Histol Histopathol (2020) 35: 849-862

http://www.hh.um.es

Histology and Histopathology
From Cell Biology to Tissue Engineering

Histological villous maturation in placentas of complicated pregnancies

Philippe Vangrieken[1], Sizzle F. Vanterpool[2,7], Frederik J. van Schooten[1], Salwan Al-Nasiry[3], Peter Andriessen[4], Ellen Degreef[5], Joachim Alfer[6], Boris W. Kramer[3,7] and Ulrike von Rango[8]

[1]School of Nutrition and Translational Research in Metabolism (NUTRIM), Department of Pharmacology and Toxicology, Maastricht University Medical Center+, Maastricht, the Netherlands, [2]Department of Reproductive Medicine, University Hospital Ghent, Ghent, Belgium, [3]School for Oncology and Developmental Biology (GROW), Department of Obstetrics and Gynaecology, Maastricht University Medical Center+, Maastricht, [4]Department of Pediatrics, Máxima Medical Center, Veldhoven, the Netherlands, [5]Foundation Laboratory for Pathology and Medical Microbiology (PAMM), Eindhoven, The Netherlands, [6]Department of Pathology, Kaufbeuren-Ravensburg, Ravensburg, Germany, [7]School for Mental Health and Neurosciences (MHeNS), Department of Pediatrics and [8]Department of Anatomy and Embryology, Maastricht University Medical Center+, the Netherlands

Summary. Chorioamnionitis and preeclampsia account for the majority of preterm births worldwide. Thus far, adequate methods for early detection or prevention of these diseases are lacking. In preeclampsia, accelerated villous maturation is believed to compensate placental insufficiency. However, little is known about the effects of placental inflammation in chorioamnionitis on villous maturation. Therefore, we established a set of morphological parameters to evaluate histological villous maturity in pregnancies complicated by chorioamnionitis and preeclampsia. Preterm placentas complicated by chorioamnionitis or preeclampsia were compared to idiopathic preterm placentas and term controls. Histological villous maturation was analyzed by means of 17 histological markers. Fourteen of these markers provided information on absolute and relative numbers of the terminal villi (TV), the extent of their vascularization (using CD31-stained sections) and their exchange capacity. In addition, the numbers of syncytial bridges, syncytial apoptotic knots and shed syncytiotrophoblasts were counted. Accelerated villous maturation in preeclampsia was demonstrated by means of histological villous remodeling and confirmed by 11 relevant markers. Chorioamnionitis, however, only showed increased area of fetal capillaries. In preeclampsia, placentas may transition from growth to maturation earlier than placentas in normal pregnancies, whereas in chorioamnionitis placental changes are more acute and therefore less elaborated at a structural level. Regression analysis suggests the number of all villi and the number of terminal villi as a percentage of all villi as parameters to evaluate histological villous maturity in preeclamptic placentas and to assist diagnosis. However, we would recommend to analyze all 11 relevant parameters to judge placental maturity in detail.

Key words: Histological villous maturation, Chorioamnionitis, Preeclampsia, Preterm birth, Scoring method

Introduction

Pregnancy specific pathologies include hypertensive disorders like preeclampsia (PE) and inflammatory related complications such as chorioamnionitis, which affect up to 8% and 4% of all pregnancies respectively

Offprint requests to: Philippe Vangrieken, School of Nutrition and Translation Research in Metabolism (NUTRIM), Department of Pharmacology, Maastrich Univeristy Medical Center+, Maastricht, the Netherlands. p.vangrieken@maastrichtuniversity.nl

DOI: 10.14670/HH-18-205

Abbreviations. AK, Syncytial apoptotic knots; AVM, Accelerated histological villous maturation; DD, Diffusion distance; HELLP, Hemolysis Elevated Liver enzymes and Low Platelets; PAMM, Foundation Laboratory for Pathology and Medical Microbiology; PE, Preeclampsia; PECAM1, Platelet and endothelial cell adhesion molecule 1; TV, Terminal villi; VSM, Vasculo-syncytial membrane.

