibrotic areas** which would also impact on placental efficiency?
- Whether there is any evidence of differences in the **conducting/stem areas** of the placental villi in the pre-eclamptic samples?

Therefore, in an attempt to address the above questions, the aims of this dissertation are:

1. To morphometrically estimate and compare placental density between normotensive and pre-eclamptic groups, and linked to this to make a comparison of the occurrence of fibrotic areas between normotensiive and pre-eclamptic groups.

2. To make a comparison in the carrying capacity of stem vessels between normotensive and pre-eclamptic groups as evidenced by luminal areas of these vessels.

CHAPTER TWO

MATERIALS AND METHODS

2.1 FORMAL HYPOTHESES

As outlined in the introduction, the main research questions of this dissertation are the following:

- Whether the increased growth of peripheral villi affects **placental density** (and therefore intervillous flow), and whether the ischaemic environment could have increased **fibrotic areas** which would also impact on placental efficiency?
- Whether there is any evidence of differences in the **conducting/stem areas** of the placental villi in the pre-eclamptic samples?

Based on this, two formal hypotheses have been formulated as follows:

1. **The increased growth of peripheral villi associated with pre-eclamptic placentas affects placental density, possibly resulting in diminished intervillous flow and contributing to an increase in the formation of fibrotic areas.**

2. **The vessels in the stem/conducting villi of pre-eclamptic placentas show diminished carrying capacity in comparison to normotensive samples.**

2.2 ETHICAL APPROVAL AND PATIENT CONSENT

The study was conducted at the Obstetric Unit, King Edward VIII hospital, and the Optics and Imaging Centre, Doris Duke Medical Research Institute, Nelson R. Mandela School of Medicine, Durban, South Africa. Ethical approval was obtained from the Biomedical Research Ethics Committee (appendix 1), University of KwaZulu-Natal. Following a full explanation, informed consent (appendix 3 and 4) was obtained from all patients by a Zulu speaking research midwife.

2.3 THE SAMPLE

2.3.1 SAMPLE SIZE

This study comprised two groups, namely, a pre-eclamptic group and a normotensive group. The latter was to act as the control group providing a norm against which to make comparisons. Under the guidance of the resident university bio-statistician, a sample size of 30 was chosen for each study group in order to ensure statistical validity. This choice took into consideration the fact that basic T-tests were to be used in making a comparison of the data of the two groups. Therefore, thirty normotensive patients and thirty pre-eclamptic patients were recruited by a trained research midwife to participate in this study as per inclusion and exclusion criteria outlined below. Demographic and clinical data, including maternal, placental and neonatal data was also collated as an important aspect to the project.

2.3.2 INCLUSION CRITERIA

Patients included in the normotensive cohort had BP ≤ 130/80 mmHg, whilst those included in the pre-eclamptic cohort had SBP≥140mmHg; DBP ≥ 90 mmHg and demonstrating proteinuria. Patients between the ages of 18-40 years and parity 0-5 were included in the study.

2.3.3 EXCLUSION CRITERIA

When selecting patients for a study involving pre-eclampsia, one needs to ensure that exclusion criteria incorporate any medical factors which could potentially mimmick the symptoms of pre-eclampsia (such as pre-existing hypertension or seizure disorder). Failure to do so would create confounding variables. For this study, exclusion criteria included chronic hypertension, pre-existing seizure disorder, eclampsia, pre-gestational diabetes, placental abruption, gestational diabetes, thyroid disease, asthma, chronic renal disease, intrauterine death, cardiac disease and infection with HIV.

2.4 SAMPLE COLLECTION, PROCESSING AND STAINING

2.4.1 SAMPLE COLLECTION AND FIXATION

Sample collection was standardised in order to avoid confounding variables associated with the method of sample collection. The umbilical cord was clamped following delivery of the baby, and placental samples were taken immediately following delivery of the placenta. These samples of the placentas were taken from the amnion through to the decidua approximately 5cm from the umbilical cord, avoiding the larger radial vessels running just under the amnion in the chorionic plate (Fig.2,1). In order to ensure preservation of the tissue structure and prevent any autolytic changes, the samples were immediately fixed in 10% buffered formaldehyde at room temperature for 24 hours.

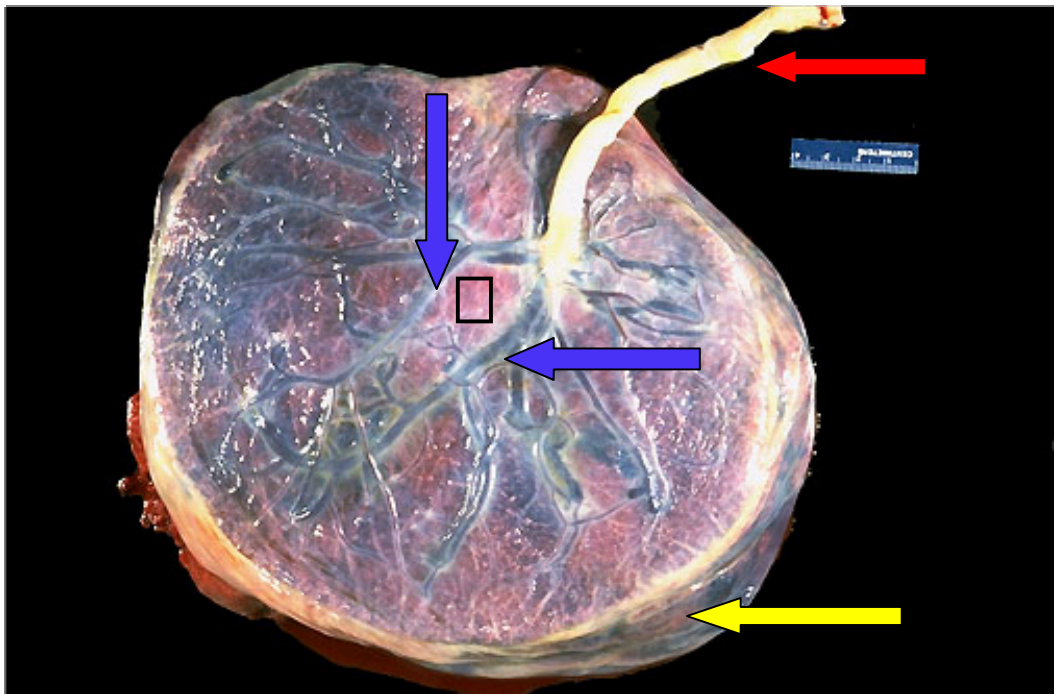

Figure 2.1: Fetal surface of human placenta with umbilical cord attached (red arrow). Large radial vessels (blue arrows) can be seen running under the transparent membranous amnion, better seen towards edge of the placenta in this image (yellow arrow). Black square indicates an area where a centralized sample could have been taken from in order to avoid the large vessels. Adapted from an image available at www-edlib.med.utah.edu/WebPath/jpeg2/PLAC031.jpg

2.4.2 TISSUE PROCESSING

Smaller pieces of the sample were cut and placed in tissue cassettes for processing. Processing of the samples was performed on a Sakura VIP 500 Automated Tissue Processor. The tissue samples were dehydrated through a series of ethanol (99% ethanol, Saarchem, SA), cleared with xylene as the intermediate solvent (AR, Saarchem, SA), and infiltrated with paraffin wax (Paraplast Plus, Sherwood Medical, St Louis, USA) as depicted in Figure 2.2 and Table 2.1 below. The wax-infiltrated tissue sections were then placed in moulds for automated wax embedding in a Leica EG1160 tissue embedding station.

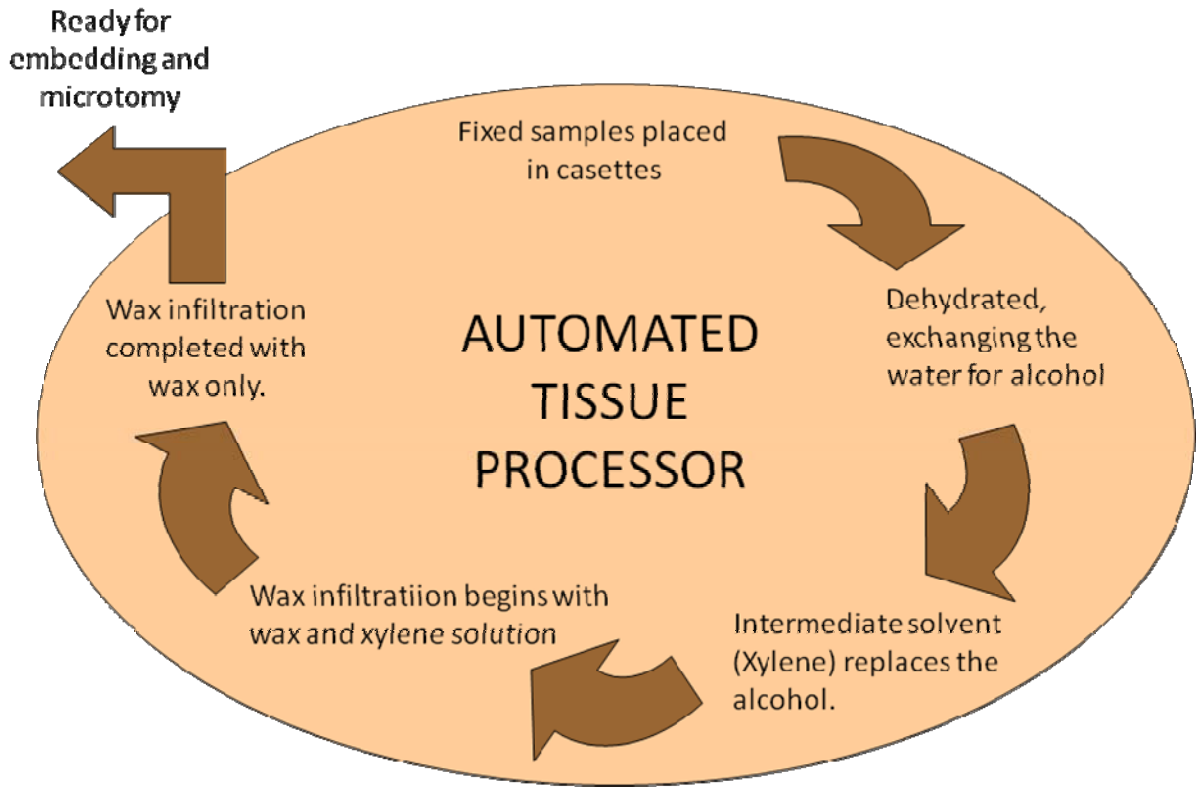

Figure 2.2 Stages of automated tissue processing.

Table 2.1 Solutions used in automated tissue processing

STEP	SERIES OF SOLUTION CHANGES	TEMP	TIME
Fixation	10% buffered formal saline	Room T^o	24 h
Dehydration	95% ethanol	24°C	1 h
	95% ethanol	24°C	1 h
	95% ethanol	24°C	1 h
	absolute ethanol	24°C	1 h
	absolute ethanol	24°C	1 h
	absolute ethanol	24°C	1 h
Clearing	xylene	24°C	1 h
	xylene	24°C	1 h
Vacuum infiltration	paraffin wax 1	60°C	2 h
	paraffin wax 2	60°C	2 h

2.4.3 MICROTOMY

Tissue sections were cut at 3-5μm thickness using sterile disposable blades on a rotary microtome (Leica Jung, RM2035). They were floated in a water bath and picked up onto polysine coated glass slides (B & M Scientific, Cape Town), and baked in a Memmert 400 oven at 60°C for 60 min prior to dewaxing and staining.

2.4.4 PROCEDURE FOR HAEMATOXYLIN AND EOSIN STAINING

The sections were deparaffinised and rehydrated using standard techniques prior to being stained with Mayer's Haematoxylin (Sigma Chemicals, St Louis) and 0.5% alcoholic eosin (BDH Lab Supplies, UK). Post staining, sections were dehydrated using alcohol and cleared using xylene prior to securing coverslips with DPX mountant (dibutyl phthalate). The process is outlined in Table 2.2 below.

Table 2.2: Procedure for Haematoxylin and Eosin staining.

STEP	SOLUTION	TIME
Dewaxing	Xylene	3 x 5 min
Rehydration	100% Ethanol	3 x 5 min
	90% Ethanol	1 x 5 min
	80% Ethanol	1 x 5 min
	70% Ethanol	1 x 5 min
	Running tap water	1 x 5 min
Staining for basophilia	Mayer's Haematoxylin	1 x 5 min
	Rinse in running tap water	1 x 5 min
Staining for acidophilia	0.5% alcoholic eosin	1 x 2 min
	Rinse quickly by immersing slides in 95% ethanol	30 sec
Dehydration	Absolute Alcohol	2 x 1 min
	Xylene	1 min
Securing coverslips	Using DPX as the mountant.	

2.5 PLACENTAL MORPHOMETRY

Placental pathological assessment together with stereological measurements, were also performed on a Nikon Eclipse E600 microscope interfaced with the Software Imaging Systems (Germany) image analysis package. A single observer did all the measurements in order to minimize inter-observer bias. The following morphological parameters were assessed:

2.5.1 DENSITY OF PLACENTAL VILLUS ARRANGEMENT

In order to measure this, four images were captured from each Haematoxylin slide using the 4X objective, one in each quadrant (120 fields per sample group). Color thresholds, saturation and intensity were individually set for each image. Binary images were created (Figure 2.3), and phase analysis was performed to determine the area of the field that was occupied by placental villi. This was then expressed as a percentage of the total field area of 11 500 000 μm^2. The average of the measurements for the 4 quadrants was determined for each slide.

