CD146 expression in early and late onset Pre-eclampsia – is there a difference?

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DECLARATION

I declare that this thesis is my original work and I have not in entirety or in part submitted this work to any University for another degree.

__________________________
Signature

Pawel Tomasz Schubert
17 June 2014
ABSTRACT

Objective: To investigate the difference in expression of CD146 immunohistochemical staining on intermediate trophoblast in early and late onset pre-eclampsia placentas as well as comparing this expression to gestational age matched control placentas.

Study Design: Retrospective case series of 100 placentas: 25 early onset and 25 late onset pre-eclampsia placentas as well as 25 early and 25 late gestational age matched control placentas. Placentas were obtained from patients delivering in Tygerberg Hospital.

Methods: Placentas were routinely fixed and processed. 2 sections of one preselected block from each case was cut and stained with CD146 and MNF116 immunohistochemical stain. The expression of the staining would be performed by means of analytical and image analysing software.

Results: The study failed to demonstrate differences in CD146 expression by the intermediate trophoblast in the pre-eclampsia and control placentas. The analytical approach was deemed to be subjective and the image analysing software had too much background staining and inaccurate identification of the intermediate trophoblast in order to produce reproducible consistent results.

Conclusion: Dual staining, using immunofluorescent staining of CD146 and MNF116 on smaller biopsies of the decidua are thought to be able to produce much better material for image analyses software.
ACKNOWLEDGEMENTS

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Prof. H Wainwright – for her amazing love of paediatric pathology.

Ms M Alblas - for her help with the Nikon imaging system and always smiling.
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<table>
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<th>Description</th>
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<tr>
<td>AT</td>
<td>Angiotensin</td>
</tr>
<tr>
<td>CAM</td>
<td>Cell Adhesion Molecule</td>
</tr>
<tr>
<td>CAW</td>
<td>Colleen Anne Wright</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster Designation</td>
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<tr>
<td>DIC</td>
<td>Disseminated Intravascular Coagulation</td>
</tr>
<tr>
<td>e.g.</td>
<td>for example.</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and Eosin stain</td>
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<tr>
<td>HW</td>
<td>Helen Wainwright</td>
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<tr>
<td>i.e.</td>
<td>that is</td>
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<tr>
<td>IHC stains</td>
<td>Immunohistochemistry / immunohistochemical stains</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ISSHP</td>
<td>International Society for the Study of Hypertension in Pregnancy</td>
</tr>
<tr>
<td>IT</td>
<td>Intermediate Trophoblast</td>
</tr>
<tr>
<td>IUGR</td>
<td>Intrauterine Growth Retardation</td>
</tr>
<tr>
<td>NHLS</td>
<td>National Health Laboratory Service</td>
</tr>
<tr>
<td>nl</td>
<td>namely</td>
</tr>
<tr>
<td>MCAM</td>
<td>Melanoma Metastasis-Associated Surface Molecule</td>
</tr>
<tr>
<td>MMed</td>
<td>Masters in Medicine</td>
</tr>
<tr>
<td>Mel-CAM</td>
<td>Melanoma Cell Adhesion Molecule</td>
</tr>
<tr>
<td>PECAM</td>
<td>Platelet/Endothelial Cell Adhesion Molecule 1</td>
</tr>
<tr>
<td>PI</td>
<td>Principle Investigator</td>
</tr>
<tr>
<td>PIGF</td>
<td>Placental Growth Factor</td>
</tr>
<tr>
<td>SA</td>
<td>South Africa</td>
</tr>
<tr>
<td>sENG</td>
<td>Soluble Endoglin</td>
</tr>
<tr>
<td>sFLT1</td>
<td>Soluble fms-like Tyrosine Kinase 1</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular Cell Adhesion Molecule</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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</tbody>
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1. INTRODUCTION and LITERATURE REVIEW

Pre-eclampsia is a pregnancy associated hypertensive disorder, occurring in 5 - 8% of pregnancies, after 20 weeks of gestation with a new presentation of hypertension and proteinuria.1-3 The disease can present earlier (prior to 20 weeks gestation), particularly if it is associated with trophoblastic disease (i.e. molar pregnancy or hydrops; disorders that typically have large bulky placentas).1

Although the disease manifests with hypertension and proteinuria it actually represents a systemic maternal inflammatory disorder with endothelial dysfunction.4-6

Pre-eclampsia also seems to be more common in patients who have microvascular disease or metabolic disorders that predispose to endothelial dysfunction (hypertension, diabetes mellitus, obesity and renal disease).7,8

1.1. Definition of Hypertensive disorders in pregnancy.

In 2000 the International Society for the Study of Hypertension in Pregnancy (ISSHP) produced a paper to try to establish a uniform classification system and diagnostic criteria for pre-eclampsia.2 Hypertensive disorders are classified as: Chronic hypertension, Pre-eclampsia-eclampsia, Pre-eclampsia superimposed on chronic hypertension.1,2 These were defined as:

**Chronic hypertension**: hypertension diagnosed prior to 20 weeks gestation. This also includes *de novo* hypertension that develops late in pregnancy and does not resolve postpartum.

