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Maastricht University

Faculteit Geneeskunde en Levenswetenschappen
School voor Levenswetenschappen
master in de biomedische wetenschappen

Masterthesis

The inter-observer variation for a new method to score placental maturity

Nadine Ndarurinze

Scriptie ingediend tot het behalen van de graad van master in de biomedische wetenschappen, afstudeerrichting klinische moleculaire wetenschappen

PROMOTOR :
Dr. Salwan AL-NASIRY

COPROMOTOR :
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Acknowledgment

This thesis is one of the fruits of my research work by Maastricht university over the past nine months. During this internship that was part of my master studies of Biomedical Sciences, I gained hands-on experience in histological sections image analysis and set my mind to be critical but just. It was a dreamed occasion for me to be able to satisfy my ambition to learn how to be a scientific researcher. It was a privilege to contribute to the validation of a useful method for scoring placental maturation in normal and complicated pregnancies by preeclampsia or infection disease. The high prevalence of miscarriages with an unknown cause is a painful event in the life of young families and a great loss for the whole humanity. In this study comparing the placental maturity among different groups of preterm and term placenta, new insight in histopathologic features of the placental syndrome have been lightened.

I take this occasion to express my gratitude to my supervisors and daily mentor for supports and comprehension expressed to me allowing the successful accomplishment of my work. It was extremely challenging to combine the duty of a researcher and taking care of my lovely young children.

My first gratitude goes to Dr. Salwan Al-Nasiry who offered me to be a member of his research team and diligently supported me in writing my thesis. Great thanks to Dr. Ulrike von Rango and Phillipe Vangrieken for all the supports by morphological analysis of placental sections and by peer review my thesis. I express my great gratitude to Pr. Niels Hellings for the advises and encouragement during my master studies.

Last but not least, I thank my family and friends for their supports and comforts and dedicate this work to them.

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Abbreviations

CA: chorioamnionitis

CD: cluster of differentiation

DD: diffusion distance

FC: fetal capillaries

IUGR: intrauterine growth restriction

Nr: number

MUMC+: Maastricht University Medical Center+

LOA: limits of agreement

PE: preeclampsia

SD: standard deviation

TV: terminal villi

VSM(s): vascular syncytial membrane(s)

μm: micrometer

Sec: second

Abstract

The inter-observer variation for a new method to score placental maturity

Introduction: Adequate placental development assures optimal nutrition exchange between mother and fetus. Maturation of placental terminal villi (TV) is pre-requisite for optimal diffusional exchange and is thought to be disturbed in *preeclampsia* (PE), a dangerous hypertensive disorder of pregnancy and in *chorioamnionitis* (CA), a bacterial or viral infection of the fetal membranes. TV are characterized by numerous peripherally located fetal capillaries (FC) in close contact with the covering syncytiotrophoblast cell layer forming the so-called vascular syncytial membrane (VSM) which favorites diffusional exchange.

Objectives: The first objective was to confirm the earlier data on disturbed placental maturation in pregnancies complicated by PE and CA in comparison to idiopathic preterm (Control) and at term (Term) pregnancies. The second aim was to evaluate the inter-observer variation of 18 parameters used in the new method to score placental maturity of the first cohort. The third aim was to determine which of CD31 or CD34 endothelial markers used to stain the fetal capillaries (FC) give the best results by scoring placental maturity of the second cohort.

Materials and Methods: The first cohort includes placentas from PE, CA, Control and Term (n=6 each) and the second cohort comprise preterm control and preeclamptic placentas (n=10 each). The placentas stained for CD31 or CD34 and Hematoxylin/eosin, were scored using 18 parameters related to placental maturity such as the number, percentage, area and circumference of TV and FC; the lengths of VSM and the diffusion distance. The Bland–Altman test followed by a One-Sample t-test or Wilcoxon Signed-Rank test was used to calculate the inter-observer variation within the groups. The data analysis of the first and second cohort was performed with One-Way ANOVA or Kruskal-Wallis.

Results: For nearly all parameters data of the earlier analysis could be confirmed ($p > 0.05$ for 16 of 18 parameters in control group). However, a significant inter-observer variation was obtained by the following parameters: *percentage TV* ($P = 0.007$ for PE and $P = 0.05$ for CA); only for PE *the circumferences of TV* ($P = 0.04$), *the area of FC* ($P = 0.04$) and *the percentage area FC/TV (*$P = 0.02$); only for Term *the area FC* ($P = 0.02$) and *circumference FC* ($P = 0.004$). In general, the CD31 and CD34 vascular staining give similar results by scoring the parameters of placental maturity in studied groups.

Discussion & conclusion: For the analysis of the first cohort, the most data were reproducible in control group. Variation between raters in others studied groups revealed that some parameters are more sensitive to observer bias. The lower number of placenta studied per group in the first cohort did not allow detecting a significant difference in most of the scored parameters between the tested groups excepted for *the number of fetal capillaries* that were increased in preeclampsia compared to preterm control. Further, the CD31 and CD34 staining for FC give similar results by placental maturity analysis of the second cohort.

1 INTRODUCTION

1.1 Placenta development in normal pregnancy

1.1.1 Placental villous formation

The placenta, present only during pregnancy, is an essential organ of the body responsible for feto-maternal nutrients and gas exchange. Nevertheless, it is least comprehended due to its complex function and development. For the growth and birth of the healthy infant, normal placentation is necessary.

After fecundation of the ovum, the zygote develops into a blastocyst which is covered with cytotrophoblast cells (figure 1A). The blastocyst adherence to the uterine wall marks the earliest stages of placental development (1, 2). Further, the cytotrophoblast cells proliferate to form a double cells layer. Cytotrophoblast composes the inner layer whereas the outer layer becomes the syncytiotrophoblast constituted of fused cytotrophoblast cells (figure 1B). In the syncytiotrophoblast, lacunae develop and the syncytiotrophoblast extends sprouts giving rise to primary villi (figure 1C) (3). Subsequently, secondary villi are formed by arising of a stroma between primary villi (figure 1D). When the fetal capillaries appear in the villi, they are termed tertiary villi. (figure 1E).

In the meantime, under the lytic influence of the syncytiotrophoblast cells, erosion of the maternal tissues ensues aiding a deep invasion of the blastocyst and remodelling of the decidual spiral vessels by the extravillous trophoblasts (figure 1E) (4). From the second month of gestation, a complex process of villous differentiation occurs and a range of different villous surges like stem villi, mesenchymal villi, immature intermediate villi, mature intermediate villi and terminal villi (TV), all with a different caliber, structure, and function. TV are still covered with a thin layer of syncytiotrophoblast cells which participate in the transport of gas waste and nutrients and possess endocrine properties (figure 1F) (5, 6).

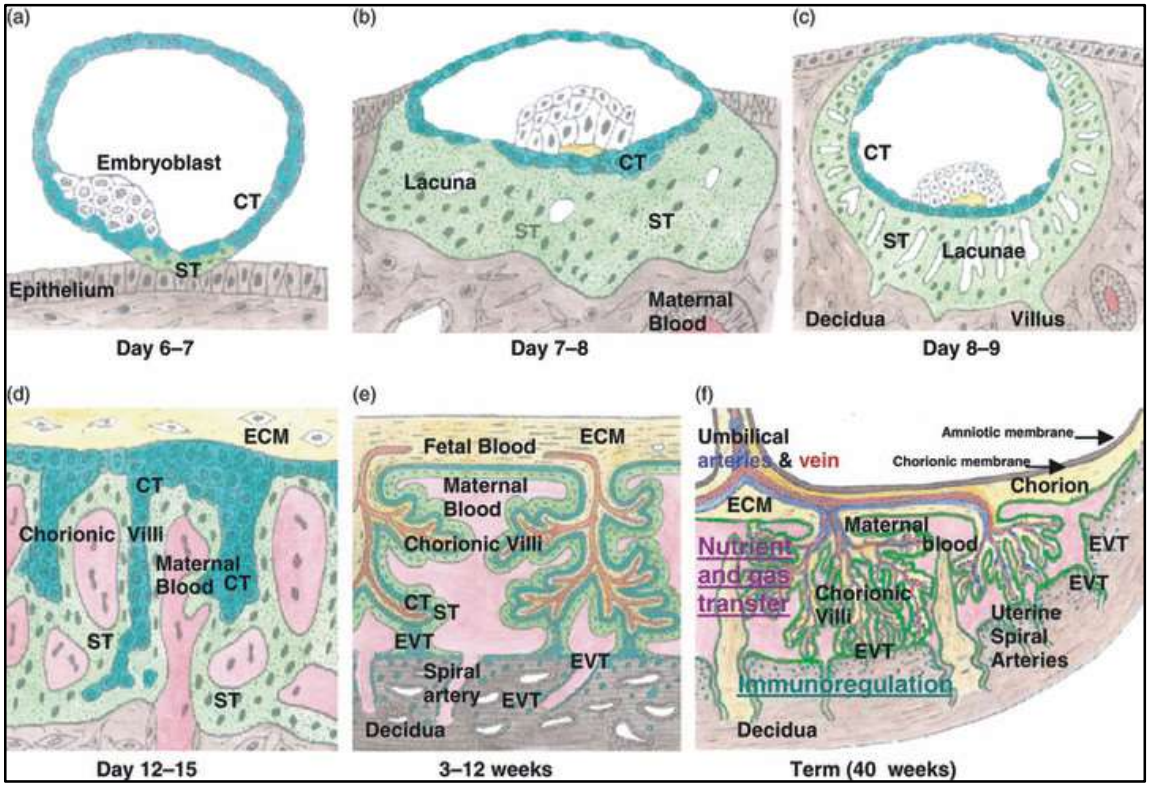

Figure 1 Early placental development

CT: cytotrophoblast, EVT: extravillous trophoblast, ECM: extracellular matrix, ST: syncytiotrophoblast.

By A, the blastocyst is surrounded by cytotrophoblast (CT, see green colored) layer, while the inner core consists of embryoblast. The blastocyst comes in contact with the uterine wall epithelium and at this site the cytotrophoblast fuse to form the syncytiotrophoblast (ST, light green colored). In B, the cytotrophoblast proliferates and forms a double layer: an inner layer of cytotrophoblast and an outer layer of syncytiotrophoblast where lacunae develop. By C, primary villi are formed by an extension of the syncytiotrophoblast into the decidua. Further, as shown D stroma develops in the primary villi, giving rise to secondary villi. By E, the fetal capillaries arise in the secondary villi (red colored) transforming them into tertiary villi. From the top of the villi, CT cells proliferate to form cell columns that migrate into the interstitial tissue in the decidua and into the spiral arteries where they integrate the endothelium. These are called extravillous trophoblasts (EVT). Reprinted from Kumpel *et al,* Vox Sang. 2012 Jan;102(1):2-12. doi: 10.1111/j.1423-0410.2011.01533.x (2).

1.1.2 **Placental villous vascularization**

In the first weeks after fetus implantation, the yolk sac and the uterine glands constitute the major source of nutrients for the fetus (7). However, fetal demands for gas exchange increases exponentially, hence, the formation of maternal and fetoplacental blood flow is crucial to ensure normal fetal development.

Placental villous differentiation is substantially accompanied by the development of fetal vessels within the villous. Fetoplacental vascularization originates from vasculogenesis and angiogenesis. During vasculogenesis, new blood vessels are created by differentiation and migration of haemangioblasts under stimulation of fibroblast growth factor (FGF2) and vascular endothelial growth factor (VEGF). This process starts in the first trimester of pregnancy when aggregates of haemangioblastic cells are formed, in which narrow intercellular spaces arise and fuse later on to form a lumen surrounded with flattened endothelial cells (figure 2) (8). Afterward, new blood vessels are created from pre-existing blood vessels by a process called angiogenesis, counting a branching and a non-branching form. Until the end of the second trimester, new blood vessels are formed by branching angiogenesis. From about the third trimester of gestation to term non-branching angiogenesis predominates.

In branching angiogenesis, new vessels are created by the generation of new branches and segments by a mechanism of sprouting from existing vessels or partitioning of a lumen of a vessel into two or more lumens. This process is stimulated by VEGF and its receptors VEGF-R1 (leading to endothelial tube formation) and VEGF-R2 (leading to endothelial cell proliferation). As pregnancy progresses placenta-like growth factor (PlGF), angiopoietins and other growth factors are upregulated to facilitate the expansion of placental vascular network by stimulating tube formation or non-branching angiogenesis. At the same time, expression of VEGF and VEGF-R2 decline steeply (9-11). Obviously, fetoplacental angiogenesis follows sequential-steps and is a process where abundant factors play a role and can modify it in various ways.