(Kramer et al., 2005; Vanterpool et al., 2016). PE is associated with histological abnormalities in the placenta but the exact pathophysiology is still largely unknown (Ruiz-Quinonez et al., 2014; Fisher, 2015). These pathologies are the primary cause of perinatal mortality worldwide and surviving newborns may face life-long complications (Lawn and Kinney, 2014).

PE and the syndrome of Hemolysis Elevated Liver Enzymes and Low Platelets (HELLP) generally occur after 20 weeks of gestation. The diseases are not simply de novo onset of hypertension and proteinuria, but rather a syndrome involving multiple organs. Their clinical severity ranges from relatively mild to life threatening, being a major cause of severe maternal morbidity and mortality (e.g., stroke, edema and liver rupture) (Haram et al., 2009; Steegers et al., 2010; Saleh et al., 2016). This suggests that the disorder has multiple etiologies and probably is multifactorial. The leading hypothesis considers disturbed placental development during the first trimester of pregnancy to be the main cause (Steegers et al., 2010). Impaired remodeling of maternal spiral arteries due to compromised invasion of extra-villous trophoblast cells is thought to precede the development of PE (Fisher, 2015; Smith et al., 2016; Huppertz, 2018). As a first consequence, reduced trophoblastic plugs may be formed in the lumen of the spiral arteries leading to premature perfusion of the placenta in the first trimester of pregnancy, which may induce oxidative stress in both placental and embryonic cells (Burton et al., 2009; Burton and Jauniaux, 2011). Secondly, the preserved smooth muscle cells in the walls of the spiral arteries and their elastic lamina will reduce dilatation of the terminal ends at the time placental blood flow starts (Burton et al., 2009). This then leads to increased vascular resistance and reduced local placental perfusion resulting in placental hypoxia and oxidative stress (Vangrieken et al., 2018). As result of the increased blood pressure at the terminal ends of the spiral arteries, the velocity of the maternal blood reaching the intervillous space will be locally increased and may initiate mechanical damage of the villous trees (Burton et al., 2009). The products of oxidative stress, such as lipid peroxides are intrinsically pro-inflammatory and initiate increased apoptosis (Borzychowski et al., 2006; Burton et al., 2009).

Chorioamnionitis is defined as an acute inflammation of the placental membranes and chorion, which may also include the cord, in which case it is called funisitis (Yoon et al., 2001). The infection is typically due to an ascending poly-microbial bacterial infection in the setting of membrane rupture or very small fastidious genital mycoplasmas such as *Ureaplasma* species and *Mycoplasma hominis* when the membranes are still intact (Eschenbach, 1993). Plasmodium vivax and/or probably also P. falciparum can cause pregnancy complications because they are able to induce syncytial damage and thus can enter the syncytiotrophoblast or even the placental villus (Crocker et al., 2004; Robbins and Bakardjiev, 2012). In cases of clinical chorioamnionitis maternal fever, uterine fundal tenderness, turbid amniotic fluid and maternal/ fetal tachycardia can be found (Williams et al., 2012). Chorioamnionitis can result in stillbirth, neonatal sepsis, chronic lung diseases, brain injury and maternal postpartum infections and sepsis (Tita and Andrews, 2010). Symptomatic clinical management is mainly directed to delay preterm birth and limit fetal and maternal morbidity and mortality (Steegers et al., 2010). Histological chorioamnionitis is diagnosed in the placenta after birth by diffuse infiltration of neutrophils in different placental sites. Intra-amniotic infection is generally considered to be the main cause of acute chorioamnionitis and funisitis. Nonetheless, a "sterile" intra-amniotic inflammation can occur in the absence of evidence of colonization by microorganisms (Romero et al., 2014).

Currently, little is known about histological villous maturation in chorioamniotic placentas. As the recurrence risk of preeclampsia and especially preterm birth with histological chorioamnionitis is high (Redline, 2015), it is mandatory to be able to identify placental aberrations and to make a definitive diagnosis of the underlying pathology.