**Pre-eclampsia-eclampsia**: hypertension and proteinuria that develops after 20 weeks gestation and normalizes post partum. Hypertension is defined as blood pressure >140/90mmHg and proteinuria as >300mg/day. If proteinuria is absent, pre-eclampsia can still be suspected if one of the following is present: renal insufficiency (creatinine ≥ 90µmmol/L or oliguria), liver disease (raised transaminases and/or severe right upper quadrant pain), hematological disturbances (thrombocytopenia, DIC, hemolysis), fetal growth restriction and neurological features [convulsions (eclampsia), hyperreflexia with clonus, severe headaches with hyperreflexia, persistent visual disturbances].1

**Pre-eclampsia superimposed on chronic hypertension**: development of features of pre-eclampsia (new onset proteinuria with the loss of blood pressure control) in a woman with pre-existing hypertension. The hypertension persists postpartum.

1.2. Problem(s) with Pre-eclampsia Diagnostic Criteria

Although there has been a tremendous amount of work put into trying to standardize the diagnostic criteria for pre-eclampsia, it is however acknowledged by the ISSHP that the criteria are somewhat ‘restrictive’ in that a woman with a blood pressure of 139/89mmHg does not qualify for the diagnosis, nor does proteinuria of 290mg/day.2 It would seem as if these cut off criteria are too restrictive.
The diagnostic criteria are also deficient in identifying the clinical phase of pre-eclampsia (i.e. the symptomatic maternal phase of the disease), and can miss the woman in the latent phase of the disease. Evaluation of raised blood pressure also is not sufficient to define the entire haemodynamic abnormality that occurs in the setting of pre-eclampsia.

As pre-eclampsia is an ominous disorder worldwide with a high mortality and morbidity rate in South Africa, it is recommended that it is better to over-diagnose this condition when trying to differentiate it from gestational hypertension as this will lead to better monitoring and more aggressive and early therapy.

There is also evidence that women with early onset pre-eclampsia (before 34 weeks of gestation) are particularly at a higher risk of developing cardiovascular disease later in life. The rationale behind this is that both pre-eclampsia and atherosclerosis share risk factors for systemic inflammation and endothelial dysfunction. These factors include: obesity, dyslipidemia, diabetes mellitus and insulin resistance, hypertension, endothelial dysfunction and a family history. Another theory also suggests that in pre-eclampsia maternal vessels also suffer damage which promotes further maternal atherosclerosis.

2. PATHOGENESIS of PRE-ECLAMPSIA

The pathogenesis of pre-eclampsia has always been ascribed to the incomplete or superficial invasion of the trophoblast into the endometrium/myometrium with incomplete “physiological” transformation of the spiral arterioles with subsequent inadequate placental perfusion. The cause for this poor trophoblastic invasion is as yet unknown. The resultant placental ischemia-reperfusion injury causes a release of biological active factors (molecules and particles) into the maternal vascular circulatory system, which results in excessive oxidative and inflammatory stress in the maternal circulation with endothelial dysfunction and manifests with the clinical phase of the disease.

In the first 10 weeks of gestation the embryo is fed by the endometrial glands and lives in a low oxygen environment. This relative hypoxia induces the cytotrophoblast to invade the decidua and myometrium and remodel the spiral arterioles. Initially the cytotrophoblast occludes the arterioles and reduces the blood supply to the embryo as the high blood pressure would induce a termination of the conceptus. Within 8-18 weeks however the spiral arterioles are remodelled with loss of their muscular walls and endothelial lining and their diameter increases five to ten fold. This modification extends up to the inner third of the myometrium. This change does not occur normally in pre-eclampsia, where some vessels are only modestly (incompletely) remodelled while others are not remodelled at all (resulting in the placental lesion called decidual vasculopathy). These changes can be found histologically in roughly half the placentas examined as frequently only the superficial decidualised endometrium is attached to the placenta and is available for examination. It is predominantly the endovascular invasion that is affected in pre-eclampsia but not the interstitial invasion of the trophoblast.

The pathological lesion of decidual vasculopathy, the most characteristic variant of which is acute atherosis, is however not limited to pre-eclampsia and can
also be encountered in placentas’ from idiopathic fetal growth restricted infants, in cases of hypertension without proteinuria and autoimmune diseases, particularly lupus erythematosus, scleroderma and anti-phospholipid antibody syndrome and rarely even in placentas from normal pregnancy. At this stage it is not understood why some placentas with incompletely remodelled spiral arterioles develop pre-eclampsia and others do not.

These vascular changes lead to ischemia-reperfusion injury to the placenta parenchyma with resultant placental oxidative and endoplasmic reticulum stress. This leads to the release of inflammatory factors into the maternal circulation, which include amongst others trophoblast derived pro- and anti-angiogenic proteins, trophoblast-derived micro- and nanovesicles and other proinflammatory products as well as activating AT1-receptor antibodies.