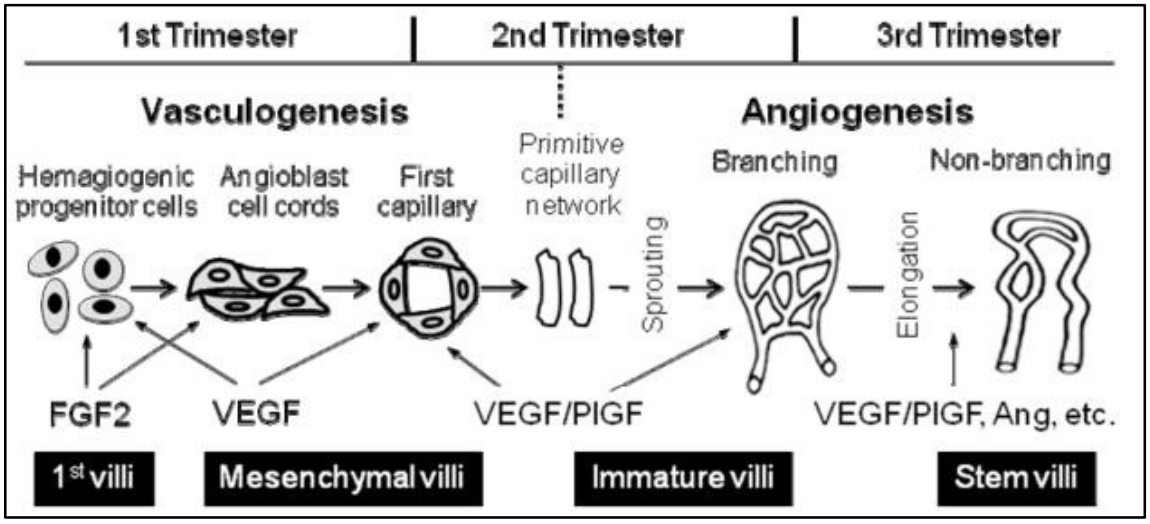

Figure 2 Branching angiogenesis and non- branching angiogenesis

Ang: angiopoietins, FGF2: fibroblast growth factor 2, PlGF: placental growth factor, VEGF: vascular endothelial growth factor. Placental vasculogenesis starts with the differentiation and migration of haemangioblasts under stimulation of FGF2 and VEGF during the first trimester of gestation. Further, haemangioblasts form cells cords and when a lumen arose within, the surrounding endothelial cells become flattened. By the mid-pregnancy, primitive capillary network is formed. Until the end of the second trimester, new blood vessels are formed by branching angiogenesis. In the third trimester of pregnancy, non-branching angiogenesis predominates. Reprinted from Dong-bao Chen *et al*, Microcirculation. 2014 Jan; 21(1): 15–25. doi: 10.1111/micc.12093 (9).

In the present study, the focus is directed towards the TV which are main sites of feto-maternal exchange. The high degree of vascularization, the thin syncytiotrophoblast cover, and the formation of vascular syncytial membranes (VSM) within the TV results in minimal maternofetal diffusion distance making it the most appropriate site for the diffusional exchange (figure 3) (5, 8). At this location, which is characteristic for TV, diffusion distance is nearly zero and thus the diffusion is most efficient.

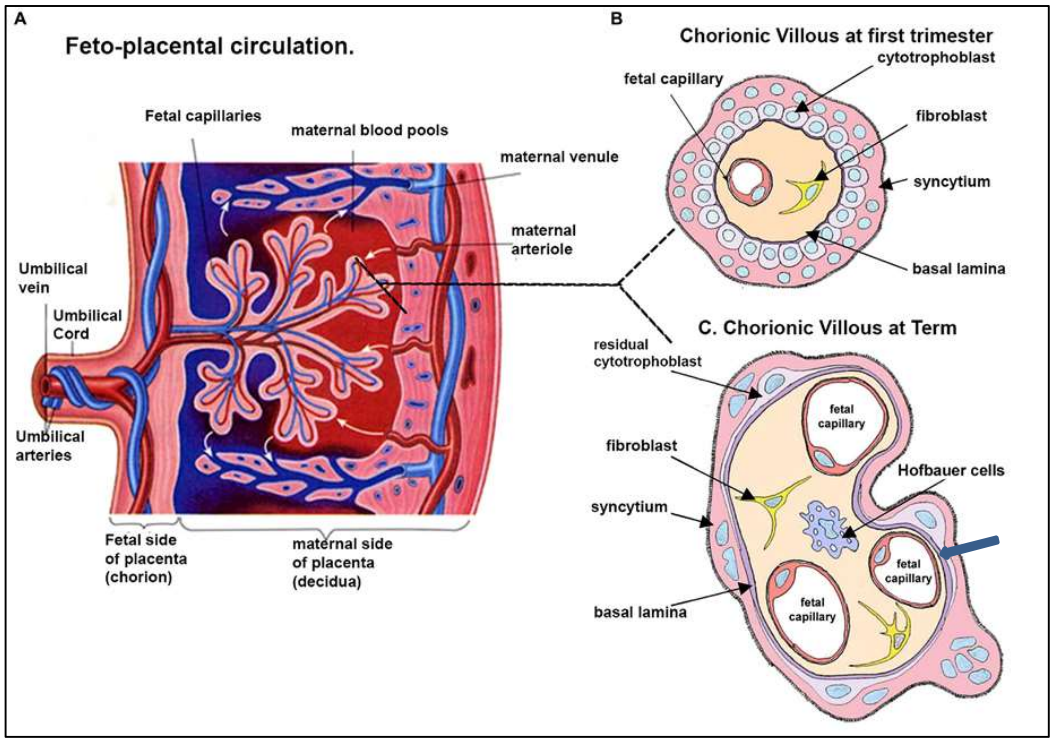

Figure 3 Schematic representation of a cross-section of human placenta

Right, A is a representative drawing of the fetal-placental circulation. The dotted line points up the cross-section through the chorionic villous at 10 weeks (B) and term (C). (B) At 10 weeks, the chorionic villous is surrounded by a basal epithelial layer of cytotrophoblast cells and an apical layer of a multinucleated syncytium of cytotrophoblasts. The connective tissue contains fibroblasts, Hofbauer cells (macrophages) and the fetal capillaries. (C) Chorionic villous at term corresponds to the *terminal villi* and at some area, the syncytiotrophoblast basal lamina comes in contact with the fetal capillary endothelium to form the vascular syncytial membrane (blue filled arrow). Reprinted from Murthi *et al* 2014 doi: 10.3389/fphar.2014.00133 (12).

1.2 Placental syndrome

A normal pregnancy is a complex physiological structure consisting of many strictly regulated inter-related consecutive changes that are extremely vulnerable to interference. Such vulnerabilities become evident in pregnancies complicated by a wide spectrum of placenta-related disorders, collectively named, placental syndrome. They include preeclampsia, intrauterine grow restriction (IUGR), placental abruption, placental inflammation, spontaneous preterm delivery and intrauterine fetal death (13, 14). Preeclampsia, a gestational complication characterized by maternal hypertension and proteinuria, represents a major health hazard among pregnant women as it accounts for 14% of maternal deaths worldwide (14, 15).

By a normal pregnancy, after implantation of the blastocyst, there is a portion of trophoblast cells which differentiate and migrate deep in the decidua part of the uterus towards the maternal spiral arteries in which they integrate. The action of this extra-villous trophoblast cells in the wall of the spiral arteries results in their wide opening which lowers the blood pressure entering the intervillous space of the placenta, hence, favoring the diffusion exchange (figure 4).

The pathophysiology of the placental syndrome is believed to start with the poor trophoblast differentiation and a failure in invasion and remodelling of maternal spiral arteries (16-18). However, various genetically and environmental factors are probably involved in processes of placental syndrome development. Investigations in these areas have enlightened some molecular pathways that could be involved (19, 20). Hence, after implantation, tolerance of paternal antigens on the extra-villous trophoblast by maternal immunological actors like the regulatory T-cells together with the decidual natural killer cells may facilitate placental growth (21). Complete failure of this recognition mechanism may result in a miscarriage while partial failure may result in poor placentation and uteroplacental perfusion dysfunction leading to preeclampsia, IUGR or both. Besides, a lower production of VEGF or PlGF or an unbalanced ratio of these factors to their soluble receptors (sVEGF-R1 or sflt-1) may also be a probable cause of abnormal placental development (22).

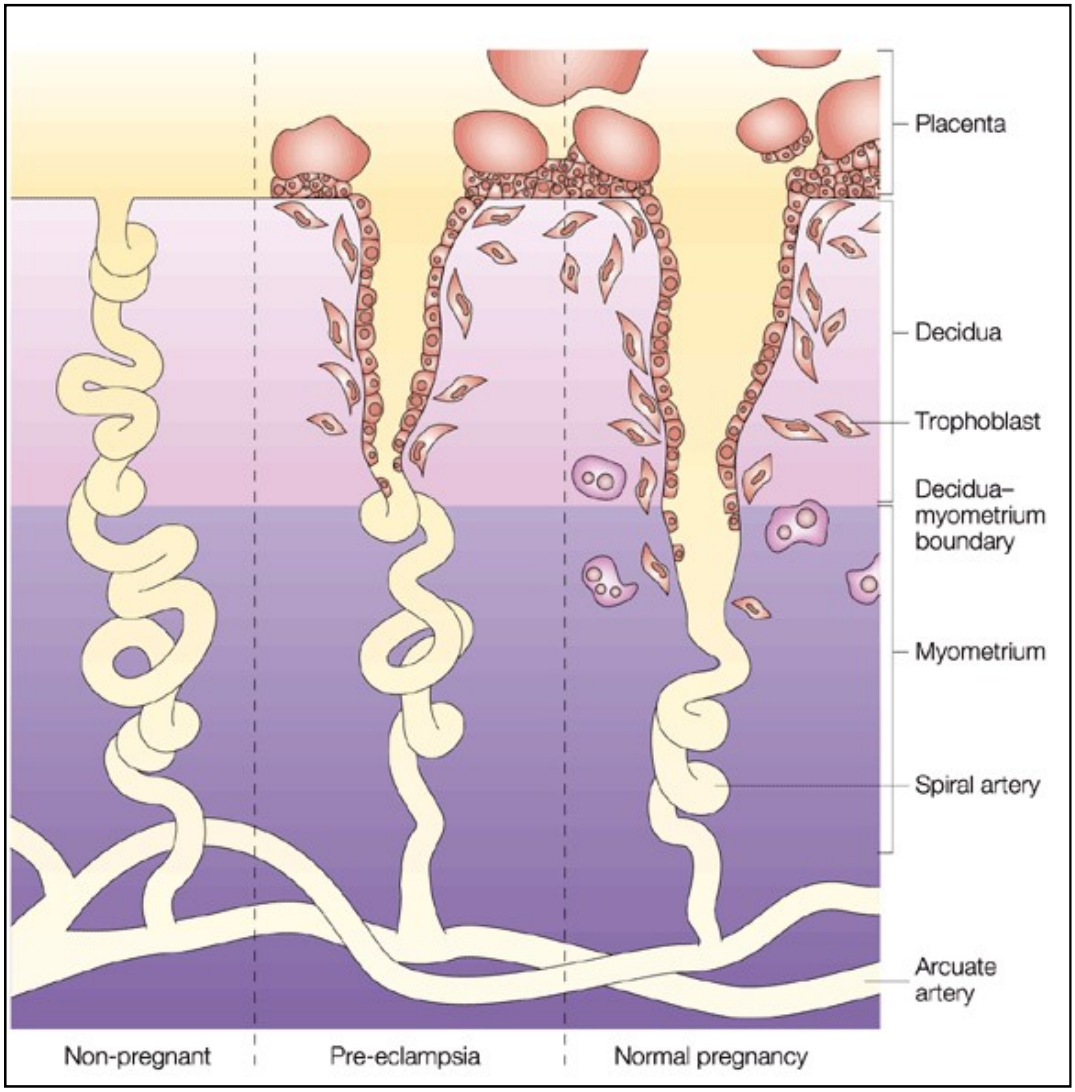

Figure 4 Illustration of spiral arteries by non-pregnant, preeclampsia and normal pregnancy In above figure is a representation of maternal spiral arteries: left, by a non-pregnant woman, in the middle by a preeclampsia complicated pregnancy and right by a normal pregnancy.
In normal pregnancy (right), trophoblast cells invade and remodel the spiral arteries resulting in their wide opening which lower the blood pressure entering the interstitial space of the placenta. By preeclampsia (middle), the poor trophoblast invasion results in a poor remodelling of spiral arteries. Here, the blood enters the placenta with a high pressure.
Reprinted from Elaine Bell E *et al*, December 2004 Nature reviews. Immunology 4(12):927-927, doi 10.1038/nri1514.