The morphology of the placenta changes as pregnancy progresses in order to increase the efficiency of the exchange of nutritional compounds and O_2/CO_2 between mother and child. Placental maturation is generally associated with an increase in diffusion surface and a decrease of the diffusion distance (Ruiz-Quinonez et al., 2014). The degree of differentiation of terminal villi (TV) accounts for the placental efficiency as they contain large coils of fetal vessels. Their vascular endothelial basal membrane is partially fused to the basal membrane of the syncytiotrophoblast forming the so-called vasculo-syncytial membranes (VSM). The number and length of these VSM as well as number and circumference of the fetal capillaries are useful parameters to assess placental efficiency (Fig. 1) (Burton et al., 2009; Haram et al., 2009; Steegers et al., 2010; Fisher, 2015).

In addition to the markers mentioned above which focus on the efficiency of placental exchange, syncytial knotting is widely accepted as a reflection of placental maturation and accelerated histological villous maturation (AVM) (Kaufmann and Huppertz, 2007; Loukeris et al., 2010; Coleman et al., 2013; Turowski and Vogel, 2018). Syncytial knotting has been attributed to pathologically increased compensatory villous branching (Huppertz et al., 2006; Kaufmann and Huppertz, 2007; Burton and Jones, 2009). It represents the extent of tangential sectioning of syncytiotrophoblast (and therefore probably the extent of villous branching) as well as the number of syncytial apoptotic knots (AK) as the final event of the trophoblast turnover cascade. Therefore, syncytial knotting is an important characteristic of mature placentas (Mayhew, 2001). AK eventually are shed into the maternal circulation (Huppertz et al., 2006; Kaufmann and Huppertz, 2007;

Burton and Jones, 2009).

It is already suggested that adverse pregnancy and fetal outcome are linked to placental senescence (Gomez-Lopez et al., 2017; Maiti et al., 2017; Sultana et al., 2017, 2018). Changes in histological villous maturation may be a placental adaptation in response to the pathology e.g. maternal vascular mal-perfusion (Turowski and Vogel, 2018; Christians and Grynspan, 2019). Histological maturation in placentas of adverse pregnancy outcomes such as preterm birth after PE or chorioamnionitis have not been analyzed in detail in the context of the underlying pathology. However, detailed analysis of histological villous maturation may reveal the pathophysiology. Knowledge of what happens to the placenta during pregnancy may improve follow up of both the mother and child and reduce recurrence risk.

The present study therefore aims to increase the knowledge of specific placental aberrations/adaptations by evaluating a set of parameters of TV maturation that can be used to assess placental maturity and link changes in histological villous maturation to a specific pathology.

Materials and methods

Placental specimen

Preterm placentas from a total of 306 singleton pregnancies were recruited from the public hospital Máxima Medical Center in Veldhoven, The Netherlands between January 1st 2009 and December 31st 2010. PE was diagnosed as new onset hypertension (blood pressure >140/90 mmHg or mean arterial pressure >105 mmHg recorded on at least two separate occasions) after 20 weeks gestation accompanied by proteinuria (>300 mg/24h) (Smit et al., 2015). As suggested by the Amniotic Fluid Infection Nosology Committee, placental and umbilical cord samples were assessed for signs of histological chorioamnionitis by determining the presence of polymorphonuclear cells present in the chorionic plate or membranous chorionic connective tissue and/or the amnion. The diagnosis of histologic chorioamnionitis with funisitis included any of the following features: chorionic vasculitis, umbilical phlebitis, umbilical (pan) vasculitis, (sub-acute) necrotizing funisitis, or concentric umbilical perivasculitis (Redline et al., 2003; Vanterpool et al., 2016). Spontaneous idiopathic preterm pregnancies, used as preterm control, were defined as pre-term birth with no known clinical abnormalities. For this study a cohort of 100 preterm placentas from pregnancies complicated by chorioamnionitis (+/- funisitis) (29±2 weeks), early onset PE (+/- HELLP) (30±2 weeks) and spontaneous idiopathic preterm pregnancies (30±2 weeks) could be included. Exclusion criteria were:

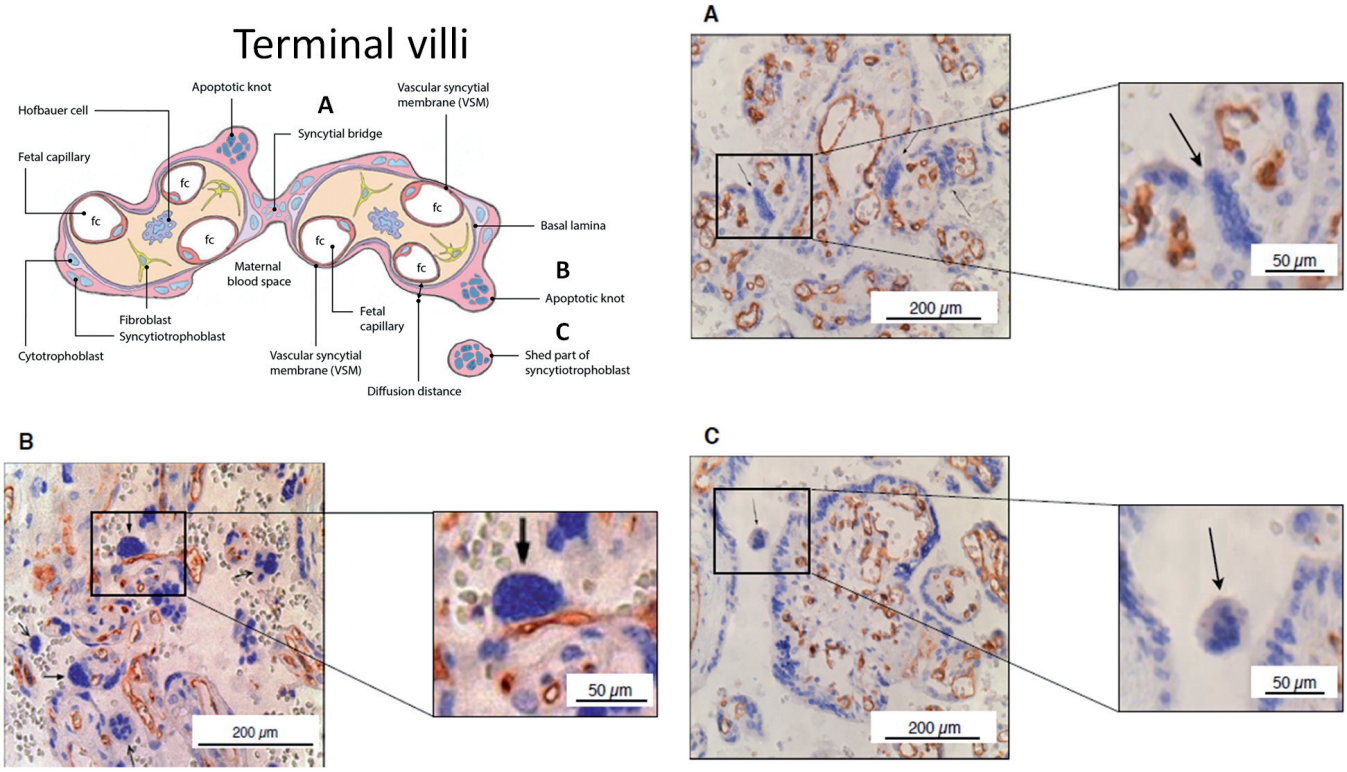

Fig. 1. Schematic example of a cross-section of a terminal villi including all investigated parameters. An example of a Syncytial bridge **(A)**, Apoptotic knot **(B)** and Shed part of syncytiotrophoblast **(C)**, all indicated with arrows.

missing of relevant clinical data, multiples, presence of other pathologies, insufficient amount of suitable tissue or poor morphological quality. For accurate morphometric analysis of TV, the assessment of the quality of the samples used for detailed morphometric analysis was stricter in comparison with the assessment of syncytial knotting. Therefore, only 70 samples were suitable for detailed histological analysis of TV. In addition to the preterm cohort, a group of 15 singleton term placentas (39±1.2 weeks) from singleton pregnancies was collected at Maastricht University Medical Center, 2015-2016 as a positive control for normal complete maturation (see table 1 for clinical data). Placentas of both cohorts were collected immediately after each delivery and fixed for 24-48 hours. The placenta was cut in slices of 1-2 cm to determine the presence of macroscopic lesions. Three blocks of normal placental parenchyma (sampled from the central area of the placenta) were embedded in paraffin, processed and stained with hematoxylin and eosin (Vanterpool et al., 2016). Blocks showing the best tissue morphology were used for further analysis.