3. PRE-ECLAMPSIA: two variants of the disease

Clinically pre-eclampsia can be distinguished into two patterns of presentation, an early onset form (<34 weeks gestation) and a late onset form, >34 weeks gestation.

Early onset pre-eclampsia, which represents 5-20% of all pre-eclampsia cases, is regarded as the more severe form of the disease, while late onset, which is the more common form, appears to be a milder form of the disease with a better maternal and perinatal outcome.

The early form is characterized by an early onset of sympathetic dominance in the maternal cardiovascular system with elevated circulation markers of endothelial dysfunction, inadequate trophoblast invasion and transformation of spiral arterioles, abnormal Doppler waveforms of the uterine arteries with notching. This form more commonly results in elective pre-term birth and birth of small for gestational age infants.

The late onset pre-eclampsia is more commonly superimposed upon a pre-existing maternal condition associated with increased cardiovascular or metabolic risk of endothelial dysfunction (e.g. obesity, insulin resistance, diabetes mellitus, chronic hypertension and renal disease). The infants born to these mothers have a normal or slight higher birth weight than those of gestational age matched controls. The Doppler waveforms show much milder abnormalities in the uterine arteries than in the early onset form.

Pathologically the early onset form shows more vascular lesions (decidual vasculopathy) with more placental infarction, accelerated parenchymal maturation and smaller placentas.

Haemodynamically the early onset form shows a hypovolemic state with decreased cardiac output and increased total peripheral vascular resistance and on echocardiography the left ventricle size appears smaller. This denotes an underfilling state with a pressure overload. Comparatively the late onset form shows a hyperdynamic state with increased cardiac output and decreased total peripheral vascular resistance. On echocardiography the left ventricle shows a large diameter measurement with a higher stroke volume. This denotes an overfilling state without a pressure overload.
4. PATHOLOGICAL CHANGES in the PLACENTA of PRE-ECLAMPSIA

4.1. Macroscopic Morphology

Placentas of pre-eclampsia tend to have a more oval / oblong shape (Figure 1) rather than the circular shape (Figure 2) of normal placentas. This is due to the irregular trophoblast invasion of the decidua with the resultant ischemia and oxidative stress that results in villous necrosis and this leads to the irregular shape of the placenta. Also due to the above process, placentas from pre-eclampsia tend to be somewhat thicker than their gestational age matched controls.7

The umbilical cords tend to be thin, <1cm in diameter as compared to gestational age matched controls.12

4.2. Weight

The trimmed weight of the placentas tends to be lower than gestational age matched controls. This is particularly true for the more severe form, the early onset pre-eclampsia. Typically cases display placental weight below the 10th percentile for gestational age.7,20
4.3. Histological features:

The histological features associated with pre-eclampsia are similar to those seen in placentas from pregnancies with early intrauterine growth retardation (IUGR), namely those of reduced placental perfusion. These following changes are more prominently expressed in early onset pre-eclampsia than in the late onset group.

**Parenchymal features** include accelerated maturation of the villi (when compared with that expected for gestational age), distal villous hypoplasia (these are small, slender villi which show reduced branching with very small terminal villi which lie in an expanded intervillous space and contain aggregated clusters of syncytiotrophoblastic nuclei)(Figure 3), increased syncytial knots (Figure 4), increased intervillous space and placental infarction (multiple and diffuse)(Figure 5).12

![Histological features of distal villous hypoplasia (H&E 100x).](image-url)
Figure 4: Parenchyma with increased syncytial knots (H&E 100x).

Figure 5: Placental parenchymal infarction right of centre with viable parenchyma left of centre (H&E 40x).
**Decidual vasculopathy** includes incomplete vascular remodelling of spiral arterioles and acute atherosis (fibrinoid necrosis). Incompletely remodelled arterioles retain their muscular collars (Figure 6) and predispose to spontaneous vasoconstriction and intermittent perfusion of the intervillous space, generating ischaemia-reperfusion injury.  

![Image of decidual vasculopathy](image_url)

**Figure 6**: Incomplete vascular remodelling of spiral arterioles, with the spiral arterioles maintaining their muscular coats and with perivascular lymphocytic infiltration (H&E 200x).

The lesion of acute atherosis lesions have lipid-laden foamy cells (macrophages) being deposited beneath the endothelium with fibrinoid necrosis of the wall and occasionally leukocyte infiltration of the affected vessel (Figure 7). The foamy cells are CD68 positive macrophages. This lesion does not affect all spiral arterioles in the placenta, and seems to affect the decidual part of the spiral arteriole in particular. It does not affect the entire length of the arteriole and occasionally only part of the circumference is affected. It is thought that atherosis only is present in 20-40% of pre-eclamptic placentas.
This change causes subsequent narrowing of the lumen, thrombosis of the vessels and downstream ischemia and infarction. These lesions are restricted to the uterus and do not effect systemic maternal vessels in pre-eclampsia.

It is currently thought that the lesion of acute atherosis, which has a close histological resemblance to the early lesion of atherosclerosis, represents an inflammatory lesion rather than a lesion caused/induced by hypertension.