1.3 Effect of placental syndrome on placental development

Although the placental syndrome is a major cause of maternal and perinatal morbidity and mortality(4), there is still much to be elucidated regarding its pathophysiology. Under placental syndrome, are categorized preeclampsia and IUGR. Preeclampsia alone affects about 3% of pregnancy and up to 28% of the case, it is accompanied by IUGR (23-25). The imbalance between placenta produced factors and maternal response and its adaptation is believed to be the origin of preeclampsia. There is a pre-clinical stage of preeclampsia which is symptomless, while in the clinical phase is characterized by the onset of proteinuria and hypertension by the pregnant woman which suffers from headaches, swelling of hands or feet (26, 27).

During pregnancy, the uteroplacental blood supply originates from spiral arteries which branch from the maternal uterine artery. A major factor in determining the blood flow to the placenta is the physiologic transformation of spiral arteries influenced by the trophoblast insertion in their endothelial muscle wall (18). Therefore, the spiral arteries enlarge their diameter and via their wide opening flows the blood in the intervillous space between the placenta villi at a low velocity which favorites the nutrients and gas exchange (figure 5A left)(28).

In preeclampsia, spiral arteries remodelling is inadequate, and the spiral arteries maintain their vascular smooth muscle wall (29, 30). Therefore, maternal blood enters the intervillous space through narrow blood vessels at high velocity in a pulsatile intermittent manner exposing the placenta to alternating oxygen levels ranging from hypoxia to normoxia resulting in the ischemia-reperfusion injury of the placenta (figure 5A right) (6, 30).

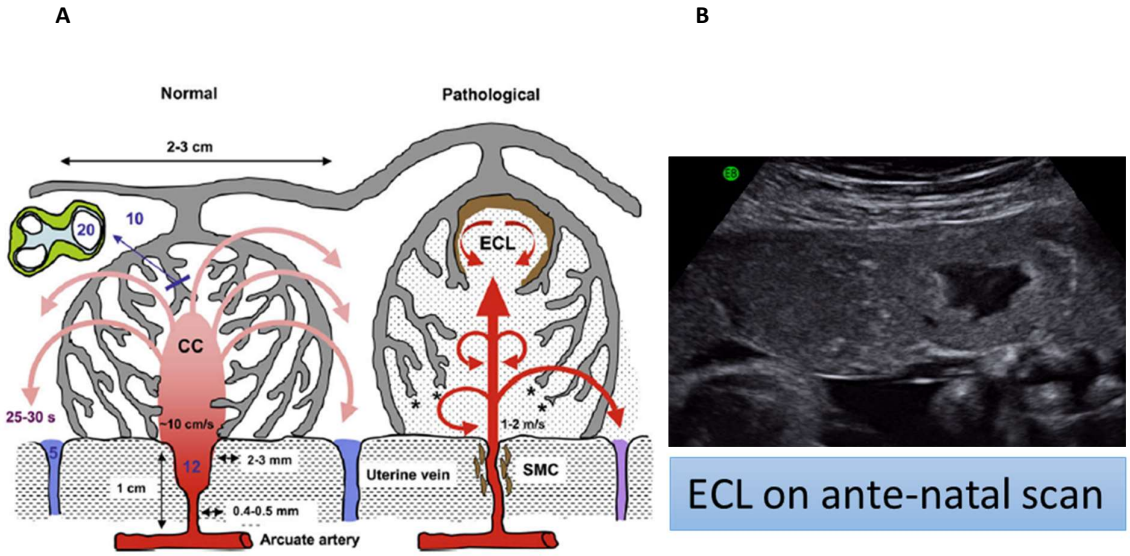

Figure 5 Effect of spiral artery remodelling failure on blood flow in intervillous space of placenta
In this figure, A is a schematic representation of the architecture of a placental lobule where is shown the effect of spiral arteries remodelling on the maternal blood inflow in intervillous space of placenta. The left part of this figure A represents the situation in normal pregnancy where the end segment of converted spiral arteries have 2 to 3 cm of diameter and the blood flow into the central cavity (CC) at a speed of 10 cm per second. The blood disperses further in other regions of the intervillous space and returns to uterine vein after 20-25 sec of transit permitting the oxygen and nutrient exchange. The blood pressure in mmHg is indicated in blue numbers in the figure A: 12 by the dilated segment of spiral arteries and 5 in uterine vein, 10 in intervillous space and 20 in fetal capillary as illustrated on a section of the villous segment (blue arrow, upper corner left of figure A). On the right part of the figure A is shown the pathological conditions such as in preeclampsia where the unconverted spiral arteries give a blood flow at a speed of 1 to 2 m per second producing enough force to rupture anchored villous in nearby (asterixis) and displaces others to form echogenic cystic lesions (ECL) lined by thrombus (brown). Here there is a turbulent blood flow indicated by circular arrows and the blood transit time in the intervillous space will be reduced impairing adequate oxygen exchange. The microparticulate debris (dots in intervillous space) originating from trophoblast may be extricated from the villous surface, leading to maternal endothelial cell activation. The remaining of smooth muscle cells (SMC) around the spiral artery will increase the risk of unprompted vasoconstriction and ischemia-reperfusion damage. Reprinted from GJ Burton *et al*, Placenta 30 (2009) 473-482
Figure B is a real picture of an ante-natal scan where ECL is seen as a dark hole. Reprinted from Dr. S. Al-Nasiry (unpublished data).

To some extent, hypoxia is required for placental angiogenesis (8, 31). However, uteroplacental hypoxia seems to be increased in preeclampsia and results in increased angiogenesis resulting in hyper-capillarization (figure 6, by A) (32).

Growth restriction of the fetus arises when it does not receive enough nutrition via the placenta and consequently does not achieve its genetically determined growth potential. It is not fully understood what restricts the nutritional supply, but the placenta is widely believed to be a key factor. By IUGR, there can be a high resistance in the umbilical blood flow from the fetus to the placenta resulting in fetal hypoxia, the oxygen does not reach the fetus and accumulates in the placenta, resulting in a placental hyperoxic state (3, 32). Contrary to

preeclampsia alone, hyperoxia by IUGR inhibits villous angiogenesis, resulting in placental villous hypo-capillarization (figure 6 by C) (5, 33).

Bacterial or viral infections in pregnancy, such as chorioamnionitis (CA) which affect fetal membranes have been described to have also an influence on placental growth and function (34). In the infected placental membranes, there is an ongoing inflammation in order to eliminate the present pathogens. Hence infected placental cells including trophoblast cells could be the target of the immune cells (34, 35). Therefore, it is interesting to investigate the influence of placental inflammation on villous maturation.

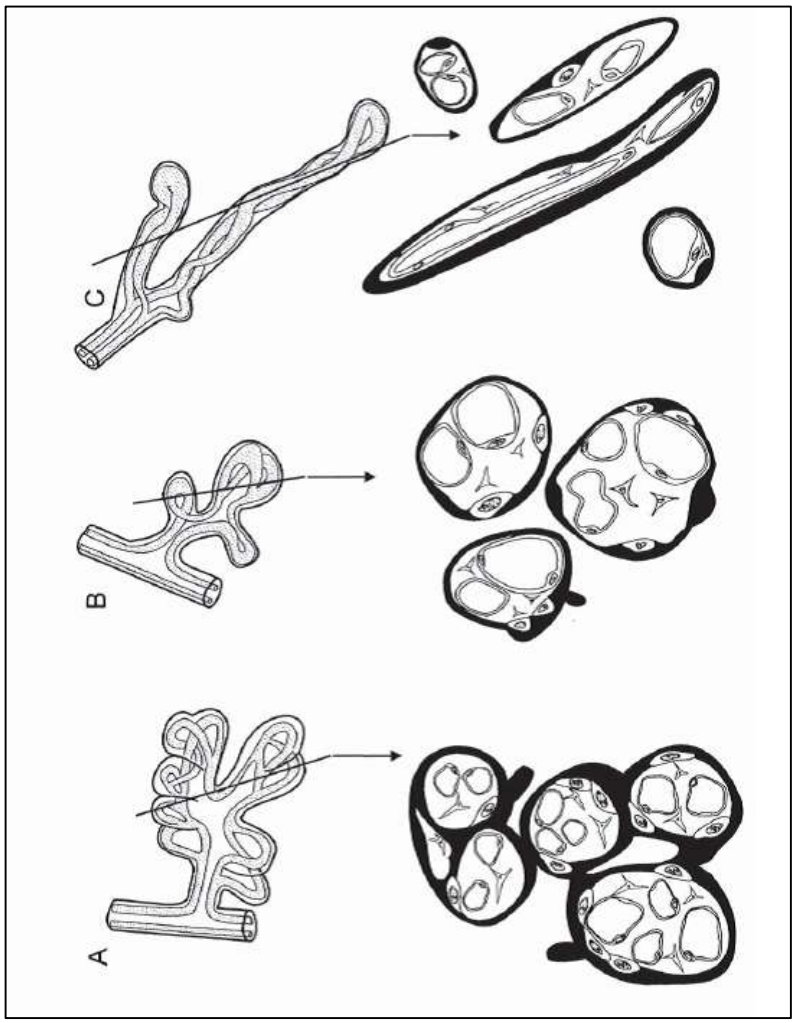

Figure 6 different patterns of villous capillarization

In normal placental villous vascularization, there is a balance of non-branching and branching angiogenesis. This figure illustrates at the left in A, B and C different form of villous capillarization and at right the corresponding image obtained by cross section. A symbolizes a hyper-capillarization of terminal villous as is seen in preeclampsia. B represents the normal capillarization of terminal villous by placenta at term. C shows the terminal villi capillarization where the branching angiogenesis dominated as it occurs in intrauterine growth restriction. Reprinted from Kingdom, J.C.P., Kaufmann, P, Placenta, Volume 18, Issue 8, November 1997, Pages 613-621.

1.4 Research objectives

This research project focus on the validation of an early developed new method to score placental maturity. The first objective was to confirm the earlier data on disturbed placental maturation in pregnancies complicated by preeclampsia and chorioamnionitis in comparison to placental maturation in idiopathic preterm control (negative control) and term (positive control) pregnancies of the first cohort.

The second aim was to evaluate the inter-observer variation for the 18 parameters used to score maturity of the placenta from the first cohort originating from idiopathic preterm control, term, preeclampsia and chorioamnionitis pregnancies.

For the visualization of fetal capillaries in placental sections, vascular markers for endothelial cells such as the cluster of differentiation (CD)31 and 34 are currently used in research. CD31 is a transmembrane protein that plays a role in adhesive interactions between adjacent endothelial cells as well as between leucocytes and endothelial cells. CD34 is transmembrane glycoprotein expressed on hematopoietic precursor cells and on capillary endothelial cells and functions as cell-cell adhesion factor to facilitate cell migration. Both markers are used in parallel in our research laboratory, so our third objective was to evaluate which staining between CD31 and CD34 will give best results by scoring placental maturity in the second cohort comprising preterm control and preeclamptic placenta.

2 MATERIALS AND METHODS

2.1 Selection of placenta samples used for placental maturity analysis

2.1.1 First cohort of placenta used for inter-observer variation

Recently a new method for placental maturity analysis has been developed by the Department of Embryology & Anatomy research group using placentas obtain by preeclampsia (PE), chorioamnionitis (CA) and idiopathic preterm (control) and term pregnancies.
The placentas were from a cohort of pregnant women which delivered at public Hospitals (Maxima medical centrum by Veldhoven and Maastricht University Medical Center+ (MUMC+), The Netherlands). From each placenta, we analyzed a series of 3 pictures (20x magnificence) of hematoxylin/eosin and CD31 stained placental sections used by the first observer during the implementation of the new method to score placental maturity. Six placentas from each placental group (PE, CA, control, term) were randomly chosen to be analyzed by a second observer using the same method as the first observer. Both observers performed a blind analysis of placental maturity by three pictures from each placenta. Thereafter the results were categorized according to the group they belong to.

2.1.2 Second cohort of placenta for CD31 and CD34 fetal capillaries staining

Twenty placentas that originate from patients who delivered in MUMC+ were included for placental maturity analysis (second cohort). From each placenta, two identical serial sections took on the same placenta block and that has been recently stained for CD31 and CD34 after Hematoxylin and eosin staining in the DAKO Immunologic staining automat (protocol in supplement 3), were included for placental maturity analysis of the second cohort. We want to find which fetal capillary staining markers between C31 and CD34 give the best results by placental maturity analysis. Four groups were analyzed respectively, CD31 controls, CD34 controls, CD31 PE and CD34 PE and n=10 placenta per group. By each placenta, three pictures that were approximately taken in the same region of successive sections but differently stained (CD31 versus CD34) were analyzed at 20x magnification to score placental maturity as in the first analysis.

2.2 Parameters used to measure placental maturity

Morphometric analysis at 20x magnification on three picture fields per placental section is performed using the Leica QWin standard V3.5.1 image processing software. The placental maturity was measured by means of stereological quantification on placental tissue sections where villous vasculature quantification was the most important. A schematic overview of criteria for inclusion of villous to analyze per placental section view is given in figure 7 and figure 8 shows how some parameters are measured.