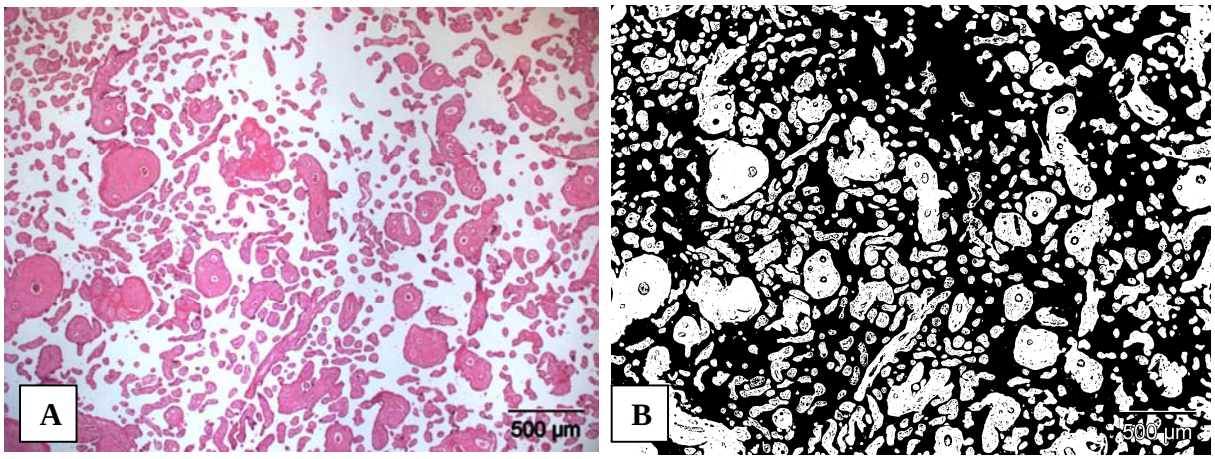

Figure 2.3: A: Light micrograph of H and E stained placental tissue. B: Binary image of micrograph A. The combined area occupied by white in micrograph B was expressed as a percentage of the total field area.

2.5.2 PERCENTAGE OF STEM VILLUS AREA OCCUPIED BY VESSEL LUMINA

Two stem villi were selected in each section on the basis of clarity of morphology for measuring purposes (60 stem villi photographed per sample group). Cross sections were preferentially selected. Stem villi at different levels of branching (and therefore different calibers) were included. For each stem villus, the lumina were outlined and the sum of all luminal areas calculated. This combined area was then expressed as a percentage of total villus area (Fig.2.4).

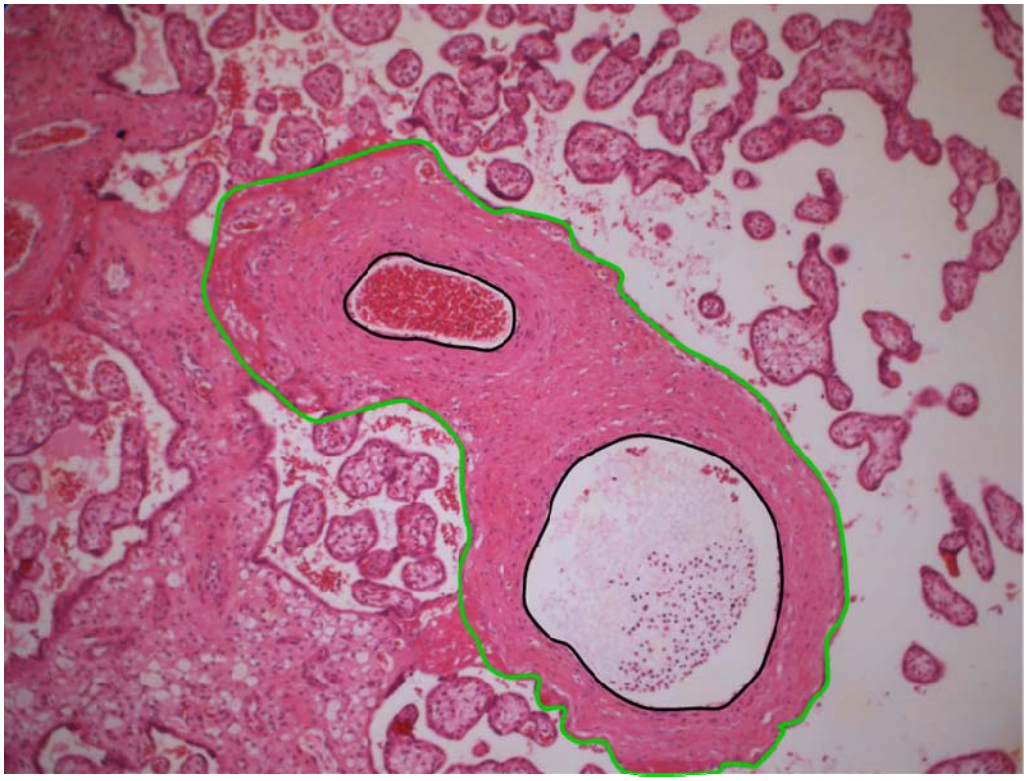

Figure 2.4 Photomicrograph of a stem villus showing outline of stem villus area (green) and perimeters of vessel lumeni (black), the latter expressed as a percentage of the former as a measure of carrying capacity. Initial mag 100X.

2.5.3 RELATIVE THICKNESS OF STEM ARTERIAL WALLS

Following the parameters outlined in 2.4.2, the main arterial vessel was selected in each image (60 stem arteries measured per sample group). The area of the artery was demarcated from its outer adventitial boundary. The lumen area was subtracted from the latter in order to ascertain the area occupied by arterial wall. This wall area was expressed as a percentage of total vessel area, in order to compensate for differing calibers of stem villi (Fig.2.5).

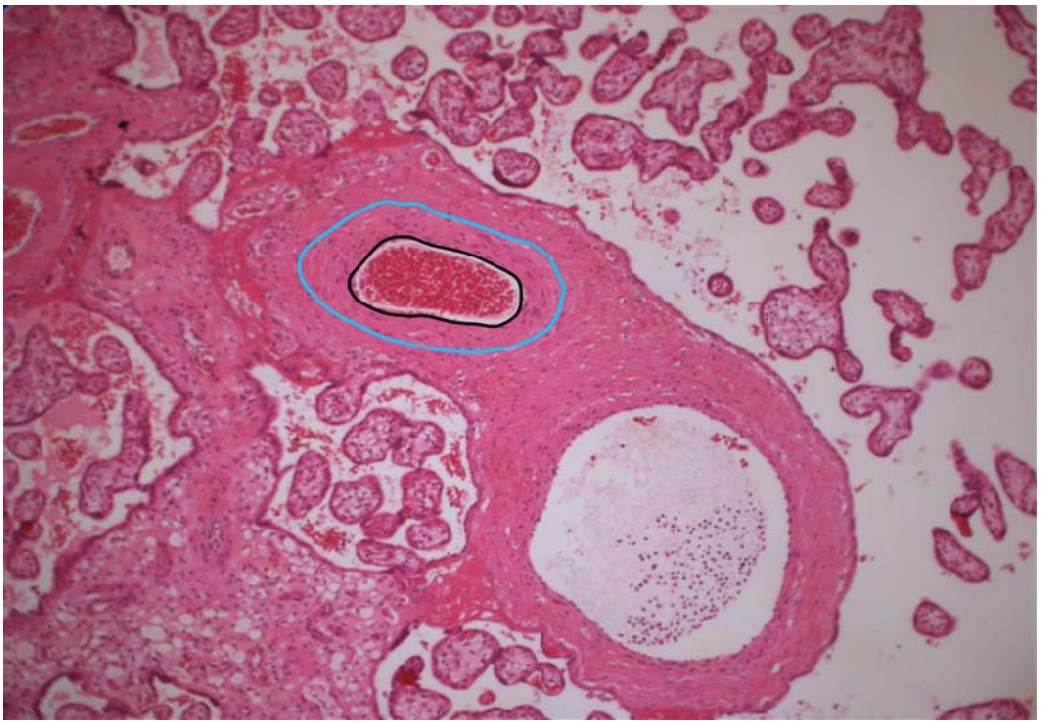

Figure 2.5 Photomicrograph of a stem villus showing outline of main stem artery/artery area (blue) and outline of the arterial lumen (black), the latter was subtracted from the former to get a measure of area occupied by the wall, then expressed as a percentage of vessel area. Initial mag 100X

Calculation done as follows:

Area of vessel = area inside adventitial boundary

Area occupied by arterial wall = area of vessel - lumen area

Percentage of arterial wall area was then calculated as follows: (Area of artery wall/Area of vessel) X 100

2.5.4 EXTENT OF FIBROSIS

Samples were examined utilizing the 10X objective, and the area of greatest fibrosis was selected in each quadrant (4 fields per specimen or 120 fields per sample group and 240 fields in all). All fibrotic areas in the field of view were outlined (Fig.2.6) and the sum of all these areas of fibrosis was expressed as a percentage of total field area for each photomicrograph.

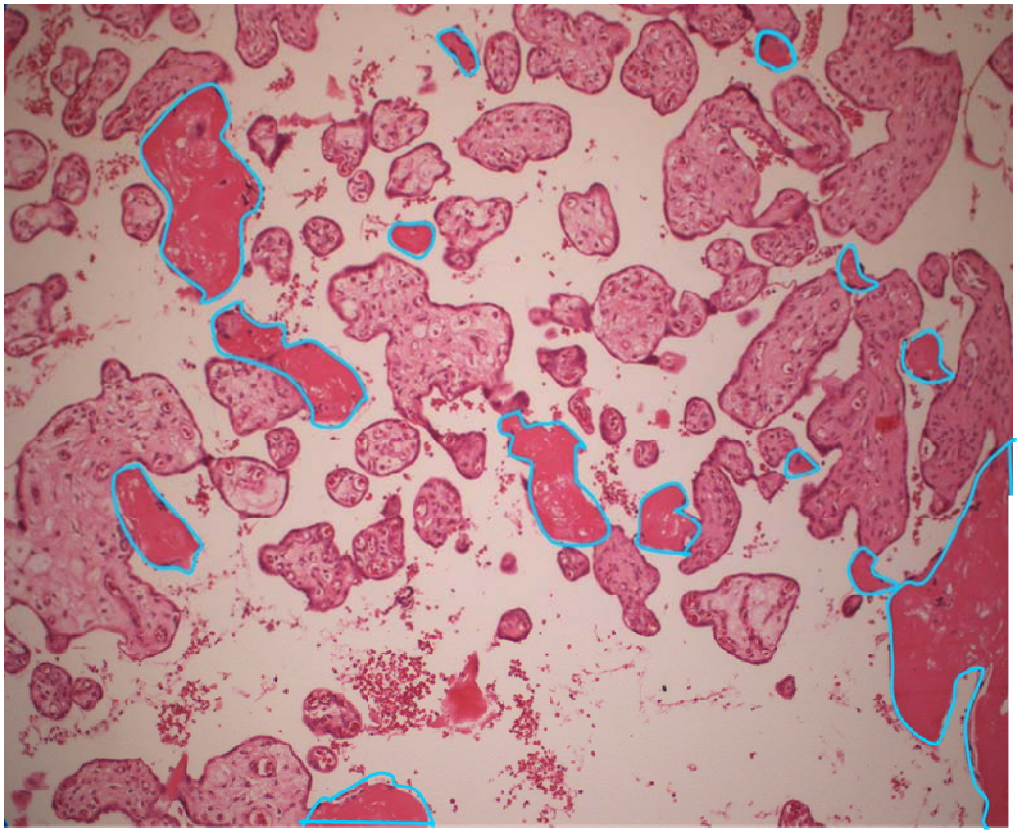

Figure 2.6: Photomicrograph of villi showing outlines of fibrotic areas (blue), the latter expressed as a percentage of the total field area as a comparative measure of fibrotic change. Initial mag 100X.

2.6 STATISTICS

Mean ± standard deviation were calculated for clinical and morphological quantitative variables by group. Statistical significance was determined using t-tests ($P<0.001$). Comparisons of categorical variables between groups were achieved using the Pearson Chi-square test, with a $P<0.05$. Correlations of mean arterial pressure (MAP) *vs* placental weight, baby weight *vs* placental weight, baby weight *vs* weeks gestation and baby weight *vs* MAP were performed using two tailed Pearson correlation coefficient (r) with $P<0.01$ indicating statistical significance.

CHAPTER THREE

RESULTS

3.1 MATERNAL, PLACENTAL AND FETAL CLINICAL DATA

The maternal clinical characteristics of the normotensive and pre-eclamptic groups of this study are shown in Table 3.1. They are expressed as mean ± standard deviations and *P* values for t-tests. As expected, blood pressure parameters were significantly different between groups. Systolic pressures were 117.71±10.319 for normotensive and 156.94±15.131 for pre-eclamptic ($P<0.001$). Diastolic pressures were 72.19 ±8.064 for normotensive and 100.42±8.101 for pre-eclamptic ($P<0.001$). Mean arterial pressures were 87.366±7.745 for normotensive and 119.263±9.206 for pre-eclamptic ($P<0.001$). Platelet counts were significantly lower in the PE group at 212.47±94.748 compared to 285.90±100.688 in the normotensive group ($P=0.005$). Thirty six percent of the pre-eclamptic mothers had platelet counts under 175 X $10^9/\ell$. There was, however, no evidence of haemolysis ($P=0.346$).

Baby and placental data are shown in Table 3.2. Birth outcomes were $n=31$ and $n=33$ for the normotensive and pre-eclamptic groups respectively, due to twin pregnancies. Twenty of the normotensive babies and thirteen of the pre-eclamptic babies were females, whilst eleven of the normotensive babies and twenty of the pre-eclamptic babies were males. The mean gestational age of the normotensive babies was 38.87±1.432 compared to 36.58±2.840 in the pre-eclamptic group ($P<0.001$). Baby weight at delivery was lower in the the PE group at 2.74±0.813 compared to the normotensive group at 3.16±0.402 ($P=0.012$). The placental weight in the normotensive group was lower (573g ± 98.336) compared to the PE group (635g ± 172.722), However, this difference was not statistically significant ($P=0.094$). Pearson 2-tailed correlation (Table 3.3) showed a moderate positive correlation between baby weight and placental weight (r=0.623) and weeks of gestation (r=0.743). Baby weight weakly correlated with mean arterial pressure (r= -0.380).