**Placental abruption** is also associated with pre-eclampsia, occurring with a higher frequency than in healthy patients. There does not appear to be a difference in the incidence of abruptio placenta between patients with pre-eclampsia and in placentas from patients with IUGR without hypertension. The risk of placental abruption is similar in early and late onset pre-eclampsia. However there is a statistical difference in the occurrence of abruption between late onset pre-eclampsia and gestational age matched controls.

Pathologically an abruption is seen as a retroplacental blood clot covering at least 15% of the disk surface area. Histologically the appearance of the haematoma varies with its duration. If an abruptio happens acutely and the placenta is delivered relatively quickly, there may be no histological evidence seen at all. If some time passes between development of the retroplacental haematoma and delivery then the haematoma can be seen to either to:
i) Dissect into the overlying parenchyma (this is due to pressure of the blood clot forcing it’s way into the placental parenchyma or

ii) Cause overlying parenchymal infarction, due to cessation of maternal blood flow to that part of the parenchyma (Figure 8).

iii) An retroplacental haematoma has been present for a considerable period of time (days to weeks) can cause an accumulation of hemosiderin laden macrophages to be present in the decidua or the haematoma can leach into the amnionic cavity causing hemosiderin laden macrophages to be seen in the chorionic plate of the placenta, so called chronic abruption. Intravillous haemorrhage is also a characteristic feature of an abruptio placenta.23

These pathological findings that are encountered in most pre-eclampsia cases are not always present. In fact the placentas can appear grossly and histological appropriate for gestational age, even in cases with associated eclampsia.12 Therefore although these findings can be confirmatory/supportive of pre-eclampsia, their absence cannot be used to exclude the diagnosis.12

Figure 8: Retroplacental haematoma with early infarction of the basal villi (H&E 40x)

5. SOLUBLE FACTORS of PRE-ECLAMPSIA

The syncytiotrophoblast in response to oxidative stress secretes a host of substances that affect the maternal circulatory system. Important substances are soluble vascular endothelial growth factor receptor-1 (sVEGF or sFLT-1), soluble endoglin (sENG) and placental growth factor (PIGF).4 A healthy placenta secretes
a balanced amount of sFLT which leads to normal levels of provasodilatory and anticoagulant factors being available for binding to sFLT1 receptors on endothelial cells. In ischaemic placentas there is more sFLT and sENG secreted with less PIGF which bind to more circulatory factors leaving less of these factors to bind to endothelial cells, leading to systemic maternal endothelial dysfunction.4,23

The use of pro- and anti-angiogenic factors has mainly been studied in early pre-eclampsia.

6. IMMUNOHISTOCHEMICAL PROPERTIES of INVADING TROPHOBLAST

The invasive trophoblast is highly dependant on the expression of appropriate adhesion molecules on its cell surface for adequate invasion of the decidua and vascular tissues as well as for vascular remodelling.24 Failure to express adequate cell surface markers is indicative of a deficit in the invasive abilities of the intermediate cytotrophoblast.24 Zhou et al demonstrated that the cytotrophoblast, in pre-eclampsia, lacks expression of such adhesion markers, including VCAM-1, PECAM-1, VE-cadherin and αβ3 integrins.25,26 Q Liu et al investigated the expression of CD146 (Mel-CAM) on invasive cytotrophoblast and found a decreased expression of this cell surface molecule in pre-eclamptic placentas when compared to normal controls.24 CD146, also known as melanoma cell adhesion molecule (Mel-CAM) is a member of the immunoglobulin gene superfamily.27 It is detected in only a limited number of normal tissues and tumours, namely melanoma, vascular endothelial cells, smooth muscle cells, implantation site intermediate trophoblast and activated T lymphocytes.24,27 In melanoma its expression level is associated with invasive and metastatic potential of the melanoma cells.28

Furthermore CD146 takes part in interendothelial interactions as a calcium independent cell adhesion molecule. Participating in the outside-in signalling pathway that is associated with endothelial cell migration.29,30 CD146 is expressed on invasive intermediate trophoblast, but not on cytotrophoblast or syncytiotrophoblast.24 It appears to regulate the migration of intermediate trophoblast on smooth muscle.31 Liu Q et al investigated whether the expression of CD146 would be the same in the intermediate trophoblast in placenta from normal and pre-eclamptic placentas.24 They found that in normal placentas, CD146 was restricted to the endometrial vessels with the decidual cells exhibiting some background staining on the maternal side.24 On the fetal side CD146 stained the intermediate trophoblast in the uterine decidua as well as in the trophoblastic columns of anchoring villi. The staining was ‘strong’ and uniform, independent of the depth of trophoblastic invasion. CD146 was not detected in the syncytiotrophoblast or cytotrophoblast of the anchoring or floating villi.24 This contrasted with pre-eclamptic placentas where the staining was remarkably reduced or undetectable in the invasive trophoblast in the decidua as well as the trophoblastic columns from anchoring villi, basal plate and remodelled maternal vessels.24
7. HYPOTHESIS (aims & objectives)

Based on the work performed by Liu Q et al, it was decided to perform similar research on pre-eclamptic placentas. Van der Merwe et al performed a study at Tygerberg Hospital comparing early and late onset pre-eclamptic placental morphology to appropriately gestation-matched controls to establish any differences. It was decided to use this cohort of placentas for the following further research study.