Counting total number of villi and TV in placental sections provide an estimate of the degree of placental development. Measuring surface and circumference length of TV gives an idea of their growth. TV are defined as having 30% of their area occupied with FC. Cross-sectional areas, perimeters and shape coefficients (circumference/area of TV) in turn assess caliber and shape of villous. Villous vascularization is described by estimating capillary volume, surface and length densities within villi, and capillary villous surface and length ratios. All the parameters used for the measurement of placental maturity are summarized in table 1.

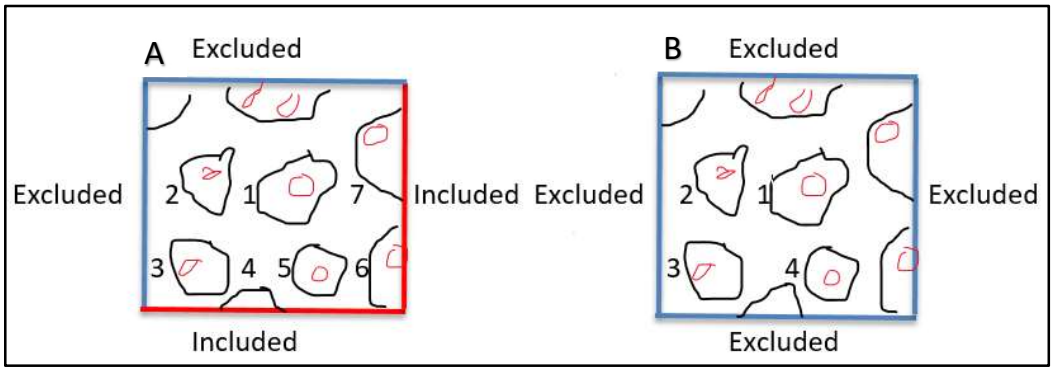

Figure 7 An overview of criteria for in- or exclusion villi for analysis

Left, A illustrates how the mean number of villi per view was determined by the parameter *number of all villi*: all villi were included except for the villi which touched the left and upper edge of the field. Right, B show how the number of villi located fully in the view was counted per field: only villi situated in the field which make no contact with any edge are included and further investigated to determine which one is a terminal villous.

Table 1 Placental maturity parameters

Parameters	How measured	Significance
1. Number all villi	All villi, villi touching right, and lower extremity edge of the view included	Indication placenta villous development
2. Number all villi fully in view	Only villi that do not encounter extremity edges are counted	All other parameters of placental maturity are measured on villi fully in view
3. Number TV	villi by which at least 30% of the area is covered with FC and where 2 VSM's are present	Indication of the degree of placenta tree branching
4. % TV	Number TVx100/Number all villi	Indication potential for gas and nutrient exchange
5. Number FC	Counted FC per TV	Degree of vascularization
6. Number VSM's	Counted VSM per TV	Indication of how smooth is the diffusion
7. Tot length VSMs/ TV (um)	Sum VSMs length per TV	Indication how smooth is the diffusion
8. Tot length DD/ TV	Sum of diffusion distance (DD) per TV	Indication diffusional barrier length
9. Mean DD length/ TV (-VSM)	Sum DD length (not counting VSM) /Number DD	Indication diffusional barrier length
10. Mean DD / TV (+VSM)	Sum DD length (including VSMs as null DD) /Number FC	Indication diffusional barrier length
11. Mean DD/ FC	Number DD per TV /Nr FC	Indication diffusional barrier length
12. Circumferences TV	Perimeters of TV	Size of TV
13. Area of TV	Surface of TV	Surface covered with TV
14. Circumferences of FC	Sum of perimeters of all FCs per TV	Degree of villous vascularization
15. Area of FC	Measured surface of all FCs	Degree of villous vascularization
16. % Area FC / TV	Ratio of surface of all FC to the surface of TV	Degree of villous vascularization
17. % circumferences VSM / TV	100 x sum length of VSMs/ perimeter TV	Indication how smooth is the diffusion
18. Circumferences TV / Area TV	Circumferences TV / Area TV	Round shape indication

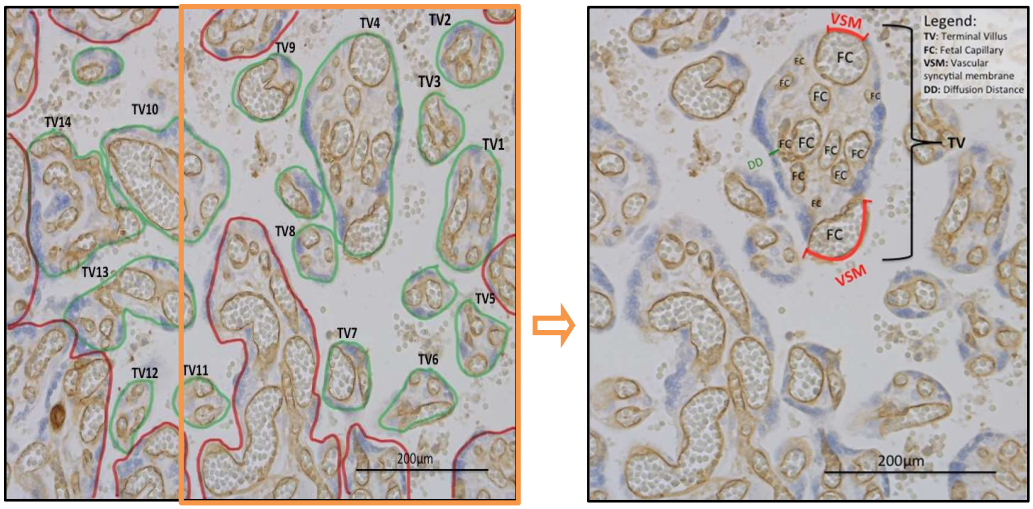

Figure 8 An illustration of placental maturity parameters measurement

In figure 8 show a placenta section picture morphometric analyzed by the light microscopy, after successful immunohistochemical staining of the vascular endothelium (brown borders) for CD31 and counterstained with hematoxylin/eosin. The bluish cells are nuclei of syncytiotrophoblast cells. By the left picture, all villi that are not completely in the view, shown here by red margins are excluded from analysis. All placental villi completely in the view contain a green margin. All TV in view enumerated (TVn) are counted and their area measured. Right is the same picture where parameters measured on TV4 are illustrated. Fetal capillaries (FC) are counted their area summed per each TV. On the right picture, the vascular syncytial membrane (VSM) length formed in the TV are demarcated with rood line and the diffusion distance (DD in green) is measured by FC that form no VSMs. Reprinted from Vonhögen I.G.C.(36).

2.3 Statistical analysis

2.3.1 Clinical characteristics of first and second cohort

With Kruskal-Wallis, we tested if there were a difference between clinical characteristics medians of the first cohort. For the second cohort, Mann Whitney test comparing medians of two groups PE and Control were used. P-value < 0.05 were considered as significant indicating that compared medians rank were different.

2.3.2 Analysis of groups differences in placental maturity scores of the first cohort

When the mean scores variances of analyzed placental maturity parameters were normally distributed, One-way ANOVA test was used to determine if these means were different, followed with Tukey's multiple comparisons to determinate which groups were different. Otherwise, Kruskal-Wallis, a non-parametric test was performed followed by Dunn’s multiple comparisons test. P-value < 0.05 were considered as significant meaning that the compared mean scores were significantly different.

2.3.3 **Inter-observer variation**

Means of scored parameters for placental maturity were used in the statistical analysis using Prism 5.1. The data obtained from the two independent observers were recorded in two different columns. For the statistical analysis of the data, we chose the Bland-Altman method of comparison (Difference (A-B) versus Average (A+B./2)), the bias (mean of differences) and 95% limits of agreement were calculated. A scatter plot was generated showing the spreading of the differences of means scored between the two observers for each parameter of placental maturity versus their average. Further, we investigated if the variances between observers scores were normally distributed using Kolmogorov-Smirnov test. Our hypothesis was that the variances between the two observers were equal to zero. Therefore, a One-sample t-test is performed to test if the variance were significantly different than zero. When the observers' scores variances followed the Gaussian distribution, results of the One-Sample t-test were considered, otherwise we used Wilcoxon Signed-Rank test a non-parametric test where a normal distribution is not necessarily assumed.

2.3.4 **Statistical analysis of second cohort results by CD31 and CD34 staining**

For the second cohort, the difference in results obtains from studied groups were analyzed with One-way ANOVA followed with Tukey's multiple comparisons test. Using One-way ANOVA gives us information if there were a difference in the four groups' mean scores for the parameters of placental maturity. The Tukey's P-value < 0.05 were considered as significant and was given for the comparison of the mean scores of each pair of groups studied (CD31 controls, CD34 controls, CD31 PE and CD34 PE and n=10 placenta per group).

3 RESULTS

3.1 Clinical characteristics of studied cohorts

Before we proceeded with the statistical analysis of our results, we checked if there was a difference in clinical characteristics among studied groups. Here below table 2 present clinical characteristics of the first cohort such as maternal and gestational age, placental weight, birthweight of the infant, gravidity, and parity of the mother.

Table 2 Clinical characteristics of the first cohort studied by inter-observer variation

	Control (preterm)[1] N=6	CA[2] N=6	PE[3] N=6	P[5]	Term[4] N=6	P[6]
Maternal age (years)	31.5 ±6.2	33 ± 5	31.5 ± 1	0.89	35 ± 5	0.83
Gestational age (weeks)	29 ± 1.5	28.5 ± 1	29.5 ± 1.4	0.82	39 ± 0.7	0.003*
Placental weight (g)	223 ± 113	321± 65	260 ± 64	0.29	NA	NA
Birth weight (g)	1080 ± 371	1422 ± 243	1140 ± 227	0.54	3097± 495	0.68
Gravidity	1± 1	2 ± 4	1 ± 1	0.11	2 ± 2	0.07
Parity	1 ± 1	1 ± 2	0 ± 1	0.09	1 ± 1.9	0.03*

NA: not available. The data presented in table 2 are the median ± standard deviation. [1]Idiopathic preterm birth as control, [2]Chorioamnionitis, [3]Preeclampsia,[4] normotensives at term pregnancies. [5] P-value by Kruskal-Wallis comparing clinical characteristics medians of control, CA and PE groups. [6] P-value by Kruskal Wallis testing medians difference between control, CA, PE and Term groups. *By P-value < 0.05, the compared groups medians are significantly different. Maternal age, placental weight and gravidity between groups PE, CA and Control were not different. The group Term present subjects with elevated gestational age and parity than of PE, CA and Control. The gravity represents the number of time a woman has been pregnant regardless the pregnancy outcome while the parity is the number of time the women carried pregnancies till a viable gestational age (including live birth and stillbirths but excluding miscarriages).

As expected the statistical analysis of clinical characteristics of the PE, CA and control groups revealed no differences which could influence the placental maturity founded scores. By group term, the gestational age is higher than the other groups which preterm delivery. Information concerning weights of term placenta was not available for this cohort.

Further, table 3 expose the clinical characteristics of the second cohorts used to examine which of the CD34 and CD31 vascular staining give best results by placental maturity analysis. The data presented are the medians with standard deviation for maternal age, gestational age, birthweight of the infant, and gravidity.

Table 3 Clinical characteristics of the second cohort used for CD31 and CD34 fetal capillaries staining

	Control[1] N=10	PE[2] N=10	P[3]
Maternal age (years)	35±4.6	28.5±5.7	0.06
Gestational age (weeks)	30,73±4	30,3±4	>0.99
Birth weight (g)	1576±762	1281.5±754	0.36
Gravidity	3±3	1±4	0.01*

The data presented in table 3 are the medians± standard deviation.
[1]Idiopatic preterm as control, [2]PE: preeclampsia. [3]P-value by Mann Whitney test. *P-value < 0.05 is significant.
The median gravidity of the control group was higher than that of the PE group.

When comparing the clinical characteristics recorded for groups control and preeclampsia of the second cohort, there were no other significant differences found except by gravidity. The control group presents a higher gravidity than preeclampsia group.

3.2 Placental maturity analysis in the first cohort

After placental maturity analysis of placental sections from the control, preeclampsia, chorioamnionitis and term groups, we compared the obtained scores between the studied groups (figure 9 and 10). The scores of following parameters were not different between the groups ($p > 0.05$): *the number of all villi, the number of villi fully in view, the number TV, the percentage TV, the number of VSMs, the mean diffusion distance per terminal villous or fetal capillary, the TV or FC circumferences, the TV or FC surface area, the ratio of TV area on TV circumferences*. Only preeclampsia group presented elevated ***number fetal capillaries*** than the control group ($P = 0.02$, figure 9C). In preeclampsia group, *the number and percentage of terminal villi*, *the fetal capillaries circumference* tend to be elevated than in other groups, although there was no significant difference ($p > 0.05$, figure 9 B, D, and F respectively).