Table 3.1: Maternal demographic and clinical data comparisons between the N and P groups

Variable *(Units)*	Normotensive *Mean and SD*	Pre-eclamptic *Mean and SD*	P-values
MATERNAL DATA			
Age (*Years)*	25.03±6.008	25.42±6.154	0.798
Maternal weight (*kg)*	77.87±15.74	85.38±15.40	0.058
Maternal height (*cm)*	1.54±0.057	1.55±0.041	0.803
Body mass index (kg/m^2)	32.81±6.459	35.54±5.680	0.084
Systolic B.P. (*mmHg)*	117.71±10.319	156.94±15.131	<0.001
Diastolic B.P. (*mmHg)*	72.19±8.064	100.42±8.101	<0.001
Mean arterial pressure (*mmHg)*	87.366±7.745	119.263±9.206	<0.001
PREGNANCY DETAILS			
Gravidity (*No of pregnancies)*	2(1.94±0.854)	2(1.82±1.158)	0.648
Parity (*No of live births)*	1(0.94±0.854)	1(0.70±1.104)	0.339
Weeks Gestation (*Weeks)*	38.87±1.432	36.58±2.840	<0.001
BLOOD PARAMETERS			
Red blood cells ($X10^{12}/\ell$)	3.87±0.446	3.98±0.519	0.346
Haemaglobin (*g/dL)*	11.90±1.337	11.74±1.620	0.677
Haematocrit (*ratio)*	32.75±3.287	34.35±9.241	0.382
Mean cell volume (*fL)*	84.19±7.215	83.25±8.387	0.641
Mean cell Hb (*pictograms)*	30.74±3.211	30.37±4.425	0.713
Platelets ($X10^9/\ell$)	285.90±100.688	212.47±94.748	0.005
Leucocytes ($X10/\ell$)	11.62±5.480	11.11±3.803	0.664
Neutrophils ($X10^9/\ell$)	74.89±7.474	67.23±25.008	0.107
Lymphocytes ($X10^9/\ell$)	17.89±6.086	15.17±9.366	0.180
Monocytes ($X10^9/\ell$)	3.80±1.782	4.42±2.737	0.296
Eosinophils ($X10^9/\ell$)	0.89±1.193	0.73±0.993	0.551
Basophils ($X10^9/\ell$)	0.23±0.349	0.23±0.236	0.933

Table 3.2: Baby and placental clinical data comparisons between the N and P groups

Variable *(Units)*	Normotensive *Mean and SD*	Pre-eclamptic *Mean and SD*	P-values
BABY DATA			
Apgar 1 (*Score out of 10)*	8.23±1.203	7.97±1.357	0.429
Apgar 2 (*Score out of 10)*	9.48±0.851	9.36±0.783	0.558
Baby weight (*kg)*	3.16±0.402	2.74±0.813	0.012
PLACENTAL DATA			
Full placental weight (*g)*	573±98.336	635±172.722	0.094
Placental diameter (*cm)*	20.16±0.523	19.97±0.467	0.126

Table 3.3: Correlation coefficient between MAP, gestational age and placental weight

Variables correlated	N	Correlation coefficient (r)
Mean arterial pressure with placental weight	64	0.175
Baby weight correlated with placental weight	64	0.623*
Baby weight correlated with weeks gestation	64	0.743*
Baby weight correlated with mean arterial pressure	64	-0.380*

*Correlation significant at the 0.01 level.

Pearson Chi-square tests comparing categorical data between groups are shown in Table 3.4. Presence of protein in the urine was nil in 83.9% of the normotensive group, whilst the remaining 16.1% presented with traces of protein. Proteinuria varied from 1+ to 3+ in the pre-eclamptic group, only 1 patient presented with nil protein. Pearson Chi-square tests for "indication for delivery" and "method of delivery" showed that initiation and delivery were completely normal in 56.7% of the normotensive group. In the pre-eclamptic group, however, indication for delivery was predominantly associated with maternal and fetal distress (75.8%) and in 54.5% of cases, delivery was by means of an emergency caesarean (P=0.001).

Table 3.4: Comparison of categorical data between the groups

Oedema							
		0 (none)	1 (ankle)	2 (knee)	3 (groin)	4 (generalized)	Total
Normotensive	Count	31	0	0	0	0	31
	% within group	100.0%	.0%	.0%	.0%	.0%	100%
Pre-eclamptic	Count	3	5	15	5	5	33
	% within group	9.1%	15.2%	45.5%	15.2%	15.2%	100%
Protein Dipstick							
		None	+	++	+++		Total
Normotensive	Count	26	5	0	0		31
	% within group	83.9%	16.1%	.0%	.0%		100%
Pre-eclamptic	Count	1*	10	14	8		33
	% within group	3.0%	30.3%	42.4%	24.2%		100%
Indication for Delivery							
		0 Spontaneous	1 Maternal interest	2 Fetal interest	3 Maternal and fetal interests		Total
Normotensive	Count	17	11	2	1		31
	% within group	54.8%	35.5%	6.5%	3.2%		100%
Pre-eclamptic	Count	8	12	1	12		33
	% within group	24.2%	36.4%	3.0%	36.4%		100%
Method of Delivery							
		1 Spontaneous Vaginal	2 Elective Caesarean	3 Induced vaginal	4 Emergency Caesarean		Total
Normotensive	Count	17	11	2	1		31
	% within group	54.8%	35.5%	6.5%	3.2%		100%
Pre-eclamptic	Count	8	12	1	12		33
	% within group	24.2%	36.4%	3.0%	36.4%		100%

*Although the dpistick of one patient was normal, the 24 hour protein was 1g.

3.2 PLACENTAL MORPHOMETRY

INTERVILLOUS SPACE: Histological examination of placentas revealed a denser arrangement of villi in the pre-eclamptic samples (Fig 3.1), when compared to the normotensive samples. On calculation of the actual percentage of field area occupied by placental villi (as opposed to intervillous space), a higher percentage of the field area was shown to be occupied by placental tissue in the pre-eclamptic group (64.78±6.93) versus the normotensive group (51.89±6.19) (P<0.001). This indicates a reduction in the available intervillous space in this group (Table 3.5, Fig 3.4).

LUMINAL AREA & ARTERIAL WALL THICKNESS: Initial qualitative observations revealed a decreased carrying capacity of the stem vessels (Fig. 3.2). Stem villi morphometry demonstrated a lower total luminal area (expressed as a percentage of the villus area) in the pre-eclamptic group (8.67±8.51), when compared to normotensive group (17.20±11.78) (P<0.001) (Table 3.5, Fig 3.4). Whilst the carrying capacity of the stem vessels was reduced, the thickness of stem villi arterial walls was increased in the pre-eclamptic placentas. The morphometric comparisons indicated that the percentage of the vessel area occupied by vessel wall was substantially higher in the pre-eclamptic stem arteries (0.87±0.106) compared to the normotensive arteries (0.74±0.129) (P<0.001).

VILLI FIBROSIS: An increased occurrence of fibrosis was observed in the pre-eclamptic samples compared to the normotensive samples (Fig. 3.3). Morphometric image analysis of these fibrotic zones confirmed a higher percentage of fibrosis in the pre-eclamptic group (1.582±0.707) compared to the normotensive group (0.727±0.310) (P<0.001) (Table 3.5, Fig 3.4). Note that the percentage of fibrosis must be seen as relative rather than absolute reflections of fibrosis throughout the placenta, as the areas of *greatest* fibrosis were measured.

NORMOTENSIVE PRE-ECLAMPTIC

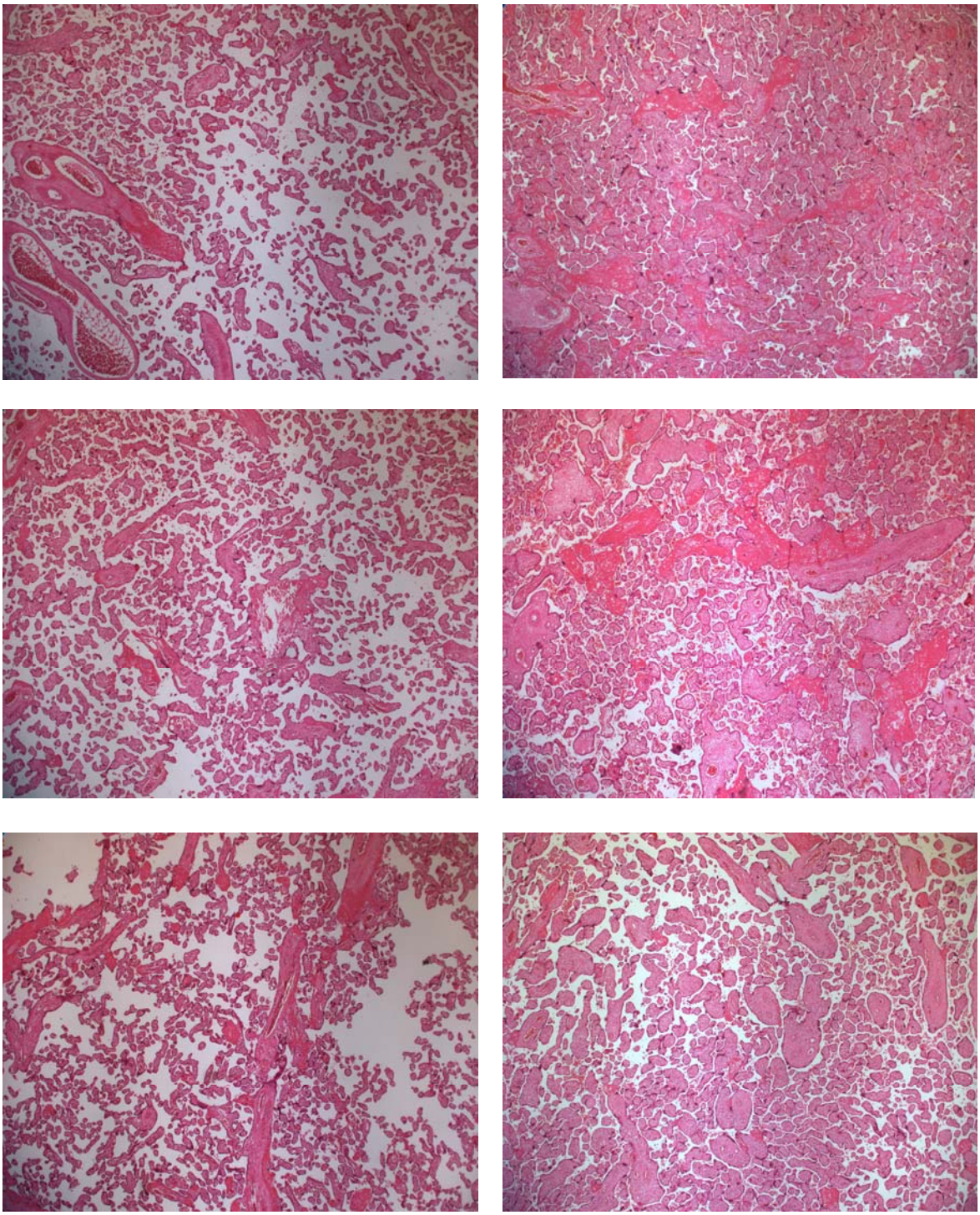

Figure 3.1: Photomicrographs showing examples of placental density of Normotensive and Pre-eclamptic placentas. Note the greater density of the pre-eclamptic samples with limited intervillous space. Initial mag 40X.

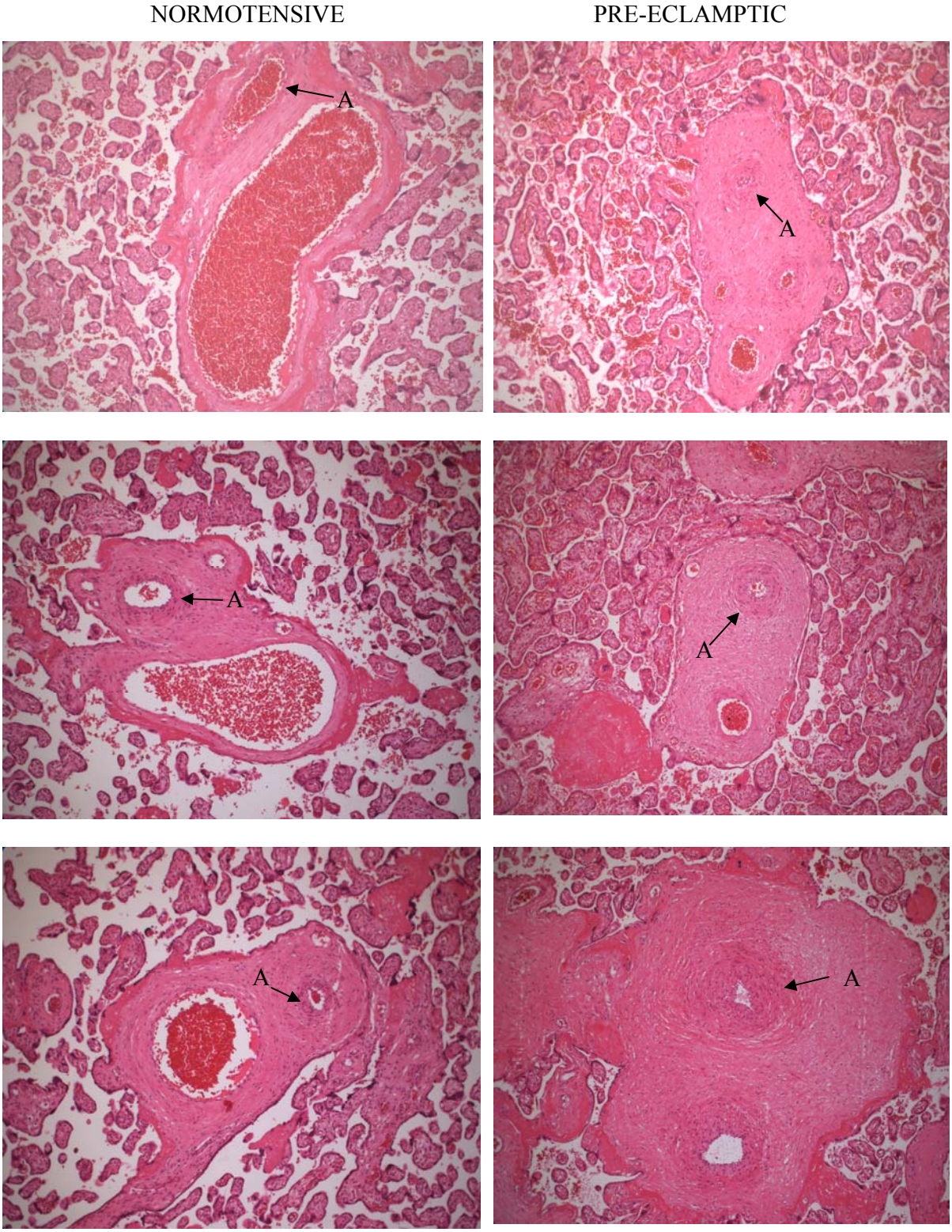

Figure 3.2: Photomicrographs showing stem villi from Normotensive and Pre-eclamptic placentas. Note the size of vessel lumina relative to villus area, as well as the thickness of the stem arterial walls (A). Initial mag 100X