7.1. CD146 expression in intermediate trophoblast in formalin fixed placentas in pre-eclamptic placentas differs when compared with gestational age matched controls.

Liu Q et al performed a similar study, but used fresh frozen tissue from placental bed and performed immunofluorescent staining, using dual labelling with CD146 and a polyclonal Cytokeratin stain. It is postulated that CD146 expression, on formalin fixed tissue, will be expressed less (weaker expression) on intermediate trophoblast in the placentas from pre-eclampsia as compared to gestational aged matched controls.

7.2. CD146 expression in intermediate trophoblast in placentas from early onset differs when compared to expression in late onset pre-eclampsia.

Liu Q et al showed that in pre-eclampsia the intermediate trophoblast shows a markedly diminished expression of CD146 when compared to intermediate trophoblast in normal placentas. However they did not compare early to late onset pre-eclampsia. Since the clinical presentation is markedly different between early and late onset pre-eclampsia, it is thought that there should be a marked difference in CD146 expression in the intermediate trophoblast in the placentas in the two forms. It is hence postulated that the intermediate, invading trophoblast in the decidua from early onset pre-eclampsia placentas will express CD146 immunostain less as compared to the late onset pre-eclampsia placenta group.

8. METHODOLOGY and MATERIALS.

8.1. Study Setting and Ethics

This study builds on the completed and published thesis of Dr JL van der Merwe. The current PI was a co-investigator in that study.
Placentas were collected as part of a case control study performed at Tygerberg Hospital, a secondary and tertiary referral academic hospital, in the Western Cape Province of South Africa.

The study recruited patients over a 7-month period. There were 4 groups of patients, each group having 25 placentas, namely early and late onset pre-eclamptic pregnant women as well as control placentas matched for gestational age for each of the two pre-eclamptic groups.

Written informed consent was obtained in writing from each of the study patients. Ethics consent was obtained from the Stellenbosch University Ethics Committee, no: N06/09/185.

Ethics extension of this current study was obtained from Stellenbosch University and ethics consent was obtained from the University of Cape Town for the current study, no 634/2013.

This study obtained funding from the NHLS Trust Fund, Grant number: Grant004_94246.

**Exclusion Criteria:**

Teenage pregnancies (<18 years of age), intrauterine deaths, neonatal deaths, multiple pregnancies and other subtypes of gestational hypertensive disorders (excluding pre-eclampsia).

Current or previous maternal diseases, including diabetes mellitus and autoimmune disease were also excluded.

Retroviral disease with a CD4 count of less than 200.

Woman with known idiopathic intra-uterine growth restriction were excluded.

**Control Group Placentas:**

Early control placentas were collected from women with idiopathic preterm labour.
Late control placentas were collected from uncomplicated normal vaginal deliveries at term or from normal vaginal deliveries after a previous Caesarean Section.

8.2. **Processing of the Placentas.**
All collected placentas were fixed in 10% neutrally buffered formalin for a minimum period of 24 hours prior to sampling (as per our laboratories standard operating procedure).
Placentas were described macroscopically and sectioned. One cassette contained two sections of the umbilical cord and a roll of the placental membranes.
Three sections of the parenchyma were taken in which a minimum of one section contained the decidual plate and one contained the fetal surface of the placenta.
After sectioning, the sections where processed as per routine practice for surgical specimens and embedded in paraffin wax blocks. 3-5µm sections where cut, placed on glass slides and stained with haematoxylin and eosin (H&E) stain and cover slipped.
All the study cases were assigned a study number from 1 to 100 in the previous study. No patient particulars were used or were known in the current study. Only after the interpretation of the immunostaining were the cases identified as to their study group (early or late control or early or late pre-eclampsia).

8.3. **Immunohistochemical Staining.**
One section of the placental parenchyma that included the decidual surface was selected for immunohistochemical analysis for each of the two-immunohistochemical stains. All immunostains where performed on formalin fixed, paraffin embedded material. 3-5µm sections were cut from each paraffin wax block for performance of the immunohistochemical stains.
**CD146 (MCAM / Mel-CAM) immunohistochemical antibody, clone N1238,** was used from Novocastra, Leica. This is a mouse monoclonal antibody and positive staining shows a membranous staining pattern. The cytoplasm and nucleus do not stain. A dilution of 1:50 was used with heat induced antigen retrieval (HIER) for 20min.
**Anti-Human Pan Cytokeratin**, clone MNF116, monoclonal mouse antibody from DAKO was used. A dilution of 1 in 500 was used with enzyme antigen retrieval technique for 10min. This stains the cytokeratin intermediate filaments of the cells and hence it is a cytoplasmic stain.
Both immunohistochemical stains were performed on the Leica Vision Biosystems Bond Immunohistochemical automated stainer. The stains were performed according the standard operating procedure (SOP) that has been optimized for these antibodies in our laboratory. The immunostaining was performed in batches. All the cases were stained prior to evaluation/analysis of the material.