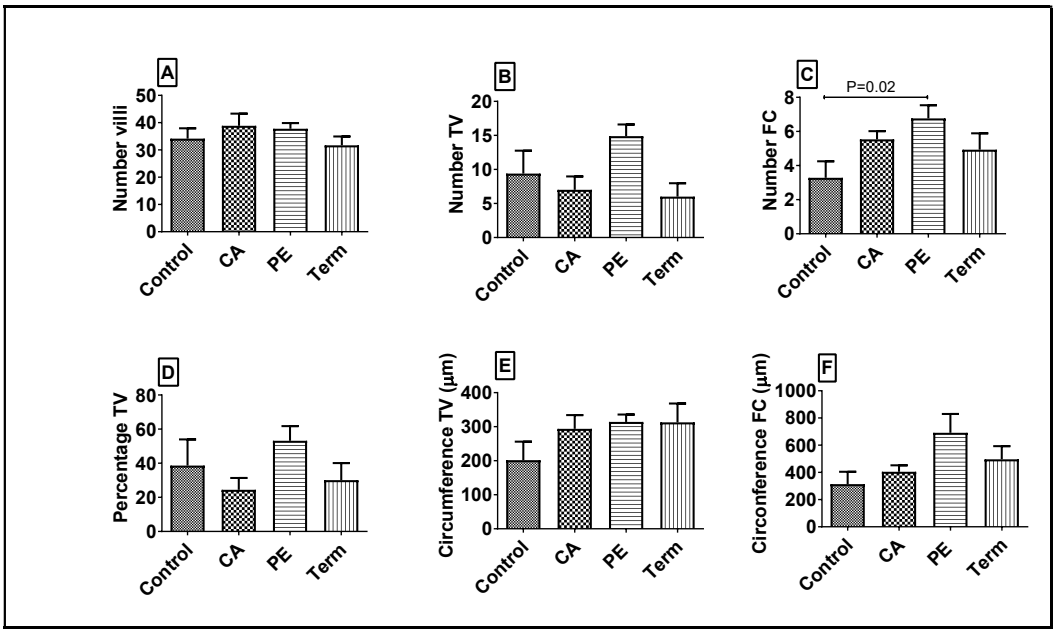

Figure 9 Various placental maturity parameters of the first cohort

CA: chorioamnionitis, control: Idiopathic preterm placenta, PE: preeclampsia, Term: placenta at term birth, TV: terminal villi, FC: fetal capillaries.

This figure shows six placental maturity parameters scored in the control, CA, PE, term groups.

The represented data are mean with standard error of the mean. Panel A shows the *number of all villi*, B presents the *number of terminal villi*, C shows the *number of FC*, D presents the *percentage of TV*, E displays the *circumferences of TV* and F shows the *circumferences of FC* respectively in control, CA, PE and Term groups studied. Only the *number of fetal capillaries* in PE is found elevated than in control group (P-value <0.05 by Dunn's multiple comparisons).

Subsequently, to evaluate the villous capillarization in control, preeclampsia, chorioamnionitis and term groups, different parameters were measured on fetal capillaries like *the FC area, the percentage of FC area per TV, the average numbers and length of vascular syncytial membranes present in TV, the average diffusion distance per fetal capillary and TV.* When calculating the mean diffusion distance per TV in one parameter (DD/TV (+VSM), the vascular syncytial membranes were counted as a null diffusion distance, whereas, by the other (DD/TV (-VSM)), null diffusion distances were not taken in account. Surprisingly, there was no difference in score of above-mentioned parameters between studied groups (P > 0,05 in all these scored parameters, figure 10).

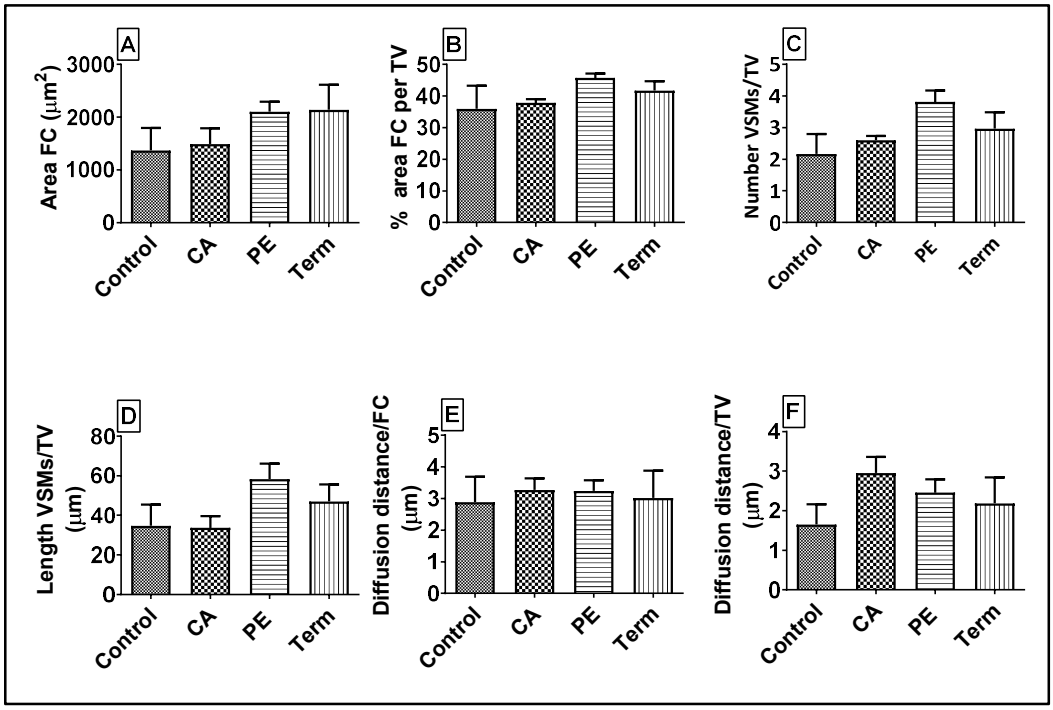

Figure 10 Placenta maturity parameters measured on fetal capillaries

CA: chorioamnionitis, control: Idiopathic preterm placenta, PE: preeclampsia, Term: placenta at term birth, TV: terminal villi, FC: fetal capillaries, %: percentage, VSMs: vascular syncytial membranes

In figure 10, the A chart shows the average of measured fetal capillaries area per terminal villi and B shows the percentage of the average area of fetal capillaries in comparison to the average area of terminal villi respectively in control, PE, CA and term groups. C chart illustrates the scores founded by the parameter *number of vascular syncytial membranes* in different studied groups. D chart shows the average *length of VSCMs* per group. E and F panels show the average *of the diffusion distance* respectively per fetal capillary or per terminal villi. There was no difference in scores of these parameters between the groups (P > 0,05 by Dunn's multiple comparisons for all illustrated scored parameters)

3.3 Inter-observer variation by placental maturity analysis

In an attempt to validate the new method to score placental maturity, too blinded observers evaluated the placental maturity for the following groups: idiopathic preterm control (negative control, n=6), preeclampsia (n=6), chorioamnionitis (n=6), and term group (positive control, n=6). The variances between the two observers were visualized by Bland-Altman plots (see figure 11, 12, and 13). The upper and lower 95 % limits of agreement were calculated as ±1.96 times the standard deviation of the mean differences. After we performed a t-test comparing the mean differences between the observers with zero.

For the parameter *mean number of all villi*, the 95 % limits of agreement (LOA) range was 0.6 to -12.3 with a bias of -5.8 for the control group and for the preeclampsia group, the LOA range was from 1.2 to -11.9, with a bias of-5.3 (figure 11 A&B). For the chorioamnionitis group this range was 9.2 to -12.3 with a bias of -2.2 for the same parameter, whereas we observed LOA range of 2.2 to -27.9 with a bias of -12.89 for the term group (figure 11C&D).

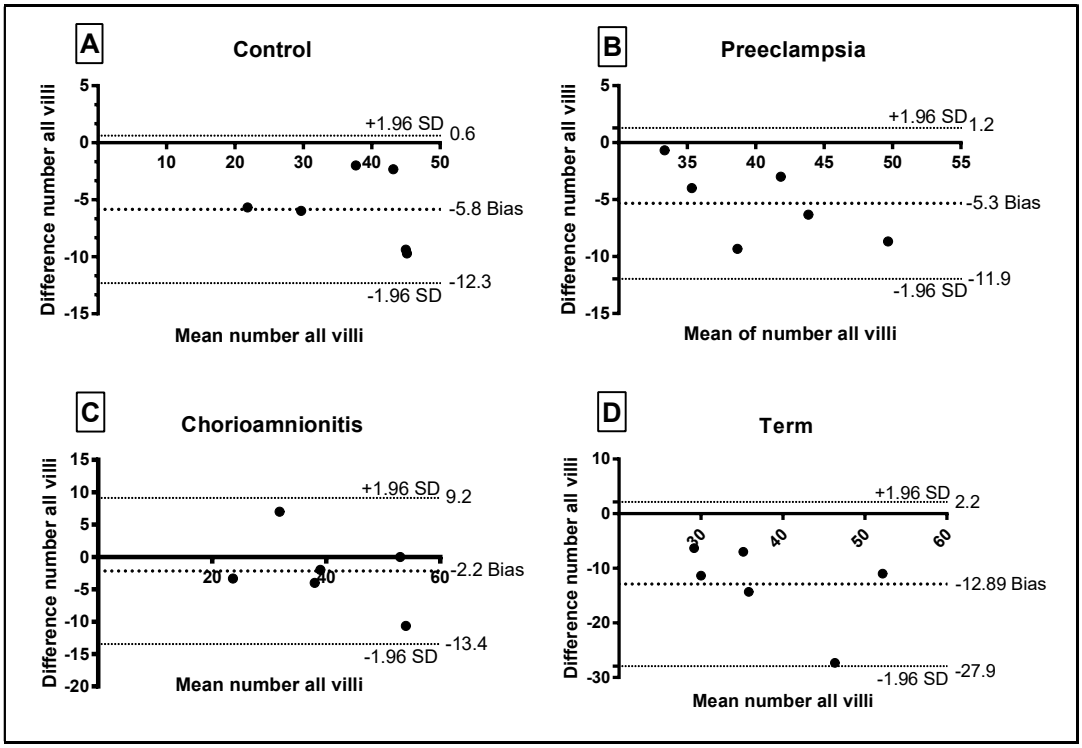

Figure 11 Inter-observer variation of number all villi parameter scores in the first cohort

SD: standard deviation. The upper and lower 95 % limits of agreement are calculated as ±1.96 SD of the mean differences. This figure displays Bland-Altman plots which visualize the dispersion of the observer's scores differences (on Y-axis) against the average of these scores (on X-axis), for the *number of all villi* parameter of placental maturity for different groups of the first cohort (n=6 for each group). Smallest 95% limits of agreement denote the concordance of scoring between observers, where the difference of score is nearly zero (dots on X-axis). Graphs A, B, C & D show for the parameter *number of villi* respectively for control and preeclampsia, chorioamnionitis, and term groups, how the observes scores mean differences fluctuate as their mean rise or drop. In A, B, and D plots one observer scored consistently higher or lower than the other as all the scatter points representing the observer's mean scores differences are on one side of the X-axis.

Further, for the parameter *number of terminal villi*, the LOA range was 5.2 to -2.9, with a bias of 1.17 for the control group, while for the preeclampsia group, the LOA ranged from 3.3 to -3.6 with a bias of -0.16 (figure 12A&B). Chorioamnionitis LOA oscillated from 9.9 to -4.3 with a bias was 2.78 and the range of LOA for the term group was 4.3 and -6,4 with a bias of -1,05 (figure 12C&D).