NORMOTENSIVE PRE-ECLAMPTIC

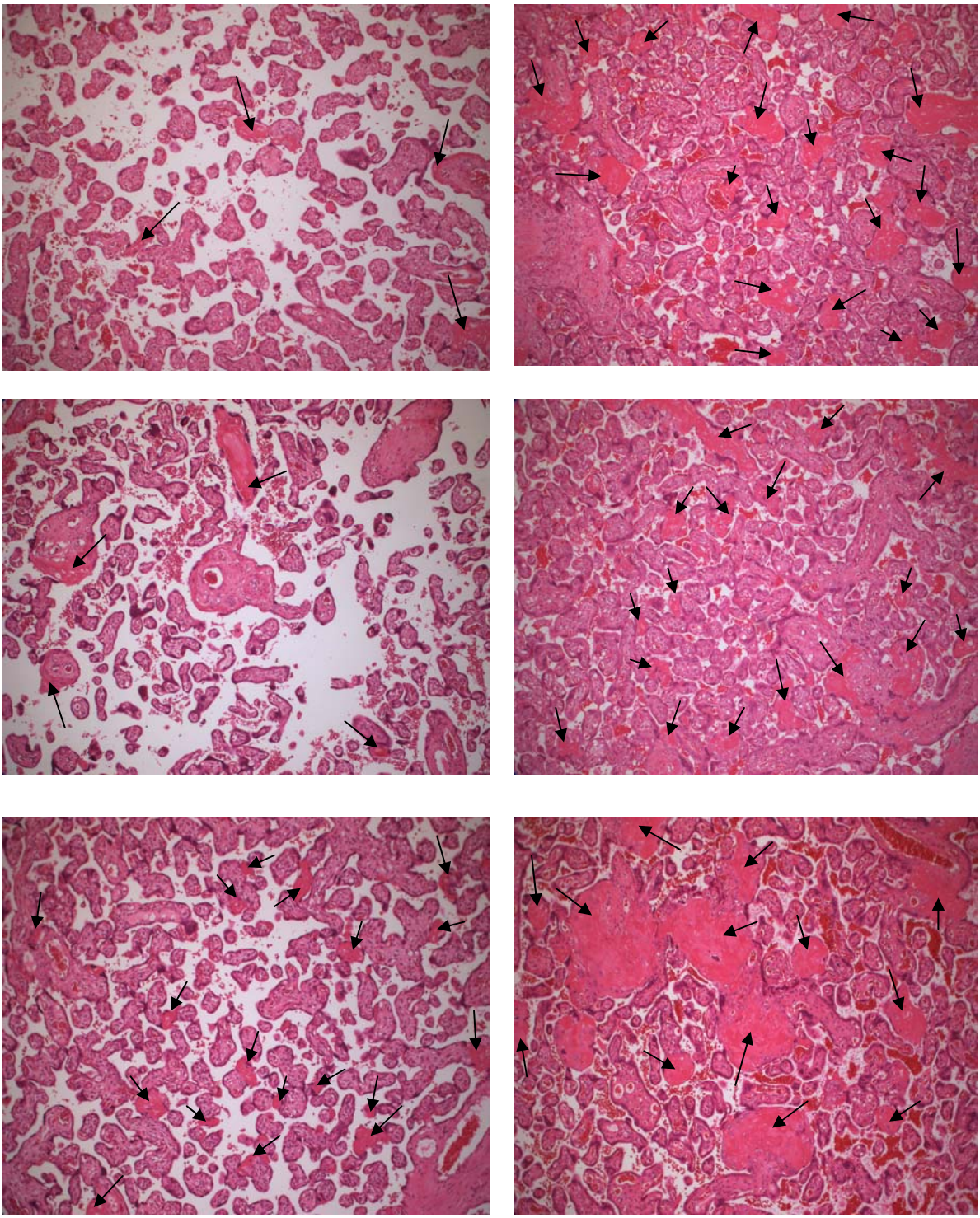

Figure 3.3: Photomicrographs showing examples of fibrin deposition in Normotensive and Pre-eclamptic placentas. Initial mags of all images 100X. Fibrin is a brighter pink compared to the maternal blood which is adarker red.

Table 3.5: Comparisons of % placental tissue, % fibrosis, % stem vessel lumina, and ratio of arterial wall

Variable	Normotensive *Mean and SD*	Pre-eclamptic *Mean and SD*	P-values
% placental tissue in field	51.89±6.19	64.78±6.93	*P*<0.001
% fibrosis in field	0.727±0.310	1.582±0.707	*P*<0.001
% of stem villus occupied by stem vessel lumina	17.20±11.78	8.67±8.51	*P*<0.001
Ratio: Area occupied by stem villus arterial wall/ total area occupied by the artery	0.74±0.129	0.87±0.106	*P*<0.001

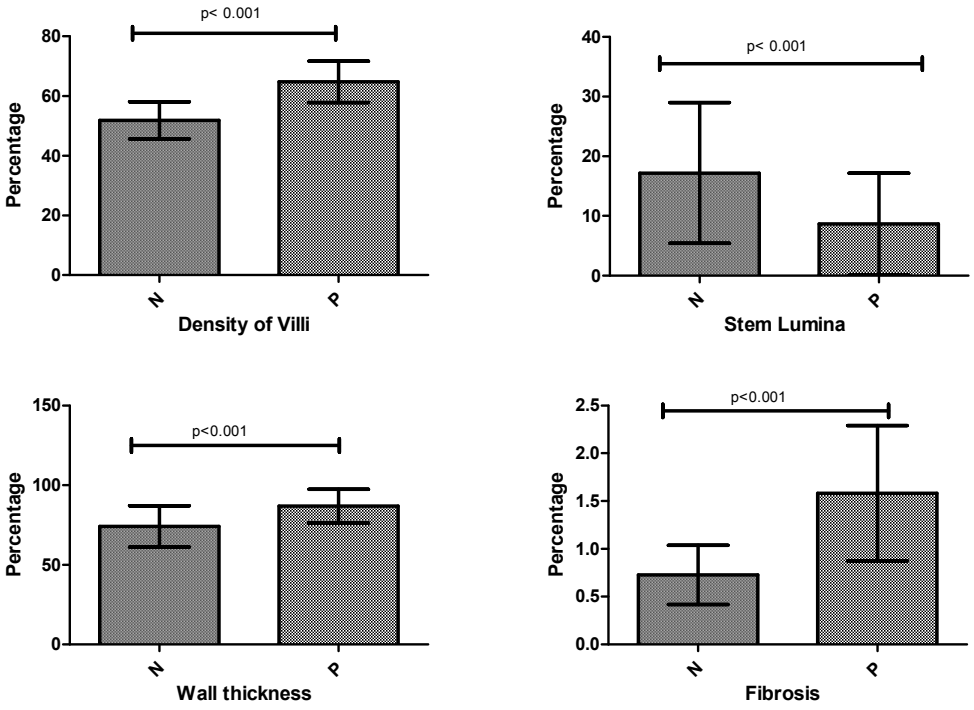

Figure 3.4: Bar graphs illustrating comparisons of means of morphometric data of Normotensive (N) and Pre-eclamptic (P) groups. (Note that the percentage of fibrosis must be seen as relative rather than absolute reflection of fibrosis throughout the placenta, as the areas of *greatest* fibrosis were measured in both groups).

CHAPTER FOUR

DISCUSSION

As one of the leading causes of maternal and fetal morbidity and mortality(Baker and Kingdom, 2004; Moodley, 2008; Sahin, 2003), pre-eclampsia continues to be a challenge for obstetricians at the bedside as well as scientists in the laboratory. It offers additional challenges in the management of pregnancy and birth, and the scientific community continues to debate the complexities of its precise aetiology. The complex puzzle it presents has resulted in the development of different yet interrelated fields of research, including maternal, fetal and placental areas of specialisation. Differences of opinion regarding its causes and effects make for a dynamic and growing niche of medical science, with a growth in collaboration between clinicians and scientists in order to answer the many remaining questions.

4.1 MATERNAL AND FETAL CLINICAL DATA

In this study 51 % of the normotensive group went into labour spontaneously, and delivered by normal vaginal delivery, whereas this scenario only occurred in 24% of the cases in the pre-eclamptic group, with the highest percentages being emergency caesarean (53.3%) resulting from both fetal distress and maternal danger (40%). These results confirm the increased medical danger, and therefore the concern of the medical profession associated with the management of pre-eclampsia.

The incidence of pre-eclampsia was not related to either parity or gravidity of the mothers in this study. The risk of pre-eclampsia has been well documented as being higher in a first pregnancy, with 75% of women with pre-eclampsia being nulliparous (Chesley, 1984; Misra and Kiely, 1997). However, further studies also showed that pre-eclampsia can occur in subsequent pregnancies if there is a new sexual partner. The possibility that pre-eclampsia may be a problem of primipaternity rather than primigravidity was suggested by Robillard *et*

al. (1993) in which they found that in 61.7% of multiparous women with pre-eclampsia, the father of the current pregnancy was different than that of the former, and that this was the case in only 6.6% of the control group ($P<0.0001$) (Robillard et al., 1993). A further Dutch study found that 22-25% of women presenting with pre-eclampsia had new partners compared to 3.4% of the normotensive mothers (Tubbergen et al., 1999). It has been shown that the paternal genes which are expressed via the fetus are as important in contributing to the risk of pre-eclampsia as the maternal factors (Terje Lie et al., 1998). Whilst paternal data was not included in this study, this could be an explanation for the incidence of pre-eclampsia in differing gravida in the current study population, and would be worthwhile including in a future study.

In attempting to elucidate whether a relationship between maternal weight and the incidence of pre-eclampsia occurred, body mass index was utilized in order to correct for differences in height. Whilst initial observations indicated many higher body mass index readings in the pre-eclamptic group, this did not prove to be statistically significant. As the BMI seemed to be clinically significant, statistical significance may have been absent due to a type two error, and could possibly be revealed by a larger sample size.

Pre-pregnancy body mass index has been shown to increase the risk of pre-eclampsia (Bodnar et al., 2005). Compared to women with a BMI of 21, women with a BMI of 26 had two times the risk, and those with a BMI of 30 had three times the risk of developing pre-eclampsia. Women with a BMI of 19 had a 33% reduction in the risk. Pre-pregnancy weights were, however, not available for the current study population.

Maternal blood parameters analysed were restricted to those performed as standard of care at King Edward VIII Obstetrics unit. Biochemical tests such as liver function tests, urea and electrolytes were only performed on pre-eclamptic patients; hence these parameters could not be included in the statistical comparisons. The lower platelet count observed in some mothers of the pre-eclamptic group is characteristic of the HELLP syndrome (Haemolysis, elevated liver enzymes, and low platelet count) associated with pre-eclampsia. In this study, thirty six percent of the pre-eclamptic mothers had a decreased platelet counts ($< 175 \text{ X } 10^9/\ell$), there was, however, no haemolysis observed, although the patients with low platelet counts had borderline low red blood cell counts. The HELLP syndrome occurs in 4-12 % of patients with pre-eclampsia. Generally, 6% present with one of the abnormalities suggestive of HELLP, 12% develop two characteristic abnormalities, and 10% develop the triad referred to above (Baker and Kingdom, 2004).

The increased incidence of twins in the pre-eclamptic group was not surprising. Researchers have found a strong association between twin pregnancies and pre-eclampsia (Basso and Olsen, 2001). It is widely established that the incidence of pre-eclampsia in primigravidae women with twin pregnancies, is four or five times higher than in women with singleton pregnancies (Sibai et al., 2000). The results of this study corroborate the latter studies in that there were three sets of twins in the pre-eclamptic group as opposed to one in the normotensive group. This increased incidence of pre-eclampsia associated with multiple pregnancies could conceivably be due to the increased combined placental mass, with an increase in placentally derived factors which play a role in aetiology.

The ratios of male and female babies were also as one would expect. Pre-eclampsia development has been linked with a higher incidence of male neonates (Basso and Olsen, 2001). In the present study, eleven of the thirty-one normotensive births (35%) were male births, compared to twenty of the thirty-three pre-eclamptic births (66%). These results strongly support the hypothesis that predisposition towards pre-eclampsia in increased by male fetus conception.

James (1995) links mammalian (including human) sex ratios at birth to parental hormone concentrations at the time of conception, with high concentrations of testosterone and estrogen favoring the production of sons whilst low concentrations favouring the production of daughters. He further extrapolated his data to investigate whether pregnancy pathologies could possibly be caused or be associated with abnormal hormone concentrations either during or prior to pregnancy. Because one risk factor for hypertension in pregnancy is pre-existing hypertension , and given the fact that free testosterone concentrations have been positively correlated with diastolic blood pressure in non-pregnant women of childbearing age, James postulated that high androgen concentrations could play a role in the aetiology of hypertension of pregnancy (James, 1995).

Taking cognizance of Davey's observation that in some patients who later develop gestational hypertension there is an excessive production of estrogen (Davey, 1986), he states that "...there are grounds for suspecting that the high concentrations of ovarian hormones (androgens and estrogens) which are postulated to be responsible at the time of conception for the excess of male offspring carried by hypertensive pregnant women, may later also be causally implicated in the hypertension development itself " (James, 1995).

The positive correlation of baby weight with gestational age is not unexpected, and must be interpreted in relation to the strong association observed between the weeks of gestation and the incidence of pre-eclampsia. Pre-eclampsia carries with it a lower chance of carrying the baby to term. It is also associated with a higher incidence of growth restriction (Huppertz, 2011). Therefore the problem presents itself of distinguishing the lower baby weights due to early birth from those due to intrauterine growth restriction. In this study, multiple regression analysis confirmed a highly significant ($p<0.001$) positive association between birth weight and gestational age rather than intrauterine growth restriction. Therefore the apparent crude association between pre-eclampsia and lower birth weight was confounded by gestational age. One would perhaps need to correct for gestational age before performing correlations, unfortunately fetal weights related to differing gestational ages were not available for our local population.