8.4. **Analysis and Interpretation**
The antibodies CD146 and MNF were used to identify the intermediate trophoblast in the decidual plate.
Once the CD146 positive intermediate trophoblast was identified, an analysis was performed by counting the amount of intermediate trophoblast in the decidua. It was recorded if the intermediate trophoblast was diffusely present or in nodules of either <20 or >20 cells per nodule. The presence or absence of decidual giant cells was recorded.

An objective analysis using the Nikon Eclipse TE2000-S microscopy and NIS-Element basic research image analysis software was used to determine the amount of intermediate trophoblast in the decidua. First the surface area of the decidua was calculated using the software. Then the CD146 IT cells were selected and the total surface area of the IT staining with CD146 calculated. A ratio of IT: total decidual surface area was calculated.

8.5. Statistical Analysis
The statistics unit at Stellenbosch University was consulted to determine if there was any significant difference in the amount of intermediate trophoblast present in the decidua in the pre-eclampsia group vs. the control group. Additionally the staining of intermediate trophoblast in the early and late onset pre-eclampsia groups was analysed.

9. RESULTS

9.1. Immunohistochemical staining.
MNF116 stained the intermediate trophoblast in the decidua as well as the cyto- and syncytiotrophoblast that surround the villi. This staining was strong, continuous with a clean background (Figure 10).
Figure 10: MNF116 staining of the IT in the decidua and the syncytiotrophoblast around the villi (200x).

CD146 showed a membranous staining pattern in the intermediate trophoblast in the decidua and also stained the fetal capillaries in the villi (Figure 11).

Figure 11: CD146 shows staining of IT in the decidua with endothelial cells staining of the fetal capillaries (100x).

The CD146 stain also showed a considerable amount of granular, non-specific background staining (Figure 12). Additionally it stained a considerable number of decidual cells positive, though less intensely (Figure 13).
Figure 12: CD146 showing the granular background staining (yellow arrow) (200x).

Figure 13: CD146 IHC stain with weak staining of decidual cells (blue arrows) while the IT stained much more intensely (green arrows) (200x).
Initially it was thought that it would be possible to distinguish the non-specific decidual staining from the true intermediate trophoblast. However, it quickly became apparent that this task would be impossible to resolve without having dual immunohistochemical staining for MNF116 and CD146 performed on the same slide. The decidua contains many cells with non-specific staining.


It was not possible to perform an objective analysis of the amounts of intermediate trophoblast in the decidua that stained with both CD146 and MNF116 immunostains as was initially outlined in the method section, as this was deemed too subjective and inaccurate. There were also more cells present in the decidua than initially anticipated. It was decided by the PI and supervisors (HW and CAW) that this would be too subjective an analysis and would not be a true representation of the distribution of the intermediate trophoblast in the decidua. This form of analysis was thus abandoned.

9.3. Analysis using Nikon software.

The Nikon Eclipse TE 2000-S microscope NIS – Elements Basic Research image software were used to determine the surface area of the decidua. The problem that was encountered from the very beginning was with the selection of the intermediate trophoblast. CD146 is a membranous stain and not a cytoplasmic stain; hence it outlines the cells but does not fill in the cytoplasm (Figure 14). There is also a considerable amount of background staining and the decidual cells stain weakly. The software had to be set so that it would ‘fill in’ (colour in the cytoplasm between the brown membranous staining) in the intermediate trophoblast, but disregards the background staining and the staining of the decidual cells.
In order to achieve this, the threshold (the amount of pixels/ intensity of the staining) had to be increased in an attempt to ‘unselect’ the falsely staining decidual cells. This is not as simple as it sounds, as this process involves decreasing the amount of pixels that would be in use (more pixels, result in darker staining). In doing this one loses definition of the tissues. This also leads to a decrease in the intensity of the intermediate trophoblast staining, making the borders less distinct. Some of the less intensely stained intermediate trophoblast also became unselected in the process. Figure 15 illustrates this feature and is taken at a magnification of 200x. At this magnification it is already a problem, however when shown at 100x magnification this problem became even more severe, Figure 16.
Figure 15: CD146 stain - note the difference in intensity of the circumferential membranous staining of the intermediate trophoblast in this group (200x).

Figure 16: CD146 stain of the same region but at 100 x magnifications. Notice the difficulty in selecting cells with the same staining intensity.
Another issue that also arose was that of background staining, which also turned out to be a major problem as the software selected all the background staining as part of the intermediate trophoblast staining. The background staining had the same intensity of staining as the intermediate trophoblast, hence it became impossible to unselect the background staining. The only way to unselect the background staining was to go on a very high magnification of 200x or preferably 400x magnification. The same issue arose with the high magnification as that discussed above.