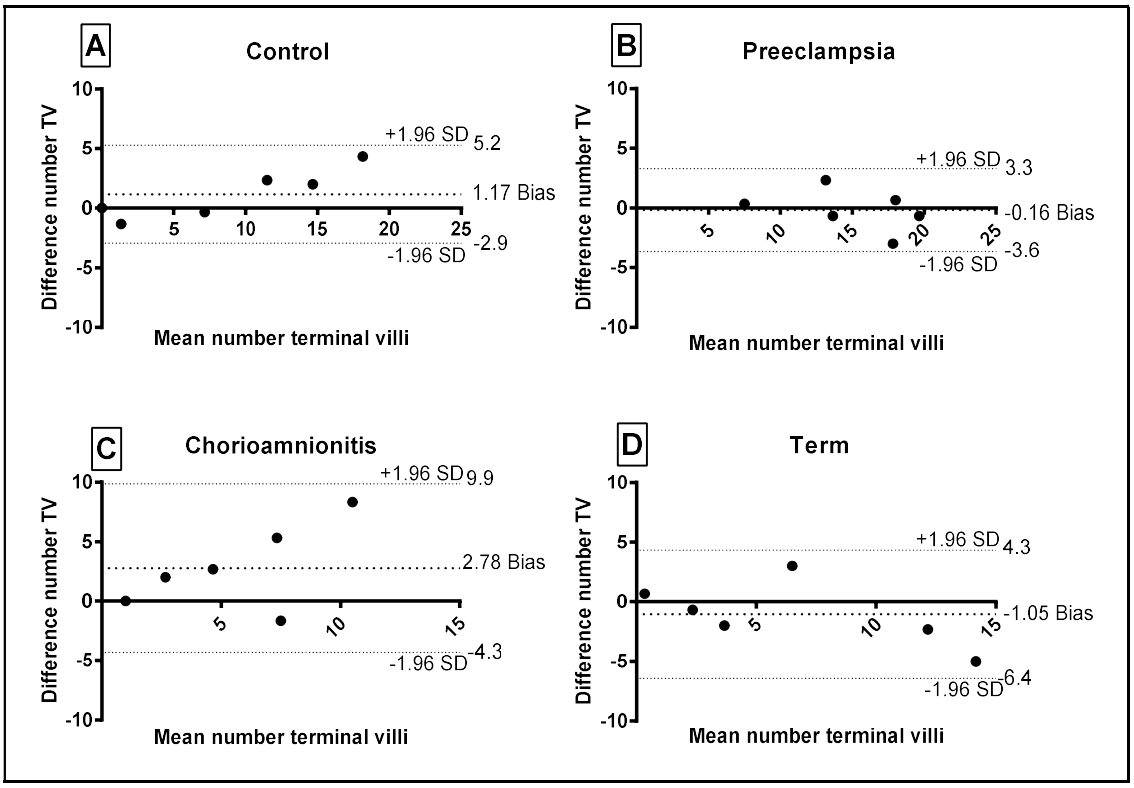

Figure 12 Interobserver variation for the number terminal villi parameter scores in the first cohort

TV: terminal villi. SD: standard deviation. The upper and lower 95 % limits of agreement are calculated as ±1.96SD of the mean differences.

Figure 12 contains Bland-Altman plots viewing the scattering of the observer's scores differences (on Y-axis) against the average of these scores (on X-axis), for the *number of terminal villi* parameters of placental maturity for control, preeclampsia, chorioamnionitis and term groups. Graphs A & B and C & D show for the parameter number terminal villi r for studied groups of the first cohort how the observer's mean variances oscillate in comparison to their averages. The range of 95% limits of agreement by control and preeclampsia for *number terminal villi* parameter were the smallest. By four samples of the six, the observer's scores differences were under the value of 1 by the *number of terminal villi* parameter for preeclampsia notice that the bias was -0.16.

The range of limits of agreement for the control and preeclampsia group for the *number of terminal villi* were smaller than those obtained for the *number all villi* parameter. Smaller LOA represent the best concordance of scoring between observers, where the difference of scores will be nearly null. Therefore, One-sample t-test is performed to test if the mean variances for the outcome between the two observers for all different parameters in different groups were comparable and thus close to zero.

For the control group, nearly all parameters' scores of the two observers were comparable, hence, the earlier analysis could be confirmed in control group: p-value > 0.05 for 16 of 18 scored parameters. In all the groups, for the *number of villi fully in view* parameter, the two observers' scores were significant different (control: p = 0.005, chorioamnionitis: p = 0.02, preeclampsia: p = 0.01 and term: p = 0,01 see data supplement 1). In addition, a significant inter-observer variation was obtained by following parameters (figure 14):

i) *percentage TV* for preeclampsia: p = 0.007 and chorioamnionitis: p = 0.01.
ii) *circumferences of the TV* for preeclampsia: p = 0.04,
iii) *area of fetal capillaries* for preeclampsia: p = 0.04, Term: p = 0.02
iv) *percentage area fetal capillaries per TV* by preeclampsia: p = 0.02
v) *circumferences of fetal capillaries* for the term: p = 0.004

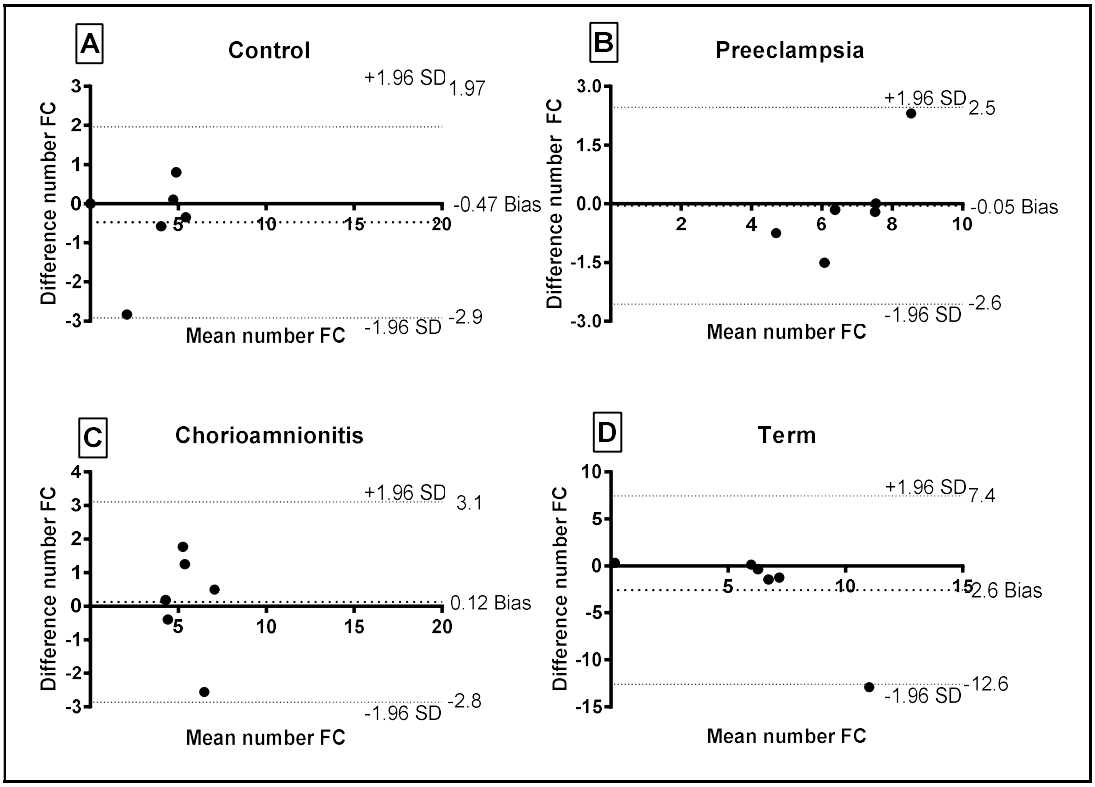

Figure 13 Inter-observer variation for fetal capillaries number in all groups of the first cohort

FC: fetal capillaries. SD: standard deviation. The upper and lower 95 % limits of agreement are calculated as ±1.96SD of the mean differences.

In this figure 13 by A, B, C and D are scattergrams of observer's scores differences versus their averages for the *number of fetal capillaries* parameter by control, preeclampsia, chorioamnionitis and term groups. The range of 95% limits of agreement by control and preeclampsia for *number fetal capillaries* parameter were the smallest and the mean average of observers scores, the bias, measured only 0.47 and -0.05 respectively.

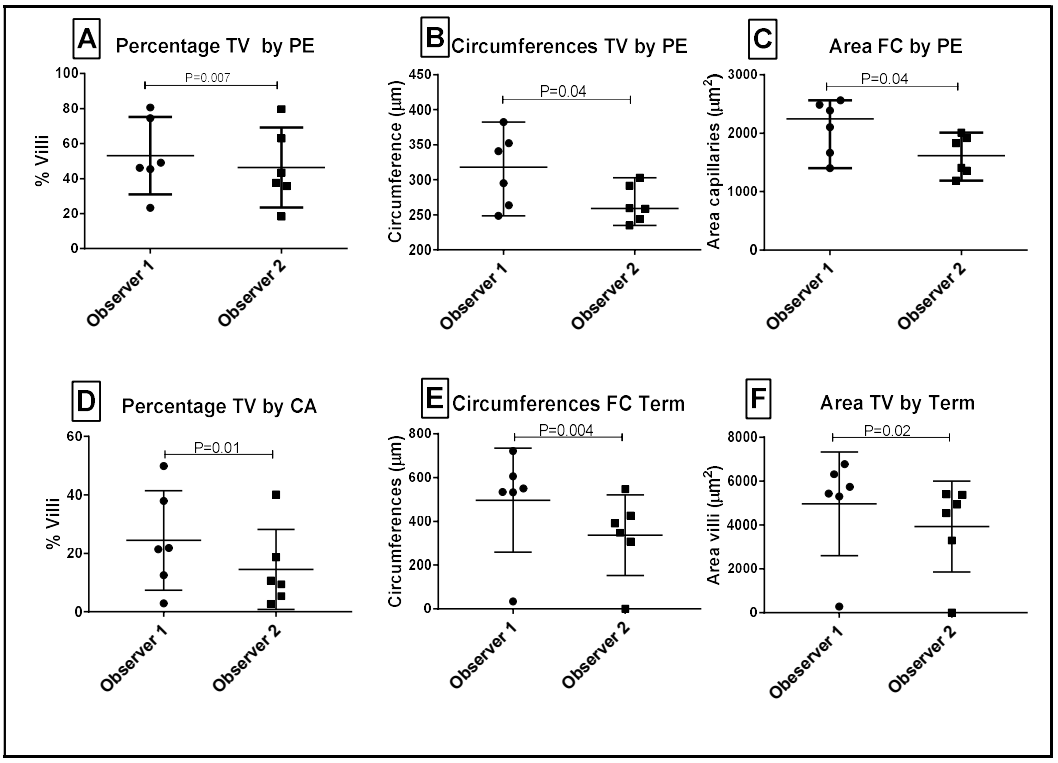

Figure 14 Observers bias detected by scoring placental maturity in the first cohort

CA: Chorioamnionitis, FC: fetal capillaries, PE: preeclampsia, TV: terminal villi. Represented data are parameters scores with mean and standard deviation. In this figure, the charts of parameters are presented where a significant inter-observer variation was observed. The first three charts A, B & C denote the preeclampsia group where respectively the *percentage terminal villi* (P=0.007), *circumferences of terminal villi* (P=0.04) and *area of fetal capillaries* (P=0.04) placenta maturity parameters were subject to significant observer's bias. D represents the *percentage of the TV* for Chorioamnionitis (P=0.01). E and F charts display by group term respectively the founded observer's bias for parameters *Circumference FC* (P= 0.004) and *area of TV* (P= 0.02). These results were obtained by One Sample t-test, p-value < 0.05 is considered as significant.

3.4 Comparison of placental maturity analysis by CD31 and CD34 staining

When we statistically analyze the scores of placental maturity in the second cohort, there were no statistically significant differences found between CD31 control and CD34 control groups, neither between CD31 PE and CD34 PE by all scored parameters. However, for the fetal capillaries related parameters measuring the diffusion distance such as the *mean total length of diffusion distance per TV, mean diffusion distance per TV* and *mean diffusion distance per FC*, we were able to detect a statistically significant difference between control and preeclampsia groups (figure 15).