Worth noting is the negative relationship between baby weight and maternal mean arterial pressure. This negative correlation indicates that the higher the mean arterial pressure of the mother, the lower the birth weight of the baby is likely to be (or based on previous arguments, the lower the gestational age at birth is likely to be).

4.2 PLACENTAL DATA AND MORPHOMETRY

Several investigators have shown a higher incidence of pre-eclampsia in pregnancies where there is a greater placental mass being carried such as twin pregnancies and molar pregnancies (Basso and Olsen, 2001; Sibai et al., 2000). Yet others have found that placental mass is decreased in pre-eclampsia (Boyd and Scott, 1985; Jain et al., 2007). In the current study, the mean placental weight was greater in the pre-eclamptic group whether the twin placentas were combined (normotensive mean = 573g and pre-eclamptic mean 635g) or separated (normotensive mean = 554g and pre-eclamptic mean = 577g). Whilst this was not statistically significant, a larger sample size could alter this result.

Vinnars *et al.* (2008) found that pre-eclampsia associated with the HELLP syndrome had a higher mean placental weight adjusted for gestational age than pre-eclampsia alone (Vinnars et al., 2008). However, utilizing only mean, SD and P-values could prove to be inadequate in the case of placental weights. A study performed on 317688 singleton pregnancies (Dahlstrom et al., 2008) found that the in *preterm* pre-eclampsia, there was an increase in smaller placentas, and a decrease in bigger placentas; whilst in *term* pre-eclampsia there was an increase in both bigger and smaller placentas. This pattern was repeated in a study looking at birth weights (corrected for gestational age) where the risk of *preterm* pre-eclampsia showed an L-shaped association with birth weight, whereas the risk of *term* pre-eclampsia showed a U-shaped association with birth weight (Vatten and Skjaerven, 2004). This U-shaped association was corroborated by a later study in which data from over 300 000 placentas was collated (Eskild and Vatten, 2010).

In the *normotensive* cohort of the current study, a large percentage (81%) of placental weights fell between 450g and 650g with the lowest placental weight being fairly close at 400g and the highest being 670g. In contrast, only 55% of *pre-eclamptic* placental weights fell within this central range, whilst the total range of placental weights was much wider, extending from240g to 960g. This suggests a tendency towards the U-shaped distribution typical of *term* pre-eclampsia. Although this U-shaped distribution is associated with pre-eclampsia, placental weight is not considered to be a useful indicator for the placental dysfunction in pre-eclampsia (Eskild and Vatten, 2010).

In this study, the weak association between placental weight and pre-eclampsia may be attributed to the inclusion criteria for pre-eclampsia diagnosis. Roberts and Catov (2008) suggest that there may be two clinical presentations of pre-eclampsia, each with completely different aetiologies, and as such may not necessarily present with the same placental dysfunction/ alterations in weight or structural anomalies (Roberts and Catov, 2008). Future studies should consider sub-categorising pre-eclampsia (early and late onset), in order to accurately examine placental anomalies, placental weights and total placental contribution to the disorder.

One of the main differences observed in the current study was the increased density of the placental tissue in the pre-eclamptic samples. This would suggest increased growth, or hyper-ramification of the villi, which is known to occur in response to hypoxia (Resta et al., 2006; Kingdom et al., 2000). This is proposed to be caused by increased branching angiogenesis together with trophoblast proliferation which occur in response to the hypoxia (Kingdom and Kaufmann, 1997). The fairly rapid branching angiogenesis also pushes the developing

capillaries against the trophoblastic barrier, causing extension and thinning of this cover (Burton et al., 1996). Thus, the placenta responds to the ischaemia by producing greater amounts of highly vascularised terminal villi, effectively increasing surface area and reducing diffusion distance.

In response to the rapidly increasing surface area described above, the cytotrophoblasts would need to divide and become incorporated into the syncytiotrophoblast layer at a greater rate. Hence cytotrophoblast proliferation warrants attention. Cytotrophoblast proliferation has been described as being upregulated by intraplacental hypoxia and downregulated by increasing intraplacental oxygen levels (Benirschke and Kaufmann, 2000). This is corroborated in the data collated by Kingdom and Kaufmann (1997) in which they demonstrate an increased amount of villous cytotrophoblast proliferation in pre-eclampsia (utero-placental hypoxia). However, Heazell *et al*. (2008) found that in *in vitro* studies using placental explants exposed to differing oxygen tensions, villous cytotrophoblast proliferation was decreased at lower oxygen concentration (Heazell *et al*., 2008). Studies by Ducray *et al* (2011) implicate an increase of the cytotrophoblast proliferation and fusion in pre-eclamptic pregnancies. In addition, the distribution of cytotrophoblasts was different to that in normotensive sections, with larger numbers of cytotrophoblasts associated with the intermediate and terminal villi. A large percentage of these cytotrophoblasts were located at areas of branching (growth) of the villous tree, and an increase was observed in the numbers of cytotrophoblasts staining positive for cleaved or activated caspase 8, which is responsible for initiating the apoptotic differentiation of the cytotrophoblasts and their fusion with the overlying syncytiotrophoblast (Ducray *et al*., 2011).

In contrast to the findings of the present study, Boyd and Scott (1985) report a significantly lower volume of parenchyma, as well as lower villous surface area in pregnancies complicated by pre-eclampsia. A study by Mayhew *et al* (2004) examining villi volumes, surface areas and lengths, found that pre-eclampsia alone had no effects on morphology, whereas pre-eclampsia with intrauterine growth restriction demonstrated abnormal growth. These findings provoke the need to clinically sub-categorise pre-eclampsia diagnosis.

Pijnenborg (2006) in discussing the purpose of the spiral arteries in normal pregnancy states that the spiral design assists in reducing the speed and pressure of the blood as it travels towards the intervillous space, as well as dampening the pulse in order to ensure a steady flow (Pijnenborg et al., 2006). It is possible that the spiral design would be insufficient to achieve these controlling effects in the absence of trophoblastic conversion of these vessels. Burton *et al.* (2009) theorises that in pregnancies complicated by pre-eclampsia, a combination of a lack of spiral artery conversion, together with smaller and fewer uterine arterio-venous shunts, will not so much result in intervillous ischaemia as opposed to a much higher velocity of the blood entering the intervillous space. They speculate that this increased momentum would damage villous architecture, even breaking the decidual attachments of anchoring villi, and propose that placental changes would be due to rheological consequences rather than chronic hypoxia (Burton *et al*., 2009). If this is the case, some areas of the villous tree would effectively be eroded by the increased pressure of the incoming blood, leading to large bare areas overlying the openings of the spiral arteries. Extending this view, morphological changes would not be uniform over the placental area, and sampling would greatly affect the observations made. It is conceivable that the research midwife in sampling for the current study may have intentionally avoided such "empty" areas. It could be helpful in morphometric studies such as this one, to

include gross biometry of the maternal aspect of the placenta. Randomised sampling would also be of greater importance in order to ensure validity of results.

The possible negative implications of a greatly increased villous volume, is two-fold. Firstly, if the villous hyperplasia is occurring at the expense of intervillous space, blood flow through the placenta (intervillous flow) will be compromised. Secondly, if there is any malfunction in trophoblast turnover and disposal, the impact on the maternal systemic circulation would be substantially increased by virtue of the increased surface of trophoblast contacting the maternal blood.

In this study, the increased placental density was also associated with more areas of fibrosis. These findings match those of Corrêa *et al.* (2008) who found that fibrin deposition was greater in all hypertensive syndromes in pregnancy. They also found that in the more severe cases, fibrin deposits changed from perivillous to intravillous, an observation also noted in the current study (Correa et al., 2008). Fibrin deposition is often observed as a defensive mechanism in vessels subjected to increased internal pressure, such as the fibrinoid necrosis of the renal vessels seen in chronic hypertension. Whilst this may protect the vessel wall itself from damage, it creates impedance to flow, further exacerbating the raised peripheral vascular resistance. Deposition of fibrin is also common in areas of sluggish blood flow with associated ischaemia, such as occurs in cirrhosis of the liver (Rippey, 1994) . The intervillous space is essentially a vascular space, and whilst it is not lined by endothelium, but syncytiotrophoblast, it is conceivable that fibrin deposition could be a defensive reaction as a result of villous damage. This villous damage could in turn be due to the physical factors of blood leaving narrowed spiral arteries as referred to by Burton (2009) above, but could also

conceivably occur in response to a sluggish flow or ischaemia. Either way, this fibrin will diminish the functional area of the villous tree, creating impedance without viable surface area.

With an established compromise to maternal blood flow, and a possible impedance to intervillous flow, it is natural to turn one's attentions to fetal or post-placental flow.
In considering the fetal contribution to exchange, factors such as the development of the villous tree and angiogenesis are of great importance and have been the subject of much investigation. Studies conducted by Resta *et al* (2005) examined the capillary loops in terminal villi of pre-eclamptic and normotensive placentas utilising confocal laser scanning microscopy. They report significant hyper-ramification together with irregular profiles and narrow lumina. Mayhew *et al.* (2004), found no difference in the capillary:villus ratio. Another study indicated that in both IUGR as well as pre-eclampsia, the vascular and non-vascular volumes appear to alter proportionately (Ong et al., 2004).

The stem villi and their vessels have not received as much attention as the intermediate and terminal villi because they are not considered to be of the same importance in exchange. Benirschke and Kaufmann (2000) state that based on the lack of fetal capillaries in the stem villi, as well as the degeneration observed in the trophoblast layer, it is generally assumed that their role in materno-fetal exchange can be considered negligible. However, if conduction between peripheral exchange areas and the umbilical cord vessels is compromised, a condition of "post-placental" hypoxia could result.

By examining the percentage of stem villus area occupied by lumina, the current study sought to compare conduction capacity of the stem villi relative to stem villus area. The lower collective luminal volume of the pre-eclamptic stem villi vessels observed in this study suggests a possible compromise to fetal flow through these villi as opposed to the stem vessels in normotensive placentas. This observation of narrow stem artery lumeni of the pre-eclamptic samples is linked to the next investigation, which examined the area of the stem arterial vessel wall in relation to the total villous area.

The higher ratios obtained for the pre-eclamptic stem arteries when relating vessel wall to total villus area, reveal a thicker arterial wall in this group compared to the normotensive group. Narrow lumina have also been identified by Las Heras *et al* (1983) together with endothelial proliferation, proliferation of smooth muscle cells within the tunica media, and within the villi stroma (Las Heras et al., 1983). This thicker wall of the stem arteries could be an compensatory mechanism to increase pressure and flow in the fetal circulation, with the resultant smaller luminal volume.

Greater amounts of highly vascularized terminal villi occurring in hypoxia (as described by Kingdom and Kaufmann, 1997) will have the effect of reducing capillary-mediated impedance to blood flow in these terminal villi. It is conceivable that this would have an effect similar to "peripheral vasodilation", with resulting diminished "venous return" towards the conducting areas of the villi. Whilst the materno-fetal system generally operates with low impedance, the reduction in impedance occurring in this greatly increased villous volume could possibly be too high. As blood moves towards the vessels of the conducting areas of the villous tree, it encounters vessels with more smooth muscle capable of providing an increase to the

impedance. If this is occurring however, the control would have to be via paracrine interactions of endothelium and smooth muscle, as the stem villi arteries and arterioles (like the umbilical cord vessels), are not innervated by the autonomic nervous system (Kingdom et al., 2000).

An interesting concept is that the muscular walls (as well as the extra vascular myofibroblasts of the villus stroma) contributes to the autoregulation of the feto-placental vascular system as a whole, and not just to the fetal flow (Demir et al., 1997). Myofibres of the vessel wall or myofibroblasts of the villus stroma which are oriented parallel to the longitudinal axis of the villous stems could possibly aid circulation of maternal blood through the intervillous space. This would be particularly so in the case of anchoring villi which connect the chorion to the basal plate thus extending across the full intervillous space, as their longitudinal contraction could decrease intervillous volume and thus increase intervillous pressure (Benirschke and Kaufmann, 2000; Krantz and Parker, 1963). However, pressure increases in the intervillous space have been postulated to reduce fetal perfusion by increasing fetoplacental impedance (Benirschke and Kaufmann, 2000; Kingdom and Kaufmann, 1997). It could be theorized that if the proliferation of tunica media of stem vessels in pre-eclampsia is in order to compensate for diminished flow through the intervillous space, this could in fact represent a maladaptation.

The field of research is still wide open with many differing opinions. There could be many reasons for the differing observations made by authors when examining the morphometry of pre-eclamptic placentas. The most likely explanation is that at present we are erring by

including conditions of several different etiologies under one banner. To explain why not all instances of pre- eclampsia are associated with placental abnormalities, Ness *et al.* propose that the disease constitutes a spectrum including 'maternal' and 'placental' forms. *Placental* pre-eclampsia would have an abnormal placenta occurring in an otherwise normal woman, and *maternal* pre-eclampsia, would have a normal placenta within a woman who suffers from a preexisting problem, such as cardiovascular disease or diabetes (Ness and Roberts, 1996). In addition, there is growing support that there are differences between the aetiology and alterations in placental morphology of placentas from *early* and *late* onset pre-eclampsia, as well as with or without *growth restriction* (Roberts and Catov, 2008). For example, Kingdom and Kaufmann (1997) propose that if pre-eclampsia manifests in later pregnancy, the situation may in fact be one of fetal hypoxia with placental hypoxia or even hyperoxia, as a result of an imbalance in placental entry and fetal extraction of oxygen.

4.3 CONCLUSION

The findings of this study, of increased villous density and associated increased fibrin deposition; as well as an apparent compromise to stem vessel carrying capacity, would indicate that *placental* and *post-placental* ischaemia are possible threats to materno-fetal exchange in pre-eclampsia, in addition to the existing *pre-placental* (maternal) ischaemia. As placental growth is a dynamic process which occurs in response to a variety of factors, including varying oxygen concentrations, it would seem that the changes observed could be adaptations of the fetal components of the placenta to the intervillous ischaemia. However, as these adaptations could in fact further compromise the fetus, they may represent maladaptations.