To perform analysis of the intermediate trophoblast surface area on a low magnification (20x or 40x) was not possible as the software either selected too much or too little intermediate trophoblast. Figure 17 illustrates CD146 staining of a decidual area. How many intermediate cells are there? The cells seem to staining strongly, however when compared to Figure 18, which illustrates MNF116 staining of the same area, there appears to be much less intermediate trophoblast than what was originally thought to be present.

Figure 17: CD146 staining a decidual area (200x)
Figure 18: MNF stain of the same areas as in Figure 17, illustrating much less IT than originally thought to be present (200x).

This would have resulted in an over- or underestimation of the trophoblast surface area. Since dual immunostaining was not performed, the problem of over- or underestimation of the amount of intermediate trophoblast could not be circumvented.

The 40x magnification does not even bring all the decidual surface area into view either; this is illustrated in Figure 19, where in this example it took 9 photos at 40x magnification to visualize the entire decidua. To overcome this problem one would have to start using magnifications of 200x or 400x, however in this setting one only looks at a small area of decidua and cells and performing this analysis would be not be representative of the entire surface area. To analyse the entire decidua at a magnification of 200x or 400x would not be possible as it would have taken thousands of photographs and man hours to analyse all the cases in this study and hence this was not a viable solution.

It was then thought to take 3 random photos of the decidua and to work out the surface area of the decidua and that of the intermediate trophoblast and to then get the ratio. However, there was no guidance in the literature as to which areas to select. Does one select hypercellular or hypocellular areas? Or must they be completely random? This was again thought, by the PI and supervisors, that this would not give an accurate representation of the intermediate trophoblast to decidua surface area ratio.
Figure 19: Nine photographs on one placental decidua taken at a 40x magnification
Help was sought from the Nikon representative for the Western Cape region. Troubleshooting was performed, but no acceptable improvement to the situation could be obtained. The software in use had not been optimized for the chromogenic immunohistochemical staining in our setting. The background immunohistochemical staining and the haematoxylin background nuclear and membranous staining turned out to impair the software. This program runs advanced protocols and just trying to understand this program was difficult in itself as it is very complicated and one has to work with this software on a daily basis to become proficient. Different methods were attempted in this program to circumvent the stumbling blocks that were encountered in trying to perform image analysis on our specimens. The steps that had to be followed became very complicated, and time consuming without additional advantages obtained.

In areas the decidual outlines were very irregular, with areas of the decidua absent and in other areas the decidua tracked/extended into the parenchyma as a septum. It also became unclear where the cut off point of the decidua was in these septae. Hence every time a decidual surface area was calculated it produced different measurements.

This was discussed in depth with the supervisors (HW and CAW) of this study and it was determined, that at this stage with the current tools at our disposal, it would not be possible to optimize this technique to make an objective, accurate and reproducible assessment/analysis of the differences in staining between the pre-eclamptic placentas and gestational age matched controls, nor between the early and late onset pre-eclamptic placentas. This study with the immunohistochemical stains for CD146 and MNF116, would have to be postponed till a future date when a more sensitive and easier to operate image analysis software was available in order to perform this analysis and prove our hypothesis.

10. DISCUSSION

Pre-eclampsia is a disease that occurs in 5-8% of pregnancies and carries a high morbidity and mortality rate for both the mother and baby, in both first and third world countries. Pregnant mothers with pre-eclampsia are predisposed to abruptio placenta, disseminated intravascular coagulation, cerebral haemorrhage, liver and kidney failure (dysfunction). The fetus is predisposed to intrauterine growth retardation, premature delivery, hypoxic damage and intrauterine or neonatal death.

At the basis of this disease is incomplete intermediate trophoblast invasion into the underlying decidua with incomplete remodelling of the spiral arterioles. This process starts early in pregnancy and continues till 20 weeks gestation. This leads to ischemic-reperfusion injuries of the placental parenchyma with resultant secretion of systemic substances into the maternal circulation that leads to the maternal phase of the disease. This has also become known as the two-stage model
of pre-eclampsia with the first stage comprising the pre-clinical placental injury/insult and the second stage referring to the clinical manifestation of the disease, that we recognize as pre-eclampsia.\textsuperscript{3,8} At the moment the diagnosis of pre-eclampsia is made at the clinical (second) stage of the disease when the clinical features of pre-eclampsia manifest.\textsuperscript{2} New research is starting to emerge on biomarkers to try and recognize/diagnose the presence of disease in the first (preclinical) stage of the disease. The forerunners of these are sFLT-1 and plasma ascorbate levels.\textsuperscript{7} sFLT-1 is a downstream marker of HIF (hypoxia response elements) activation, which is elevated, in pre-eclamptic woman with and without fetal growth restriction. Women with fetal growth restriction but without pre-eclampsia do not have elevated levels of HIF-1α or HIF-2α, nor the proteins from their downstream transcription pathway.\textsuperscript{7}