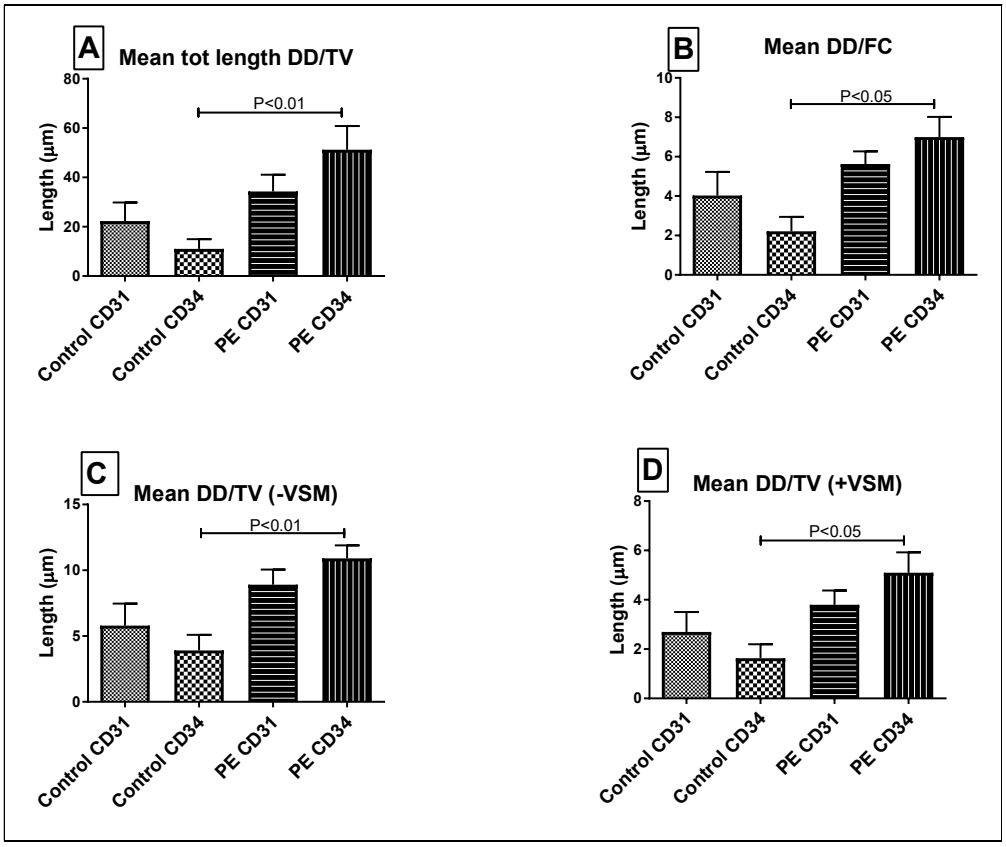

Figure 15 Comparison of CD31 and CD34 staining by scoring diffusion distance parameters

DD: diffusion distance, FC: fetal capillaries, PE: preeclampsia, TV: terminal villi, VSM(s): vasculo-syncytial membrane(s). Figure 15 shows different parameters which give an idea of the degree of diffusion per FC and TV. Graph A illustrates the scores of the *total length the diffusion distance* parameter. Graph B shows the *mean diffusion distance per FC*. Graph C shows *mean diffusion distance per TV* without counting the vascular syncytial membranes as a null diffusion distance. Graph D displays *the mean diffusion distance per TV* scores where the VSMs were taken into account. For both of these parameters illustrated in A, B, C, and D the obtained scores in preeclampsia were elevated than in control by the CD34 stained placenta and both these parameters Data are means with standard error of the mean. By one- way ANOVA followed with Tukey's multiple comparisons test P-value < 0.05 is considered as statistically different

In addition, for this placental maturity analysis of second cohort, by CD31 stained fetal capillaries, the placenta maturity parameters such as the *number of all villi* and the *number of villi fully in the view* scores were elevated in preeclampsia than in control group while this was not the case by CD34 stained fetal capillaries (figure 16).

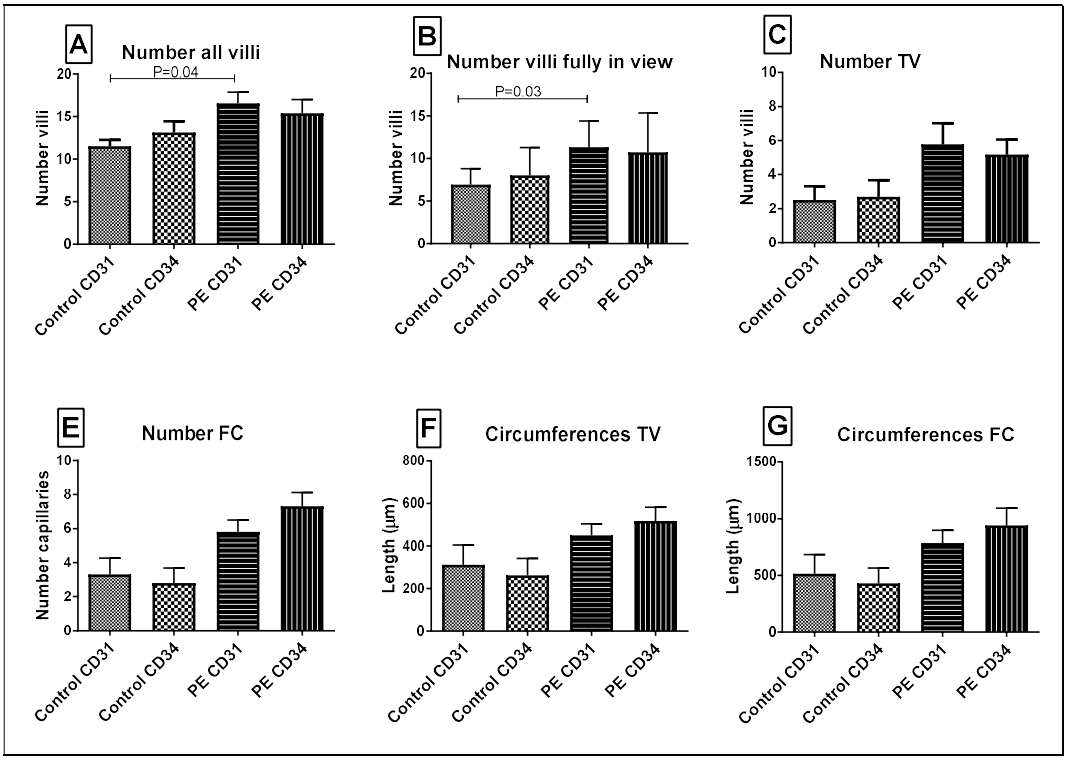

Figure 16 Comparison of CD31 versus CD34 staining results for first six parameters of placental maturity

PE: preeclampsia. Data are means with standard error of the mean. One- way ANOVA followed with Tukey's multiple comparisons P-value <0.05, is considered as significant. The graphs in figure display the scores of first six parameters for placental maturity on successive placental sections. Respectively, graphs A and B show *that the number of all villi* and *number of villi fully in view* scores are significantly elevated in CD31 PE than in CD31 control. Graphs C, D, E, F, G illustrate the obtained scores for the *number of terminal villi*, *number of FC* and *the circumferences of TV* and *FC* parameters where there were no differences found between the CD31 or CD34 stained control and PE groups.

4 DISCUSSION

4.1 Placental maturity analysis of the first cohort

The analysis of placenta maturity by the first cohort did not reveal many differences in placental maturity between preterm control, preeclampsia, chorioamnionitis, and term groups. The small number of placenta section analyzed per group (n=6 per group) and the small changes in placental maturity did not permit to detect statistically significant results. We only detect an elevated *fetal capillaries number per terminal villi* by the preeclampsia group compared to the preterm control group. In most of the case, we only saw differential trends of scored parameters among studied groups.

Comparable to our research, Corrêa *et al* 2007 conducted a retrospective study on the placental maturity of full-term and preterm placenta from hypertensive syndrome pregnancies and full-term healthy control. They found an increased number of terminal villi vessels by the group of term preeclamptic placenta (n=17) in comparison to the group of term normotensive control placenta (n=18). In addition, preterm preeclamptic placenta (n=19) had a similar number of terminal villi vessels as the full-term control placenta (37). Our findings and those of Corrêa *et al* 2007, give a hard evidence that the preeclamptic placenta adapts itself in attempt to retrieve needed nutrients and oxygen.

More recently, in 2013, Sankar and colleagues compared the histomorphological and morphometrical changes of placental terminal villi of term normotensive and preterm preeclamptic mothers (n=56 for each group), and contrary to us, they found a significantly lower density of fetal blood vessels in terminal villi of the preeclamptic placenta. However, in preeclampsia, the density of terminal villi was elevated while their diameter was lower than in term normotensive placenta. As in our findings, Sankar *et al* did not find a significant difference for terminal villi surface area in preeclampsia compared to normotensive control group (38).

In our research, we suggest the elevated fetal capillaries per terminal villi found in the preeclamptic placenta to be a result of placenta adaptation to compensate or reverse its hypoxic condition. However, these compensation changes are insufficient to limit the oxidative stress and chronic hypoxemia occurring in preeclampsia and lead to abnormal placental blood diffusional exchange resulting in exacerbation of hypoxic condition (39). Our findings are in accordance with the early developed theory arguing that to some extent, there is an increased placental angiogenesis stimulated by hypertension induced hypoxia (40). Yamakawa M. *et al* and others demonstrated that hypoxia-induced factor-1 mediates transcriptional activation of vascular endothelial growth factor (VEGF) and other hypoxia-responsive genes which favor the formation and extension of the vascular net (41, 42).

Undeniably, in a variety of conditions, such as malignant tumors, wound healing, and myocardial ischemia, hypoxia is a fundamental stimulus for angiogenesis (27, 43-45). The increased fetal capillaries density in preeclampsia occurred because of the continuous angiogenesis simulated by the resident hypoxic state of the placenta (46). It is acknowledged that in preeclampsia, maternal spiral arteries failed to adapt their endovascular wall partially because of unsuccessfully trophoblast invasion coupled with abnormal uterine natural killer (uNK) cells behavior. The failure of the trophoblast induced-spiral arteries remodelling in the placental-decidual interface implicates that the blood enters the intervillous space with a high velocity causing morphological and histological changes indicative of pathogenesis of maternal and fetal morbidity in women with preeclampsia (16, 47, 48).

Beside hypertension and proteinuria, the diagnostics of preeclampsia is based on detection of imbalanced pro-angiogenic factors by determining the ration of VEGF and PlGF on their soluble VEGR-1 (sflt-1). Zazzaro Smith *et al* conducted an immunofluorescence assay in attempt to study the expression of VEGF receptors expression by preeclampsia and normotensive placenta. Their findings revealed that there is no difference in VEGF receptors expression in preeclampsia in comparison to the normotensive placenta (49). In the future, it would be interesting to evaluate the presence of VEGF and their receptors in placental sections using VEGF and sflt-1 specific endogen staining markers and compare the results to the maturity of the respective placenta. In addition, analysis of endothelial proliferation may be useful.

4.2 Inter-observer variation of the first cohort placental maturity analysis

The presented results by placental maturity analysis of the first cohort were obtained from a second blinded observer. Earlier, the same analysis was performed on the same placenta section pictures by another blinded observer. The observer's scores acquired by measuring placental maturity parameters were compared using the Bland-Altman method. Bland-Altman plots show how variate observer's scores differences as their mean value lower or increase. The Bland-Altman 95% limits of agreement give only the range where 95% of the variances fall. It is to the researcher to determine which interval is acceptable for his analysis based on the relevance of the clinical interpretation. Our analysis was explorative, there was no consensus yet for a cutoff range of the limits of agreement and their clinical significance was not established, but the smallest range of 95% limits of agreement was wanted as it symbolizes the consistency in scoring between observers.

In the control group, the two observers were concordant in their scores by almost all measured parameters excepted for *number of all villi* and *villi fully in view* and hence confirmed the validity of this new method to score placental maturity. These results suggest that the control group was a homogenous group adequate for validation of this novel method to score placental maturity.

The observer's disagreements in scoring founded for other placental maturity parameters scored in groups preeclampsia, chorioamnionitis, and term could be due to increased observer's subjectivity arising when analyzing abnormal placental mesenchyme or by elevated villi density in the analyzed placental section image. In addition, it would be better to distinguished between mild and severe preeclampsia to underline the difference in respective placenta maturation features. Further, the fact that the observers were not as experienced when starting the analysis as they were later could too be a source of score variations. Studies for the reliability of histological analysis stipulate that even performed by pathologist experts, placenta image analysis suffer from personal subjectivity (50). Recently, a group of researchers has tried to resolve the observer bias issue by developing an automated image analyzing software program and use it to examine placenta villi and syncytial knots in histological sections. However, in their conclusion, they were aware that no automat can give equivalent interpretations as the human eye (51).

The placenta maturity analysis of the second cohort reveals similar results in general when using CD31 or CD34 fetal capillaries staining. The number of fetal capillaries did not differ between the differently stained successive placental sections. These obtained results suggest that in placental capillaries CD31 or CD34 endothelial molecules could be simultaneously expressed explaining the fact that no difference could be obtained by counting the fetal capillary stained with these markers. Likewise, by colon tumor angiogenesis studies, CD31 and CD34 were found to be equally expressed and are hence used as endothelial markers (52).

However, by the CD34 stained placenta, the *diffusion distance parameters* were elevated in preeclampsia than in control group, whereas, it was not the case for the respective groups by the CD31 stained placenta. These results suggest that the CD34 staining permit to detect subtle differences than does the CD31 staining. Nevertheless, these differences were not entirely related to the staining itself but could mainly due to the placental maturity differences of control and preeclampsia. Subsequently, there were no differences in scores of *diffusion distances parameters* measured in CD31 versus CD34 stained preeclampsia and control placenta.