Future studies should incorporate Doppler blood flow measurements spiral artery, intervillous and umbilical vessels as an adjunct to morphological studies of both the placental villi and placental bed. This would allow correlations to be made in order to elucidate pre-placental, utero-placental and fetal ischaemia and their inter-relationships. The collaboration of clinician and scientist will become increasingly important in unfolding the many complexities of pre-eclampsia.

CHAPTER FIVE

APPENDICES

5.1 APPENDIX 1: ETHICS APPROVAL AND TITLE CHANGE

UNIVERSITY OF
KWAZULU-NATAL

Research Office
BIOMEDICAL RESEARCH ETHICS ADMINISTRATION
Research Office
Room N40 - Govan Mbeki Building
University Road, WESTVILLE CAMPUS
KwaZulu-Natal, SOUTH AFRICA
Tel: 27 31 2604769 - Fax: 27 31 2604609
Email: buccas@ukzn.ac.za - Website: www.ukzn.ac.za

24 August 2007

Mrs J Ducray
Optics & Imaging Centre
DDMRI
Nelson R Mandela School of Medicine
University of KwaZulu-Natal

Dear Mrs Ducray

PROTOCOL: Morphometric image analsis of caspases 3, 8 and 9 in term placentae of normotensive and preeclamptic pregnancies. Mrs. Jennifer Ducray. Durban University of Technology. Ref: BF042/07.

The Biomedical Research Ethics Committee considered the abovementioned application and the protocol was approved at its meeting held on 15 May 2007 pending appropriate responses to queries raised. Your responses dated 13 August 2007 to queries raised on 19 June 2007 has been noted by a sub-committee of the Biomedical Research Ethics Committee. The conditions have now been met and the study is given full ethics approval and may begin as at 24 August 2007.

This approval includes the amendment to the Information to participants dated 09 August 2007.

We acknowledge receipt of the permission from the hospital manager of King Edward VIII hospital and the Zulu translations of the information and consent documents.

Please submit evidence that the translator is accredited.

This approval is valid for one year from **24 August 2007.** To ensure continuous approval, an application for recertification should be submitted a couple of months before the expiry date. In addition, when consent is a requirement, the consent process will need to be repeated annually.

I take this opportunity to wish you everything of the best with your study. Please send the Biomedical Research Ethics Committee a copy of your report once completed.

Yours sincerely

DR J MOODLEY
Chair: Biomedical Research Ethics Committee

Tel 4769 KZN to get form

UNIVERSITY OF
KWAZULU-NATAL
INYUVESI
YAKWAZULU-NATALI

12 October 2011

Professor T Naicker
Optics and Imaging Centre
DDMRI
Nelson R Mandela School of Medicine

Dear Professor Naicker

Master of Medical Science
PROTOCOL: "Immunolocalization of caspases 3, 8 and 9 in the placenta of normotensive and preeclamptic pregnancies"
J Ducray, Optics & Imaging 206526406

At a meeting of the Postgraduate Education Committee held 11 October 2011, your request for approval of protocol amendment 25 July 2011 has been noted and approved.

New title: "Morphometric comparisons of the term placenta from normotensive and pre-eclamptic pregnancies suggest maladaptions of the foetal component of the placenta in Pre-eclampsia"

Yours sincerely

Professor SJ Botha
Chair: Postgraduate Education Committee

CC. Ms JF Ducray

Biomedical Research Ethics Committee
Westville Campus

Postgraduate Education Administration
Medical School Campus
Postal Address: Private Bag X, Congella, 4013, South Africa
Telephone: +27 (0)31 260 4327 Facsimile: +27 (0)31 260 4401 Email: hsslpgs@ukzn.ac.za Website: www.ukzn.ac.za

5.2 APPENDIX 2: HOSPITAL PERMISSION

Nelson R Mandela
School of Medicine

UNIVERSITY OF
KWAZULU-NATAL

Optics & Imaging Centre
Doris Duke Medical Research Institute
Nelson R Mandela School of Medicine
Faculty of Health Sciences
✉ Private Bag 7 Congella 4013 South Africa
Telephone: +27 (0)31 260 4435
+27 (0) 31 260 4274
Facsimile: +27 (0)31 260 4435
E-mail: Naickera@ukzn.ac.za

Medical Superintendent / Hospital Manager
King Edward VIII Hospital

Dear Sir

Permission to conduct a research study: Ethics study number: BF042/07

I would like to apply for permission to conduct a masters research study entitled:

Morphometric image analysis of caspases 3, 8, and 9 in term placentae of normotensive and pre-eclamptic pregnancies.

Under the guidance of my supervisors Prof T Naicker, and Prof J Moodley, I would like to quantify levels of some key biochemical players in the apoptotic pathway of the placental villi. This is with a view to increasing our understanding of the aetiology of pre-eclampsia.

In order to do this, we require small pieces of delivered placentas to process for immunocytochemistry, and an access to patient records in order to compare clinical data such as blood pressure, weeks of gestation, maternal age, birth weight of baby etc.

A research nurse will be employed to liaise with the patients, and to collect the data as well as the placental samples. We will utilize results of tests and examinations routinely done for the patients. There will be no financial or human resource implications for King Edward Hospital.

Prof Moodley has funding available for the research nurse, who is ready to begin her work as soon as we receive full ethics approval. We have also applied to NRF and MRC for project funds (see attached documentation). Please note that this is not a clinical trial, and I am not currently involved in any other studies.

Yours sincerely

_____ Date_____

Mrs Jennifer Ducray (Student number 206526406)

PERMISSION TO CONDUCT A RESEARCH STUDY/TRIAL

This must be completed and submitted to the Medical Superintendent/s / Hospital Manager/s for signature.

For **King Edward VIII Hospital (KEH) and Inkosi Albert Luthuli Central Hospital (IALCH)** studies please submit the document together with the following:

1. Research proposal and protocol.
2. Letter giving provisional ethical approval.
3. Details of other research presently being performed by yourself if in the employ of KEH, (individually or as a collaborator).
4. Details of any financial or human resource implications to KEH, including all laboratory tests, EEGs, X-rays, use of nurses, etc. (See Addendum 1)
5. Declaration of all funding applications / grants, please supply substantiating documentation.
6. Complete the attached KEH Form - *"Research Details"*

To: Chief Medical Superintendent / Hospital Manager

Morphometric image analysis of caspases 3, 8, and 9 in term placentae of normotensive and pre-eclamptic pregnancies. BF042/07

Permission is requested to conduct the above research study at the hospital/s indicated below:

Site 1 address:
Inpatients
Obstetrics and gynaecology (maternity)
King Edward VIII Hospital

Investigator/s:
Principal: **Mrs Jennifer Ducray**
Co-investigator: **Prof T Naicker-supervisor**
Co-investigator: **Prof J Moodley-clinical**

Signature of ~~Chief~~ Medical ~~Superintendent/Hospital~~ Manager:

Date: 10 AUG 2007

MEDICAL MANAGER
KING EDWARD VIII HOSPITAL DURBAN

NOTE: Once the document has been signed it should be returned to Mrs S Buccas, Biomedical Research Ethics Administration, Room 112 Old MRC Building.

NB: Medical ~~Superintendent~~/s ~~/ Hospital~~ Manager/s to send a copy of this document to Natalia

Site 2 address: **N/A**

Investigator/s

Principal: _____
Co-investigator: _____
Co-Investigator: _____

Signature of Chief Medical Superintendent / Hospital Manager:

Date: _____

5.3 APPENDIX 3: PATIENT INFORMATION

Greeting: Good day Miss/ Mrs _____. My name is _____
Thank you for giving me the time to speak to you.
Introduction: The reason I want to speak to you today is because my colleagues and I are presently involved in a study of pregnant women. It has been noted that some women get very high blood pressure during pregnancy, and we are trying to understand more about why this happens.
I'm sure you know that when a baby grows inside you, it gets its nutrition from a piece of tissue that is attached to your womb called the placenta. People studying in this field have seen that for some reason it is the placenta that causes the high blood pressure.

When the baby is born, this piece of tissue (which is usually called the afterbirth) is also delivered. After the baby's cord has been tied and cut, the placenta is discarded. In order to learn more about high blood pressure in pregnancy, we need to study a small piece of your placenta.

Invitation to participate: We are asking for your permission to include you in our research study. We require your permission to take a small piece of your placental tissue (after it has been delivered) and to study it. This will not harm you or your baby in any way.
What is involved in the study: We will be collecting placenta samples from 30 woman whose blood pressure was normal, and 30 samples from women whose blood pressure was high. All these samples are being collected here in this hospital from South African woman and will be studied at the Nelson Mandela Medical School. The results of this will benefit medical science and be used for a masters degree. We will require nothing more from you except permission to use a piece of your placenta. The piece of placenta will be labelled, maintaining your anonymity and then processed for experimental procedures. Post-study the sample will be archived in wax. The remainder of the placenta not utilized for study purposes will be incinerated as usual by KEH.
Risks Because this sample is taken after the placenta has been discarded, it will not harm you or your baby in any way.
Participation is voluntary Please note that participation in this study is completely voluntary. You are in no way obligated to consent to us taking this sample, and should you decline you will still receive the normal hospital treatments to which you are entitled.
Confidentiality Personal information will remain confidential. Personal information may be disclosed if required by law. Organizations that may inspect and/or copy your research records for quality assurance and data analysis include groups such as the Research Ethics Committee and the Medicines Control Council (where appropriate).
Questions Do You have any questions?
Contact details of researcher/**s If you want any more information, or are unhappy about anything, you may contact the following people:**

Nalini Pillay, or Jennifer Ducray 204-2406
Contact details of BREC Administrator – for reporting of complaints / problems
Biomedical Research Ethics Committee
MRS S BUCCAS, Nelson R Mandela School of Medicine, Private Bag 7, Congella 4013
Telephone: +27 (0) 31 260 4769
Fax: +27 (0) 31 260 4609 email: buccas@ukzn.ac.za

ZULU TRANSLATION OF PATIENT INFORMATION
Ukubingelela: Sawubona Nks./ Nkz._____Igama lami ngiwu_____
Ngiyabonga ngokunginika lelithuba ukuthi ngikhulume nawe.

Isandulelo:
Into eyenza ukuba ngifise ukukhuluma nawe namhlanje yingoba mina nalabo esisebenzisana nabo senza ucwaningo ngabesimame abakhulelwe. Kuyinto esibonakele ukuthi i-BP iyanyuka kakhulu ngesikhathi umuntu ekhulelwe, esizama ukukwenza ukuthola ukuthi ngabe lokhu kudalwa yini.

Ngiyakholwa uyazi ukuthi uma umntwana ekhula ngaphakathi kuwe ukudla ukuthola ngenkaba exhumeke esibelethweni ebizwa ngokuthi umzanyana. Abakuwo lomkhakha sebebonile ukuthi into eyenza i-BP inyuke kuphathelene nawo umzanyana.

Ngesikhathi umntwana ezalwa, kunezicubu eziphumayo (umzanyana) futhi ngesikhathi ubeletha.
Emva kokuthi inkaba isiboshiwe yanqunywa, bese uyalahlwa (umzanyana) ngendlela esemthethweni. Ukuze sazi kabanzi nge-BP uma ukhulelwe, kumele sithathe ucezwana oluncane lomzanyana ukuze sifunde kabanzi ngalo.

Isimemo sokuzibandakanya: Siyakucela ukuba ube yingxenye yalolucwaningo. Sicela imvume yokuthatha isicucu somzanyana uma usulahliwe (emva kokubeletha), siwusebenzisele ucwaningo. Lokhu angeke neze kulimaze wena noma umntwana.

Okuphathelene nocwaningo: Sizoqoqa imizanyana yomame abangu-30 akade bene-BP enganyukanga, bese kuba ngeyomame abangu-30 akade bene-BP enyukile. Lemizanyana iziqoqwa ezibhedlela komame baseMzansi Afrika, izobe isiyocwaningwa esikoleni sobudokotela i-Nelson Mandela. Usosayensi bezempilo bayozuza ngemiphumela yalolucwaningo. Akukho okunye esikudingayo kuwe ngaphandle kwemvume yokusebenzisa isicubu somzanyana wakho. Isicubu somzanyana siyobhalwa imininingwane yakho engeke yaziwa ngomunye umuntu bese sisetshenziselwa indlela okuyohlolwa ngayo. Emva kocwaningo amasampula omzanyana ayogcinwa ukuze asetshenziselwe ikusasa. Umzanyana ongasebenzanga ngesikhathi socwaningo, uyoshiswa njengokujwayelekile.

Ingozi engabakhona: Ngenxa yokuthi isicubu siyothathwa uma umzanyana usubelethiwe, lokhu angeke neze kulimaze wena noma umntwana.

Ukuzibandakanya akuphoqiwe: Sicela ukukwazisa ukuthi ukuzibandakanya kulolucwaningo akuphoqelekile neze, kanti futhi uma ukhetha ukunqaba lokhu angeke kuthikameze impatho yakho esibhedlela.

Imfihlo: Imininingwane yakho izohlala iyimfihlo. Abantu abangafunyana lolulwazi yilabo abasemakomitini nezinyunyzna zocwaningo.

Imibuzo: Kungabe unayo yini imibuzo?
Imininingwane yomcwaningi: Uma udinga olunye ulwazi noma kukhona ongagculisekile ngakho, ungaxhumana nalaba abalandelayo:

Nalini Pillay, noma u- Jennifer Ducray 204- 2406.

Imininingwane kamabhalane we- BREC- ukuze wethule izinkonondo.

Ikomiti locwaningo lwezobudokotela:

NKZ. S BUCCAS, isikole sezobudokotela i-Nelson Mandela, Private Bag 7, Congella 4013. inombolo yocingo: +27 (0) 31 260 4769. I- faxi: +27 (0) 31 260 4609. i-email: buccas@ukzn.ac.za

5.4 APPENDIX 4: CONSENT FORM

Consent to Participate in Research

Morphometric image analysis of caspases 3, 8, and 9 in term placentae of normotensive and preeclamptic pregnancies (Ethics reference number BF042/07)

I understand that I have been asked to participate in a research study
I have been informed about the study by Jennifer Ducray.
It has been explained to me that because this sample is taken after the placenta has been delivered, it will not harm me or my baby in any way.
It has also been explained to me that that participation in this study is completely voluntary. I understand that I do not have to give my permission, and if I refuse, I will still receive the normal hospital treatments.
I understand that you will require nothing more from you except permission to use a piece of my placenta and to see my medical records.
I know that I may contact Nalini Pillay or Jenny Ducray at 204-2406, or the Medical Research Administration office at the Nelson R Mandela School of Medicine at 031-260 4604 at any time if I have questions about the research.
I will receive a signed copy of this document and the sheet of information about the project which has been explained to me.
The research study, including the above information, has been described to me orally. I understand what my involvement in the study means and I voluntarily agree to participate.