Invasion by intermediate trophoblast into the decidua is mediated by a complex network of soluble autocrine and paracrine factors, signalling pathways and regulatory transcription factors.\textsuperscript{13} Although little is known as yet about all these factors, it appears the epidermal growth factor (EGF), vascular endothelial growth factor (vEGF) and various cytokines (e.g. IL-11) that are secreted by the endometrial glands play a role in controlling this process in the early stages.\textsuperscript{13}

The display of adhesion molecules on surface of intermediate trophoblast also plays a critical role, as the lack of such molecules is indicative of a deficit in the ability of intermediate trophoblast to successfully invade the underlying decidua and spiral arterioles. Some of these surface molecules include, αvβ3 integrins, VE-cadherin, VCAM-1 and PECAM-1.\textsuperscript{24} CD146 is also a surface adhesion molecule that was demonstrated to be strongly expressed on intermediate trophoblast.\textsuperscript{31} Liu \textit{et al}\textsuperscript{24} decided to investigate the expression of CD146 in pre-eclamptic placentas. They took samples of decidua from the placental bed (size of sample not specified), froze the specimen and then performed dual labelled immunofluorescence for CD146 and an anti-cytokeratin polyclonal antibody immunostaining on this tissue. In this study they observed that the intermediate trophoblast demonstrated remarkably reduced to undetectable expression of CD146 when compared to the control group.\textsuperscript{15} This current study tried to determine if we could replicate these results on formalin fixed placental tissue and to determine if there would be a difference in CD146 expression between early and late onset pre-eclampsia as these two manifestation of pre-eclampsia seem to be two different diseases. Since early and late onset pre-eclampsia manifest vastly different clinical expressions\textsuperscript{3,8,20}, as described previously, we wondered if the intermediate trophoblast in these two diseases would express CD146 differently on its cell surface.

Our study differed from Liu Q \textit{et al}, in a few aspects, nl:

We used formalin fixed tissue while they used fresh frozen placental bed tissue.

We used immunohistochemical staining for CD146, Novocastra, mouse monoclonal antibody, clone N1238; while Liu Q used CD146, Santa Cruz Cooperation, CA, USA, mouse monoclonal antibody, clone AA98.
We used Anti-Human Cytokeratin, Dako, monoclonal mouse antibody, clone MNF116. Liu Q used Anti-Human Cytokeratin, Santa Cruz Cooperation, CA, USA, rabbit polyclonal antibody.

We used single immunostaining per slide; Liu Q used dual immunostaining per slide.

We stained the entire decidual surface and tried to analyse it, while Liu Q, though it is not formally stated in their article, appear to have only used a small biopsy of the decidual bed.

We wanted to determine the extent of CD146 expression in terms of volume while Liu Q used intensity of staining expression.

This current study did not have a favourable outcome due to the fact that the software could not be used to get a reliable, repeatable and accurate measurement of the ratio between surface area of the decidual surface and the volume of intermediate trophoblast present in the decidua. The variation in the intensity of staining in the CD146 in the intermediate trophoblast on the same slide complicated matters. This was further complicated by the non-specific, granular background staining and the staining of the decidual cells. It was difficult to move from the MNF slide to the CD146 slide to ensure the cells of interest were intermediate trophoblast cells and not decidual cells. Dual labelling immunostaining on the same slide would have circumvented this problem, but was unavailable to us.

This software program does not seem to work as well on chromogenic immunohistochemical staining as it does on fluorescent immunofluorescence stained slides. The immunofluorescence has a much crisper staining quality to it and is on a black background, which helps with differentiation. This seems to make the software function much better. Also the majority of immunofluorescence analysis work performed on the system (from the PI investigation) appears to be on cell cultures which include only a few cells per high field (200-400x) magnification that are being evaluated at a time. This makes it much easier to analyze the staining.

This latter point is important, as we thought that the more tissue we would have to analyse the more accurate we could be. However with the image analysis software that we used, as we were not scanning the entire slide, this was a problem as we were only looking at a restricted part of the slide and not the entire decidual surface. Again this was an unforeseen problem. In this regard had we chosen a smaller surface to stain and analyse the simpler it would have been as there would have been less variation in staining and less background staining. The entire surface could have been analysed at a much higher magnification where the analysis could have worked. Once one is forced to choose areas to analyse it becomes difficult, as there is bias in the areas one chooses.

11. CONCLUSION

The way forward is in using immunoflorescent staining with dual labelling for CD146 and MNF116 antibodies. This would ensure correct identification of the intermediate trophoblast. The analysis of the IF staining is easier as there is less background staining with more contrast as there is a black background. This makes it easier for the software to perform its analysis.
Using less tissue (decidua) will also eliminate the tremendous amount of tissue that is available for analysis. Analysing this amount of decidua as in this current study becomes a very time consuming process.

Additionally, if future attempts are to be made to try and determine the difference off expression (quantitative and qualitative) of immunohistochemical markers on IT; this should not be undertaken unless one has a good, solid knowhow of how to operate/manipulate the image analysis software that will be used for analysis.

12. REFERENCES