Ethical aspects

The collection of the placentas and the use of the placental specimens were done according to Dutch law. The use of placentas from patients of Máxima Medical Center, Veldhoven, was approved by the local Medical Ethical Committee of the Máxima Medical Center, considering that retrospective and anonymous data collection was performed (reference number 20.12.2011). The collection of the 15 term placentas were approved by the Medical Ethics Committee Academic Hospital Maastricht and Maastricht University (METC 16-4-047).

Analysis of the placental weight adjusted for gestational age

To examine whether preterm placentas show differences in weight between the three preterm groups and to validate our idiopathic preterm control group. Data reported by Kaufmann P, extrapolated from Boyd & Hamilton and O'Rahilly on the correlation between placental weight and gestational age was used to correlate placental weight in our cohort to the expected placental weight (Benirschke and Driscoll, 1967; Castellucci, 2006).

Immunohistochemistry

To visualize blood vessels, sections were stained for CD-31 PECAM1 (platelet and endothelial cell adhesion molecule 1). Placental sections were first deparaffinized with absolute Xylene and hydrated via graded ethanol series (100%-50%-DMEM-PBS (Dulbecco's Modified Eagle's Medium-Phosphate Buffered Saline)). Sections were boiled in a sodium citrate solution (10 mM, pH 6.0) for 5 minutes and washed twice in DMEM and PBS to unmask relevant epitopes. Endogenous peroxidase was blocked in 0.3% H_2O_2/Methanol for 30 minutes and 5% normal goat serum (NGS) was used to minimize nonspecific antibody binding. After the blocking procedure, placental sections were incubated with CD31 PECAM1 (DAKO clone JC70A, Dako Denmark, 1:200 in PBST (Phosphate Buffered Saline with Tween 20)/5% NGS) overnight at 4°C. After washing, a secondary antibody: goat anti-mouse (GAM-biotin Vector BA9200; 1:1000 in PBST/5% NGS) was allowed to bind for 30 minutes at room temperature. HRP coupled Avidin/Streptavidin-biotin complex (ABC (Avidin-biotin-complex) kit Elite, Vectastain PK6100, Vector Laboratories Inc., 30 Ingold Road, Burlingama, CA 94010) was used to amplify the signal and incubated for 30 min at room temperature. Sections were then incubated with the substrate 3,3'-diaminobenzindine (DAB). Staining was stopped with water and sections were counter-stained using hematoxylin. Finally, placental sections were dehydrated via a graded alcohol series (70%, 90%, 96%, 2x 100%) and covered with Entellan (Merck KGaA, 64271 Darmstadt, Germany).

Table 1. Characteristics of the patients.

Variable	Idiopathic preterm control Mean ± SD	Chorioamnionitis +/- funisitis Mean ± SD	PE +/- HELLP Mean ± SD	Non-complicated term control Mean ± SD	ND	p^1	p^2
Number placentas	24	41	35	15	/	/	/
Maternal age (years)	30±5	30±5	32±4	31±5	Yes	0.0638	0.1209
Gestational age (weeks)	30±2	29±2	30±2	39±1	No	0.0752	<0.0001
Birth weight (g)	1301±379	1292±316	1143±261	3258±380	Yes	0.0696	<0.0001
Gravidity	2±2	2±2	2±1	2±2	No	0.1802	0.1182
Parity	1±1	1±2	0±1	1±1	No	0.1244	0.1043
Fetal gender (male/female), %	29/71	46/54	63/37	47/53	/	/	/
SGA, %	13	4	11	0	/	/	/
C-section, %	42	17	97	33	/	/	/

PE, Preeclampsia; HELLP, Hemolysis, Elevated Liver enzymes and Low Platelets; ND, Normal distributed; [1]p-value including the idiopathic preterm control, the chorioamnionitis (+/- funisitis) and the PE (+/- HELLP) groups, [2]p-value including all groups, SGA: Small for gestational age.