_____ _____
Signature of Participant Date

_____ _____
Signature of Witness Date
(Where applicable)

_____ _____
Signature of Translator Date
(Where applicable)

Imvume yokuzibandakanya kulolucwaningo

Isihloko socwaningo: Ukuhlola ukuthi kumzanyana kwenzakalani nanokuthi yini eholela ekutheni kunyuke i-BP. (Ethics reference number BF042/07)

Ngiyaqonda ukuthi ngiceliwe ukuba ngizibandakanye kulolucwaningo.

Ngazisiwe ngalolucwaningo wu_____.

Ngichazeliwe ukuthi ngenxa yokuthi umzanyana uzothathwa emva kokuba sengibelethile, lokhu angeke neze kulimaze mina noma umntwana.

Ngichazeliwe futhi ukuthi ukuzibandakanya kulolucwaningo akuphoqelekile neze, kanti futhi uma ngikhetha ukunqaba lokhu angeke kuthikameze impatho yami esibhedlela.

Ngiyaqonda futhi ukuthi akukho okunye okudingayo kimi ngaphandle kwemvume yokusebenzisa isicubu esincane somzanyana wami kanye nokubona imininingwane yami yasesibhedlela.

Ngiyazi ukuthi ngingathintana no-Nalini Pillay, noma u- Jennifer Ducray kulenombolo 204- 2406, noma ihhovisi likamabhalane wocwaningo esikoleni sezobudokotela i-Nelson Mandela kulenimbolo 031 260 4604 noma ngasiphi isikhathi, uma nginemibuzo ephathelene nocwaninga.

Ngizothola ikhophi yalencwadi esayiniwe kanye nencwadi yesaziso mayelana nalolucwaningo esengichazelwe ngalo.

Ucwaningo, kanye nolwazi, ngichazeliwe ngalo ngomlomo. Ngiyaqonda ukuthi kuphathelene nani ukuzib andakanya kwami kulolucwaningo nokuthi angiphoqiwe.

_____	_____
Isiginisha yozibandakanyayo	Usuku
_____	_____
Isiginisha kafazi (uma kunesidingo)	Usuku
_____	_____
Isiginisha yomhumushi (uma kunesidingo)	Usuku

5.5 APPENDIX 5: DATA SHEET

CASPASE / PRE-ECLAMPSIA STUDY JENNY DUCRAY

Category (tick): Normotensive = N ☐ Pre-eclamptic = P ☐ **Study no:**

☺ **No exclusion criteria present** (check against list) ☐

Please place hospital sticker here

General hospital information

Admission date		KEH number			
Diagnosis (tick) if PET	MILD: BP 140-150 / 90-110 Protein +		SEVERE: BP> 160 / 110 Protein ++		

Demographics

Surname		First Name	
Date of Birth		Age	
Lifestyle (tick)	Rural	Urban	

Clinical Data

Parity	P: G:		Weeks gestation on admission			
Highest BP	Systolic:		Diastolic:			
Maternal weight			Maternal height			
Oedema (tick)	ankle	Up to knee	Up to groin	Generalised (facial)		
Lab results	proteinuria	Dipstick				
		Lab 24hr protein				
		Creatinine clearance				
	Full blood count	Red cell count		White cell count		
		Haemoglobin		Neutrophils		
		Haematocrit		Lymphocytes		
		Mean cell volume		Monocytes		
		Mean cell Hb		Eosinophils		
		Platelets		Basophils		
	Urea and	Sodium		Urea		

	electrolyte	Potassium		Creatinine	
		Chloride		Anion gap	
		CO_2			
	Liver function tests	Total protein		Alkaline phos	
		Albumin		AST	
		Globulin		ALT	
		Alb : Glob		LDH	
		Total bilirubin			

Antenatal Fetal Investigations

Type (tick)	Note any abnormalities
Sonar	
Doppler	
Electronic fetal HR	

Birth details

Weeks of gestation at time of birth				
Indication for delivery (tick one)	Maternal interest	Fetal Distress (CTG abnormal)	Combination of Maternal and fetal interest.	
	Explain above if relevant	Explain above if relevant	Explain above if relevant	
Method of Delivery (tick one)	Normal vaginal		Caesarean	
	Spontaneous		Elective	
	Induced		Emergency	
Complications in labour.	Eclampsia – related (tick)	Severe pre-eclampsia	Imminent eclampsia	
	Other (explain)			

Baby details at birth

APGAR	1 min		5 min	
Baby (tick)	Live		Stillborn	
	Perinatal death (1^{st} 7days)		Neonatal death (up to 28 days)	
Baby weight	(kgs)			

Placental details

shape	normal		abnormal	
Weight (grams)				
Diameter (cm)				
Thickness (cm)	Less than 2cm	2-3cm	More than 4	
Colour	Maternal surface	Dark Maroon	Pale	
	Fetal surface	Dark	Pale	
Infarcts (maternal surface)	Amount of infarcted tissue	clear	mild	severe
	Colour of infarcts (if present)	Pale grey	Very dark	Both
Clots (maternal surface) tick	none	few	many	
Umbilical cord	Point of attachment	central	peripheral	
	Length	Less than 30 cm	30-90 cm	Greater than 90 cm
	No of vessels	3	2	

5.6 APPENDIX 6: PEER REVIEWED PUBLICATION

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Pilot study of comparative placental morphometry in pre-eclamptic and normotensive pregnancies suggests possible maladaptations of the fetal component of the placenta

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ABSTRACT

Objective: Adequate maternal, intervillous and fetal blood flow are all necessary for fetal well-being. Compromise to any part of this exchange would be detrimental to pregnancy outcome. Pre-eclampsia is associated with reduced maternal spiral artery flow, resulting in reduced placental perfusion. This in turn creates an ischaemic environment, which may predispose to morphological changes in placental villi. This pilot study sought to assess whether there were morphological alterations in the fetal component of the placenta which could be detrimental to exchange and therefore pregnancy outcome.
Study design: This study utilized morphometric image analysis to examine some features of the fetal component of the placenta in normotensive (NT) and pre-eclamptic (PE) groups. The features examined included: density of placental villi (expressed as percentage of field area occupied by placental tissue); stem vessel carrying capacity (expressed as percentage of stem villus area occupied by vessel lumina); the thickness of the stem arterial walls relative to artery size (expressed as percentage of artery area occupied by arterial wall) and the extent of fibrosis associated with villi (expressed as percentage of field area occupied by fibrosis).
Results: There were significant differences between NT and PE placentae in density of placental villus arrangement NT: 51.89 ± 6.19, PE: 64.78 ± 6.93 ($P < 0.001$); carrying capacity of stem villi NT: 17.20 ± 11.78, PE: 8.67 ± 8.51 ($P < 0.001$); relative thickness of stem villi arterial walls NT: 74.08 ± 12.92, PE: 86.85 ± 10.55 ($P < 0.001$); and extent of fibrosis NT: 0.727 ± 0.310, PE: 1.582 ± 0.707 ($P < 0.001$).
Conclusion: These significant differences between normotensive and pre-eclamptic placentae suggest possible fetal maladaptations in response to the intervillous ischaemia, compounding the existing maternal compromise to materno-fetal exchange. Further investigations would, however, be necessary in order to make more conclusive deductions.

1. Introduction

Pre-eclampsia is a condition unique to pregnancy, characterised by abrupt onset of hypertension and proteinuria. It is associated with an increase in maternal morbidity and mortality, and with a fivefold increase in perinatal mortality [1–3]. Pre-eclampsia occurs in about 2–8% of pregnancies in developed countries, but this figure can be threefold higher in developing countries. Worldwide, pre-eclampsia and eclampsia account for over 50,000 deaths annually [3]. In South Africa, hypertensive disorders of pregnancy (HDP) account for 19% of all maternal deaths, and of these, 83% are due to pre-eclampsia and eclampsia [2]. Resolution of the disorder occurs with delivery of the placenta, hence much research has been focused on the placental role in this syndrome.

As far back as 1939, researchers had associated the development of pre-eclampsia with reduced placental perfusion [4]. During early placental development, extravillous trophoblast invasion into the placental bed is inadequate, resulting in failure of the physiological conversion of the spiral arteries that is characteristic of normal pregnancy [5–13]. This in turn leads to decreased perfusion of the intervillous space. In this ischaemic environment one would expect structural compensations of the villi such as increased surface area or decreased diffusion distance. Alternatively, there could be increased damage such as fibrotic deposition or necrotic areas.

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Studies on the comparative morphology of pre-eclamptic versus normotensive placentae have demonstrated interesting and sometimes conflicting results. Villous surface area has been shown to be altered disproportionately to volume in pregnancies associated with certain hypoxic conditions, including pre-eclampsia [14]. Intrauterine growth restriction (IUGR) has been associated with changes in placental morphology such as decreased surface areas, impoverished growth of peripheral villi and decreased fetal angiogenesis. This was not the case for pre-eclampsia alone, however [15–18]. Significant hyper-ramification of the capillary loops in terminal villi of pre-eclamptic placentae has been observed, together with irregular vessel profiles and narrow vessel lumina [19,20]. The prevalence of inflammation, infarction, ischaemia, haemorrhage, and syncytial knots was found to be increased in both pre-eclampsia and IUGR [21], and it has long been established that the placentae from pre-eclamptic pregnancies display increased syncytial knotting [22,23].

Clarity in the relationship between placental function and morphology on the one hand, and pre-eclampsia on the other hand is still lacking. Kingdom and Kaufmann suggested that changes in placental morphology will depend on intraplacental oxygen status, which in turn depends on both maternal supply and fetal extraction [24]. Pre-eclampsia is generally recognized as being characterized by reduced maternal flow with *uteroplacental* hypoxia (inadequate oxygenated blood entering the intervillous space). This, however, is only one aspect of the equation. If the villous elements of the placenta do not transport available oxygen to the fetus efficiently, a situation of *post placental* hypoxia could arise, in which a compromised villous flow compounds the reduced maternal flow. Added to this, the efficiency of exchange could also be affected by any impedance to intervillous flow (such as could result from an increase in density of villi/reduction of intervillous space/fibrosis of villi).

Utilizing morphometric image analysis, this study compared some villous parameters which could affect the haemodynamics of materno-fetal exchange. This was with a view to investigating possible morphological changes in the villi occurring in response to, but also compounding, the effects of the placental ischaemia associated with pre-eclampsia.

2. Materials and methods

2.1. Patient selection and sample collection

The study was conducted in Durban, South Africa, as an offshoot of a larger pre-eclamptic study. Following institutional ethical approval and informed consent, thirty normotensive (BP $\leq$ 130/80 mm Hg) and 30 pre-eclamptic (SBP $\geq$ 140 mm Hg, DBP $\geq$ 90 mm Hg, proteinuria) African patients were selected by a research midwife. Patients between the ages of 18 and 40 years and of parity 0–5 were included in the study. Exclusion criteria included chronic hypertension, pre-existing seizure disorder, eclampsia, pre-gestational diabetes, placental abruption, gestational diabetes, thyroid disease, asthma, chronic renal disease, intrauterine death, cardiac disease and infection with HIV. Demographic, clinical and neonatal data were collated.

2.2. Sample preparation and morphometry

Placental samples were immediately fixed in buffered formaldehyde and processed using a Sakura VIP 500 Automated Tissue Processor. A single 3–5 μm section from each sample was prepared for general morphological examination using a Mayers Haematoxylin and Eosin stain [25]. Numerous photomicrographs were taken at different levels of magnification, and utilized for the morphometry using a Nikon Eclipse E600 microscope interfaced with the Software Imaging Systems (Germany) image analysis package. A single observer did all the measurements in order to minimize inter-observer bias. The following morphology parameters were assessed:

(i) *Density of placental villus arrangement*: Using the 4× objective, the four quadrants of each slide were photographed. Colour thresholds, saturation and intensity were individually set for each image, binary images were created (Fig. 1A and B), and phase analysis was performed to determine the area of the field that was occupied by placental villi. This was then expressed as a percentage of the total field area. The average of the measurements for the four quadrants was determined for each slide.

(ii) *Percentage of stem villus area occupied by stem vessel lumina*: Utilising the features of stem villi outlined by Benirschke and Kaufmann [26], two stem villi were selected in each section on the basis of clarity of morphology for measuring purposes. Stem villi at different levels of branching (and therefore different calibres) were included. For each stem villus, the sum of all the vessel luminal areas was expressed as a percentage of total villus area.

(iii) *Relative thickness of stem arterial walls*: Utilising the micrographs from (ii) above, the main arterial vessel was selected in each. The area of the artery was established using the outer adventitial boundary. The lumen area was subtracted from this in order to ascertain the area occupied by arterial wall. This was expressed as a percentage of total vessel area in order to compensate for differing calibres of stem villi.

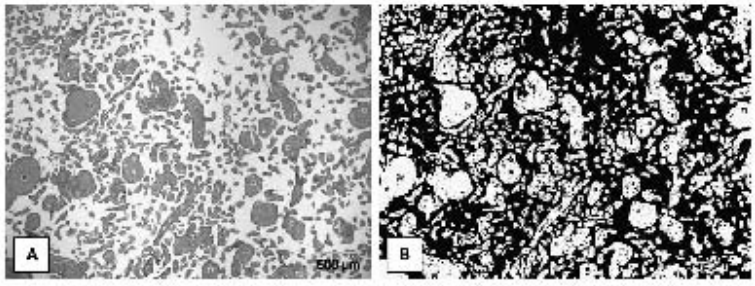

Fig. 1. (A) Light micrograph of H and E stained placental tissue. (B) Binary image of micrograph (A). The combined area occupied by white in micrograph (B) was expressed as a percentage of the total field area.

Table 1
Demographic and clinical data.