Further, in CD31 stained placenta, preeclampsia group showed an elevated *number of all villi* and *number of villi fully in view* than control. However, these findings were not related to the CD31 staining but were mainly due to the abnormal hyper-maturation of the villous tree in preeclampsia. Normally, as the analyzed placenta were successive serial sections, we should be able to reproduce the same finding concerning the *number of all villi* and *villi fully in view* parameters by CD34, however, this was not the case. The obtained controversial results show the complexity and difficulties of obtaining the same results in image analysis. In the future, an intra-observer variation may as well be evaluated.

5 CONCLUSION

Our research focused on the inter-observer variation to validate a new method for scoring placenta maturity. Only two of the eighteen placental maturity parameters scored suffered from observers bias in control group. In addition, the limited observers score variabilities in others different studied groups by placenta maturity analysis of the first cohort permit us to conclude that the new method of scoring placental maturity is valid. However, use of a large cohort should be performed to test the degree of reliability of this new method when analyzing placenta that present pathological changes as in preeclampsia and in chorioamnionitis.

Even though in our analysis and that of others the number of terminal villi did not differ by normotensive and preeclampsia placenta, we believe that terminal villi function diminished in accordance with the severity of the preeclampsia as it was revealed in our first and second analysis were respectively *the number of fetal capillaries* and *the diffusion distances per fetal capillary* in preeclamptic placenta were elevated in comparison to the normotensive placenta. The placenta maturity analysis of the second cohort reveals no obvious difference between CD31 and CD34 fetal capillaries staining. The number of fetal capillaries did not differ between controls groups by these differential staining, neither in preeclampsia groups. However, increased diffusion distances in preeclampsia in comparison to control group was more remarkable by CD34 staining. Further, as there were no other scores differences for the parameters measured on fetal capillaries by differently stained successive placental sections, we can conclude that CD34 and CD31 vascular staining give similar results by placenta maturity analysis.

Interpretation of obtained results should take into consideration the limitations of this study. For example, the control groups contained idiopathic preterm placenta where unknown factors such as chromosomal aberration leading to preterm delivery were not ruled out and hence could have influenced the scores of placental maturity analysis.

Supplementary, follow-up studies should be conducted in order to find early precursor markers that correlate to the development of pathological changes that occurs within placental disorder in general. In that perspective, blood markers like pro-angiogenic factors or metabolic effectors produced by trophoblast, which correlate with abnormal placental maturity could be then associated with the current method for a better diagnostic of preeclampsia and hence improve the management of mother an child health care.

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7 APPENDIX

7.1 Supplement 1: Table of raw data by an inter-observers variation for placental maturity parameters of the first cohort.

Groups	Control		CA		PE		Term	
Parameters	**ND[1]**	**P-value [2]**	**ND[1]**	**P-value [2]**	**ND[1]**	**P-value [2]**	**ND[1]**	**P-value [2]**
Number all villi (villi touching right and lower edge included)	Yes	**0.007**	Yes	0.39	Yes	**0.01**	Yes	**0.01**
Number all villi fully in view	Yes	**0.0005**	Yes	**0.02**	Yes	**0.01**	Yes	**0.01**
Number TV	Yes	0.3	Yes	0.2	Yes	0.8	Yes	0.4
% TV	Yes	0.06	Yes	**0.01**	Yes	**0.01**	NO	0.2
Nr FC	Yes	0.39	Yes	0.85	Yes	0.93	NO	0.16
Nr VSM's	Yes	0.53	Yes	0.91	Yes	0.43	Yes	0.38
Tot length VSMs/ TV (μm)	Yes	0.94	NO	0.84	Yes	0.84	Yes	0.40
Tot length DD/ TV (μm)	Yes	0.39	Yes	0.89	Yes	0.57	NO	1.0
Mean DD length/ TV (-VSM) (sum DD/Nr DD)	Yes	0.29	Yes	0.26	Yes	0.12	Yes	0.77
Mean DD / TV (+VSM) (sum DD/Nr FC)	Yes	0.18	Yes	0.90	Yes	0.3	Yes	0.22
Mean DD/ FC	Yes	0.13	Yes	0.07	Yes	0.4231	Yes	0.82
Circumferences TV (μm)	Yes	0.59	Yes	0.30	Yes	**0.04**	NO	0.44
Area TV	Yes	0.24	Yes	0.45	Yes	0.16	Yes	**0.01**
Circumferences FC's (μm)	Yes	0.31	Yes	0.24	Yes	0.11	Yes	**0.004**
Area FC's(μm^2)	Yes	0.2	Yes	0.53	Yes	**0.03**	Yes	0.65
% Area FC / TV	Yes	0.85	Yes	0.1984	Yes	**0.02**	Yes	0.84
% circumference VSM / TV	Yes	0.33	Yes	0.11	Yes	0.25	Yes	0.58
Circumferences TV / Area TV	NO	0.13	Yes	0.44	Yes	0.99	NO	0.44

[1]ND: Normal distribution by Kolmogorov-Smirnov test. control: idiopathic preterm placentas (n=6), CA: chorioamnionitis (n=6), a membrane disease affecting terminal villi development, PE: preeclampsia, DD: diffusion distance, Nr: number, (μm): micrometer .[2] P-value > 0.05 is significative. By normally distributed variances of means score per parameter between the observers, a One-Sample t-test was performed otherwise Wilcoxon Signed-Rank test was performed.

7.2 Supplement 2: CD31: Endothelial immunostaining on paraffin-embedded histological sections. (Manually staining).

Authors: Dr. Ulrike von Rango & Paul van Dijk

1. PRINCIPLE

Deparaffinized tissue sections. Anti-retrieval on formalin fixated tissue sections to retrieve epitope binding sites and to inactivate endogenous peroxidase. Primary antibody incubation to bind an epitope of the target protein. Incubation with LINK-biotin labeled polymer to bind to the primary antibody. Incubate with streptavidin-peroxidase followed by DAB solution to visualize target protein.

2. AREA OF APPLICATION / LIMITATIONS

Histological paraffin-embedded tissue sections

3. DEFINITIONS AND TERMS

RT: room temperature.

CD31: primary antibody

PBST: phosphate buffer with Tween 20

DAB tablet: diaminobenzidine tablet

FCS: fetal calf serum

4. SAFETY

Safety procedures:

- Work according to the general laboratory safety protocols as described on the site from ARBO & Milieu.

http://www.unimaas.nl/default.asp?template=werkveld.htm&id=KTUN21U677L015143J6F&taal=nl

5. Hazardous chemicals

Chemical	*Risk*
Xylene	Carcinogenic.
Ethanol	Cancer if exposed to high concentrations
DAB diaminobenzidine chromogen	carcinogen in contact with skin and if swallowed
Methanol	Toxic
Hydrogen peroxide	Harmful

6. Waste

Information can be found at:

- The site of ARBO & milieu: http://www.unimaas.nl/default.asp?template=werkveld.htm&id=KTUN21U677L015143J6F&taal=nl
- Information poster chemical waste at the weighing room 2.111A

Solution	*Waste group*
Xylene	3
Ethanol	3
DAB (diaminobenzidine)	6
Methanol	3

7. REAGENTS, STANDARDS, AND CONTROLS

Chemical	*Firm*	*Product no.*
CD31 antibody against human clone JC70A	DAKO	M 0823
LINK kit biotinylated	DAKO	K 0690
DAB chromogen kit	DAKO	K 3468
Sodium citrate	Merck	1.06448.1000
Sodium dihydrogen phosphate monohydrate	Fluka	71507
Sodium phosphate dibasic	Sigma	S7907
Xylene	J.T.Baker	
Entallan	VWR	

8. MATERIALS AND EQUIPMENT

Materials	*Manufacturer*	*Product no.*
Eppendorf tube 1.5 ml	Eppendorf	0030 120.086

Equipment	*Manufacturer*	*Location*

MS1 mini shaker	IKA	2.102
Eppendorf pipette 0.5-10 µl	Eppendorf	2.102
Eppendorf pipette 100-1000 µl	Eppendorf	2.102

9. PROCEDURE
    - Deparaffinize sections with 2 times 5 minutes xylene.
    - Hydrate sections via grated ethanol series 100%-50%.
    - Wash in demineralized water.
    - Wash in PBS buffer.
    - Anti retrieval (AR) for formalin fixated tissue to retrieve epitope binding sites and to inactivate endogenous phosphatase. Boil sections in sodium citrate (10mM, pH 6.0). Bring the solution to boil before slides are putting in (microwave 850 watts). After putting the slides in the solution, bring the solution back to boil by starting the microwave. If the solution is boiling again set the microwave at 80 watts for 5 minutes after that let sections stay in the same solution and cool down for 30 minutes at RT.
    - Wash 2X in demineralized water.
    - Wash 5 minutes in PBS.
    - Peroxidase block with 3% H2O2/ Methanol 10 minutes.
    - Wash 3X 5 minutes in PBS.
    - Pre-incubate with 2% FCS /PBS for 30 minutes.
    - Incubation sections overnight with CD31 1:100 dilute with 2% FCS/PBS.
    - Wash 3X 5 minutes in PBS.
    - Incubate for 30 minutes RT with LINK-biotin
    - Wash 3X 5 minutes in PBST.
    - Incubate for 30 minutes with Streptavidin peroxidase (kit)
    - Wash 3X 5 minutes in PBS.
    - Develop staining with substrate chromogen solution (kit: 1ml substrate buffer + 20µl DAB). Follow staining intensity with a microscope.
    - Stop reaction by washing slides in demineralized water.
    - Counterstain if necessary with hematoxylin for 10 seconds.
    - Wash with running tap water 5 minutes.

- Dehydrate sections via grated ethanol series 50%-100% and 3 times xylene.
- Cover up sections with Entallan and coverslip

10. QUALITY CONTROL
    - Positive control: always take a positive known sample within the staining series.
    - Negative control: let a section follow the procedure but without the primary antibody.
    - Put some secondary antibody in an Eppendorf tube with the substrate, the solution must become brown within 10 minutes.
11. QUALITY CONTROL
    - Positive control: always take a positive known sample within the staining series.
    - Negative control: let a section follow the procedure but without the primary antibody.
    - Put some secondary antibody in an Eppendorf tube with DAB substrate, the solution must become brown within 10 minutes.

7.3 Supplement 3: Protocols for CD34 and CD31 paraffine section staining on immunostainer

CD34 staining

- Heat-induced epitope retrieval with PT module in DAKO pH 9 buffer, 10 min 97 ºC
- Rinse/wash slides in Wash buffer (1x5 min in Dako wash buffer + 0.1% Tween20)
- Load the DAKO machine and rinse slides with squirt bottle containing DAKO wash buffer + 0.1% Tween20
- Incubate slides with Dako H2O2 block for 5 min.
- wash
- Incubate primary antibody, diluted in Dako antibody diluent for 20 min at RT
- Wash
- Incubate slides with Envision flex/HRP for 20 min at RT
- wash
- Incubate slides with DAB for 10 min
- Wash
- Remove slides from the machine and rinse with distilled water.
- Dip slides for 30 sec. in hematoxylin
- Put slides directly in container with **tap** water
- Put container under water tap and wash for 5 minutes
- Dehydrate slides:
    - 1 min 70% Ethanol
    - 1 min 96% Ethanol
    - 1 min 96% Ethanol
    - 5 min 100% Ethanol
    - 5 min 100% Ethanol
    - 5 min xylene
    - 5 min xylene
- Covers the slides with coverslip machine.

Primary antibody: CD034 RTU Dako clone QBEnd10
Control Colon

CD31 staining

- Heat-induced epitope retrieval with PT module in DAKO pH 9 buffer, 10 min 97ºC
- Rinse/wash slides in Wash buffer (1x5 min in Dako wash buffer + 0.1% Tween20)
- Load the DAKO machine and rinse slides with squirt bottle containing DAKO wash buffer + 0.1% Tween20
- Incubate slides with Dako H2O2 block for 5 min.
- wash
- Incubate primary antibody, diluted in Dako antibody diluent for 20 min at RT
- Wash
- Incubate slides with Envision flex/mouse link for 15 min at RT
- wash
- Incubate slides with Envision flex/HRP for 20 min at RT
- wash
- Incubate slides with DAB for 10 min
- Wash
- Remove slides from the machine and rinse with distilled water.
- Dip slides for 30 sec. in hematoxylin
- Put slides directly in container with **tap** water
- Put container under water tap and wash for 5 minutes
- Dehydrate slides:
    - 1 min 70% Ethanol
    - 1 min 96% Ethanol
    - 1 min 96% Ethanol
    - 5 min 100% Ethanol
    - 5 min 100% Ethanol
    - 5 min xylene
    - 5 min xylene
- Covers the slides with coverslip machine.

Primary antibody: CD031 RTU Dako clone JC70A Control Colon

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Ik/wij verlenen het wereldwijde auteursrecht voor de ingediende eindverhandeling:
The inter-observer variation for a new method to score placental maturity

Richting: **master in de biomedische wetenschappen-klinische moleculaire wetenschappen**
Jaar: **2018**

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