Variable (Units)	Normotensive Mean ± SD	Pre-eclamptic Mean ± SD	*P*-values
Parity (no. of live births)	1 (0.94 ± 0.854)	1 (0.70 ± 1.104)	0.339
Gravidity (no. of pregnancies)	2 (1.94 ± 0.854)	2 (1.82 ± 1.158)	0.648
Systolic blood pressure (mmHg)	117.71 ± 10.319	156.94 ± 15.131	<0.001
Diastolic blood perssure (mmHg)	72.19 ± 8.064	100.42 ± 8.101	<0.001
Mean arterial pressure (mmHg)	87.366 ± 7.745	119.263 ± 9.206	<0.001
Body mass index	32.77 ± 6.57	35.15 ± 5.75	0.180
Weeks gestation (weeks)	38.87 ± 1.432	36.58 ± 2.840	<0.001
Baby weight (kg)	3.16 ± 0.402	2.74 ± 0.813	0.012
Full placental weight (g)	573 ± 98.336	635 ± 172.722	0.094
Placental diameter (cm)	20.16 ± 0.523	19.97 ± 0.467	0.126

(iv) *Extent of fibrosis*: Samples were examined utilizing the 10× objective, and the area of greatest fibrosis was selected in each quadrant. The sum of all areas of fibrosis in the field was expressed as a percentage of total field area.

2.3. Statistics

Mean ± standard deviation was calculated for clinical and morphological quantitative variables by group. Statistical significance was determined using *t*-tests ($P < 0.001$). Comparisons of categorical variables between groups were achieved using the Pearson Chi-square test, with a $P < 0.05$. Correlations of mean arterial pressure (MAP) *vs.* placental weight, birth weight *vs.* placental weight, birth weight *vs.* weeks gestation and birth weight *vs.* MAP were performed using two-tailed Pearson's correlation coefficient (r) with $P < 0.01$ indicating statistical significance.

3. Results

The clinical characteristics of the normotensive (NT) and pre-eclamptic (PE) groups are shown in Table 1. Blood pressure parameters, viz. systolic ($P < 0.001$), diastolic ($P < 0.001$) and mean arterial pressure ($P < 0.001$) were significantly different between groups. Platelet counts were significantly lower in the PE group ($P = 0.005$), with 36% of the pre-eclamptics having platelet counts $\leq 175 \times 10^9\ l^{-1}$. The gestational age ($P < 0.001$) and birth weight ($P < 0.01$) were lower in the PE group compared to the NT group. Table 2 shows a moderate positive correlation between birth weight and placental weight ($r = 0.623$), birth weight and weeks of gestation ($r = 0.743$) and birth weight and MAP ($r = 0.380$).

3.1. Placental morphometry

The percentage of field area occupied by placental villi (as opposed to intervillous space) was higher in the PE group ($P < 0.001$). This indicates a reduction in the available intervillous space in this group (Fig. 2A and B). Stem villi morphometry demonstrated a lower total luminal area (carrying capacity) in relation to the villus area in the PE samples), compared to NT samples (Fig. 2C and D)($P < 0.001$), concomitant with an increased thickness of stem villi arterial walls in the PE group (Fig. 2C and D). This was attributable to an increase in the muscle tissue where morphometric comparisons indicated that the percentage of the vessel area occupied by vessel wall was substantially higher in the pre-eclamptic stem arteries ($P < 0.001$).

Fig. 2(E and F) shows an increase in the occurrence of fibrosis in the PE samples, which was confirmed by morphometric image analysis of these fibrotic areas ($P < 0.001$). Fig. 3 shows a bar graph illustrating the results of the placental morphometry.

Table 2
Correlation coefficient between mean arterial pressure, gestational age and placental weight.

Variables correlated	*N*	Correlation coefficient[a] (r)
Mean arterial pressure with placental weight	64	0.175
Birth weight correlated with placental weight	64	0.623*
Birth weight correlated with weeks gestation	64	0.743*
Birth weight correlated with mean arterial pressure	64	−0.380*

[a] Correlation significant at the 0.01 level.

4. Comment

4.1. Acknowledged shortcomings in the study

There are arguments both for and against the theory that what we currently diagnose as pre-eclampsia in fact represents more than one disorder [27,28]. In order to achieve complete accuracy when studying the relationship between pre-eclampsia and placental morphology, the idea is to sub-categorise the disorder into early and late onset as well as PE with or without IUGR, as done, for example, by Egbor et al. [29]. In developing countries such as South Africa, however, researchers are unable to do this as patients generally present to the hospital at the initiation of labour. The hospital in Durban at which the study was done is no exception, in that patients are often diagnosed with pre-eclampsia for the first time on admission. Ascertaining the time of onset of the condition is generally not possible. To complicate matters, no local population norms have been established for fetal weights related to gestational age, and patients are generally unsure as to their last menstrual period. These factors make isolation of pregnancies with small for gestational age (SGA) babies difficult. Multiple regression analysis confirmed a highly significant ($P < 0.001$) positive association between birth weight and gestational age, rather than intrauterine growth restriction. However, as the researchers cannot be sure that the PE cohort is free of IUGR, interpretation difficulties arise. The results therefore can be used to give insight into possible changes and to suggest possible future investigations, but not to make conclusive statements.

4.2. Image analysis

The *maternal* contribution to materno-fetal exchange is already established as being compromised in pre-eclampsia at the level of the spiral arterioles. The histological comparisons performed in this study involve *fetal* components of this exchange, investigating possible structural adaptations, maladaptations or even ischaemic damage that may occur in pre-eclampsia.

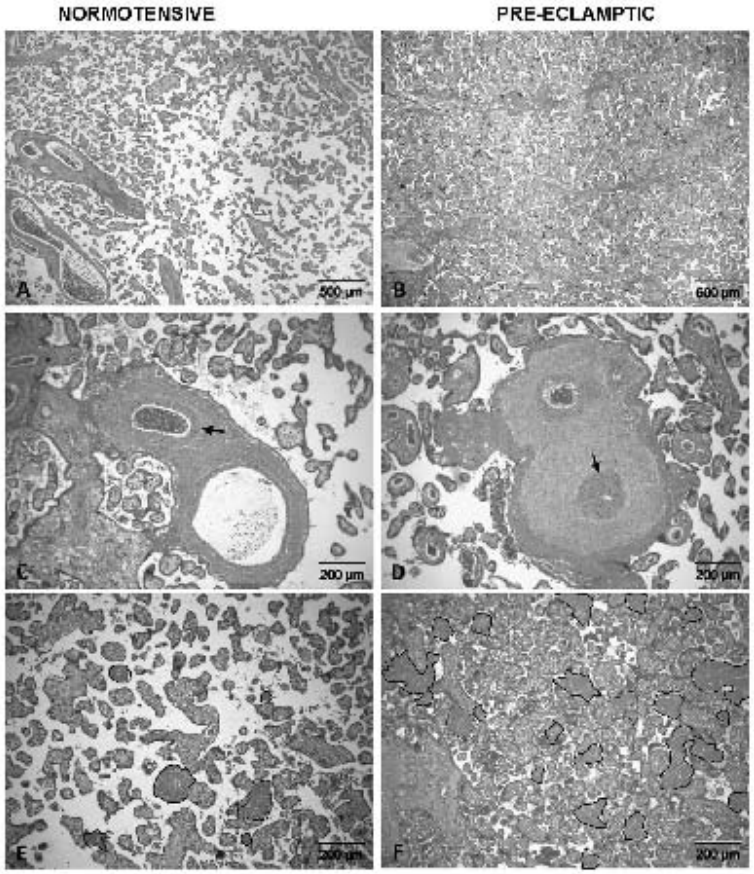

Fig. 2. Light micrographs of placental tissue from normotensive and pre-eclamptic pregnancies. Sections (A) and (B) show the density of placental tissue (villi), with the denser arrangement of villi seen in the pre-eclamptic placenta (B). Sections (C) and (D) illustrate stem villi (arteries indicated by the arrow). The collective area of all the stem vessel lumina is seen to be higher in the normotensive villus (C). The relative arterial wall thickness is higher in the pre-eclamptic sample (D). Sections (E) and (F) show the higher occurrence of fibrosis in pre-eclampsia. (F) The fibrotic areas should not be confused with the normal intervillous maternal blood which stains a much darker red.

The increased density of the villous arrangement observed in the pre-eclamptic group of this study suggests increased growth or hyper-ramification of the placental villi. This response would increase the interface available for materno-fetal exchange, and is known to occur in hypoxic conditions. These hypoxic conditions could be pre-placental such as maternal anaemia, or uteroplacental such as retention of vasoreactivity by the maternal spiral arteries [24,30]. An increased density of the villous arrangement will diminish the intervillous space, thus impacting on flow in this area. So whilst the villous surface area would be increased, flow would decrease, possibly compromising materno-fetal exchange. Boyd and Scott [31], however, found significantly lower volume of parenchyma, as well as lower villous surface area in pregnancies complicated by pre-eclampsia. A study by Mayhew et al. [16] found that a reduced volume of intervillous space was associated with IUGR, but not with pre-eclampsia, once again emphasizing the benefits of sub-categorisation in study design.

To date, many of the studies looking at placental villous morphometry have focused on the *peripheral* (intermediate and terminal) villi, as these villi form the surface for actual materno-fetal exchange. The vessels in the stem villi are for conducting blood to and from these intermediate and terminal villi. In conducting fetal blood to the peripheral villi, stem vessels will affect haemodynamics at the exchange sites, including fetal extraction of oxygen. In conducting the oxygenated blood back to the umbilical veins, they will affect umbilical haemodynamics (blood transfer back to the fetus). Any compromise to their carrying capacity will in turn compromise materno-fetal exchange. Although stem villi play this critical role in fetal circulation, studies examining them are limited.

Resta et al. [19] examined the capillary loops in terminal villi of pre-eclamptic and normotensive placentae utilising confocal laser scanning. They reported significant hyper-ramification together with irregular profiles and narrow lumina. Las Heras et al. [32]

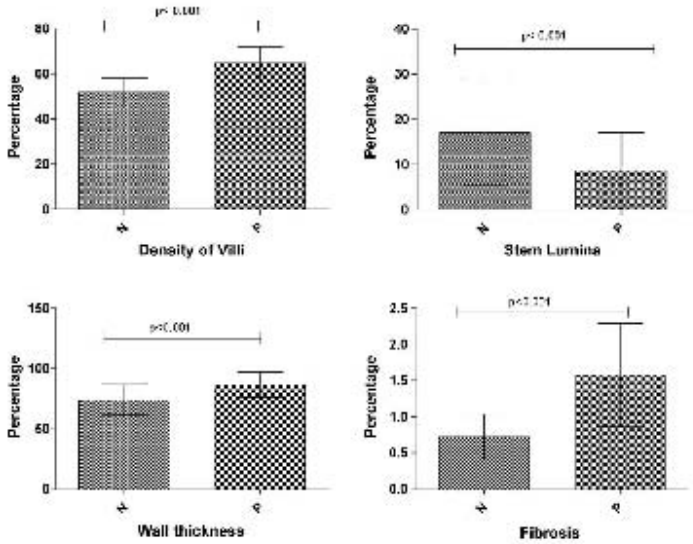

Fig. 3. Bar graphs illustrating comparisons of means of morphometric data for normotensive (N) and pre-eclamptic (P) groups. (Note that the percentage of fibrosis must be seen as relative rather than absolute reflection of fibrosis throughout the placenta, as the areas of *greatest* fibrosis were measured in both groups).

examined stem vessels in different hypertensive disorders of pregnancy, including pre-eclampsia. They found endothelial proliferation, as well as proliferation of the subendothelial and smooth muscle cells of the medial layer. The stem vessel lumina were narrowed. In this study the lower collective luminal area of the pre-eclamptic stem villi vessels (relative to villus area) suggests a compromise of fetal blood flow through these villi as opposed to the stem vessels in normotensive placentae.

The percentage of stem artery area occupied by the vessel wall was higher in the pre-eclamptic group, revealing thicker arterial walls. This may be due to *circular* muscle hyperplasia arising from a need to increase pressure and flow in the *fetal* circulation. The thicker wall could, however, also be due to the *longitudinal* elements of the media. Longitudinal myofibres of the media as well as the villus stroma which are oriented parallel to the longitudinal axis of the villous stems are postulated to aid circulation of maternal blood through the intervillous space, thus contributing to the *fetoplacental vascular system as a whole*, and not just to the fetal circulation [33]. This would be particularly so in the case of anchoring villi which connect the chorion to the basal plate, thus extending across the full intervillous space, as their longitudinal contraction could decrease intervillous volume and thus increase intervillous pressure [26,34]. Pressure increases in the intervillous space have, however, been postulated to reduce fetal perfusion by increasing fetoplacental impedance [24,26]. It may be hypothesized that in pre-eclampsia the medial hyperplasia of the stem artery wall occurs as a compensatory mechanism to the reduced flow through the intervillous space. As the increased size of the media could be the reason for the decreased total carrying capacity of the stem vessels, this medial hyperplasia could in fact represent a maladaptation. Kingdom and Kaufman [24] suggest that in IUGR there is a failure in the transport of oxygen from the intervillous space to the umbilical vein. Thus whilst the fetus is hypoxic, the intervillous space remains relatively "hyperoxic". As the release of growth factors such as vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) is oxygen-dependent, the hyperoxic environment would alter their secretion and resulting effects. This would account for differences in placental morphology between IUGR and pure pre-eclampsia.

The morphometric observation involving areas of fibrosis showed a greater incidence of fibrosis in the pre-eclamptic group. These findings corroborate those of Correa et al. [20], who found that fibrin deposition was greater in all hypertensive syndromes in pregnancy, and that the deposition changed from perivillous to intravillous in the more severe cases, an observation noted in the current study. Fibrin deposition could be a reparative process in response to infarction or inflammation. Jain et al. found both inflammation and infarction to be increased in pre-eclampsia [21].

In conclusion, the morphological and morphometrical evaluations of this study showed increased villous area in pre-eclampsia, associated with increased perivillous and intervillous fibrinoid. Stem villi morphometry revealed a significant reduction of stem vessel lumina, with a concomitant increased media. These results suggest a compromise in villous carrying capacity, thereby affecting placental transport and haemodynamics, compounding the ischaemia resulting from the reduced maternal spiral artery flow. Further investigations would be necessary in order to determine whether the features observed are associated with IUGR, and to determine whether the time of onset of gestational hypertension impacts on them in any way.

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CHAPTER SIX

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