For negative controls, the first antibody was replaced by buffer.

Analysis of the placental sections

Sections were examined with a light microscope (Leica DMRXA, Leica Microsystems GmbH, Ernst-Leitz-Strasse 17-37, 35578 Wetzlar, Germany).

Before the analysis, three different areas of each section were systematically randomly sampled. This was done by selecting 3 adequate regions at a 10x magnification including 2 peripheral (left and right corner) and one central region of the placental specimen. Then from each region a random picture was made at a 20x magnification, which was used for our analysis. First, the mean number of all villi and TV cross sections was counted giving a first indication of the degree of villous branching. From these data, the relative amount (%) of TV were calculated. Based on the definition of Kaufmann et al. 1976, a villus was defined as TV if at least 30% of its cross sectional surface was covered by fetal capillaries and if it contained at least two VSM (Fig. 1) (Benirschke and Driscoll, 1967). Villous cross sections fulfilling the criteria of a TV and being fully located in the high power field were included in further evaluation routines including area, circumference and distance measurements, using the Leica-QWin standard software. For each TV cross section, the number of fetal capillaries was counted and their cross sectional area was measured. This allows the calculation of the area of the high power field covered by TV and percentage of area occupied by fetal capillaries. In addition, per TV, the number of VSM was counted and their length was measured. Using these data and the measurement of the circumference of the TV cross section allowed us to calculate the percentage of the TV circumference that was covered by VSM (Fig. 1, Table 2). The mean diffusion distance per TV represents the average of the shortest distance between the fetal capillaries and the TV membrane (Fig. 1).

In addition to the detailed structure of the TV, syncytial knotting as a maturation characteristic of the syncytiotrophoblast was analyzed. Syncytial knots were defined as aggregates of syncytial nuclei at the surface of terminal villi (Kaufmann and Huppertz, 2007; Loukeris et al., 2010). Syncytial bridges appear as inter-villous bridges and tangential flat sectioning according to Jones et al. (Fig. 1A) (Jones and Fox, 1977). True AK were defined as isolated round or elliptic structures containing at least 10 strong accumulated syncytiotrophoblast pyknotic nuclei projected from the cross sectional villous surface and showing densely packed chromatin according to Johyansen et al. (Fig. 1B) (Johansen et al., 1999). If the syncytial/apoptotic knot could not be clearly assigned to a villus (at least one fetal capillary has to be present in the villus) it was classified as a shed syncytiotrophoblast according to Askelund et al. (Fig. 1C) (Askelund and Chamley, 2011). Syncytial knots, syncytial bridges, AK and shed parts of the syncytiotrophoblast all together represent syncytial knotting (see Figs. 1, 2 for examples).

Subclasses of syncytial knotting were characterized by two independent blinded investigators to minimize bias. Values of the Intraclass Correlation Coefficient calculated to evaluate inter-observer agreement were: 0.92 for the shed parts of the syncytiotrophoblast, 0.87 for the AK and 0.78 for the syncytial bridging. Landis and Koch classify scale values of 0 as “no agreement”,

Table 2. Morphometric analysis of the terminal villi.

Expressed in mean/view	Idiopathic preterm Mean ± SD	Chorioamnionitis +/- funisitis Mean ± SD	PE +/- HELLP Mean ± SD	Non-complicated Term Mean ± SD	ND	p^1	p^2
Number placentas	18	30	22	15	/	/	/
Number all villi	35.2±10.8	31.3±10.1	44.8±8.4	38.5±9.3	No	<0.0001	0.0001
Number TV	4.7±4.5	5.3±4.2	13.5±8.3	8.9±5.0	No	0.0001	<0.0001
TV, %	13.9±12.5	23.5±17.5	38.6±23.5	31.9±18.3	No	0.0018	0.0015
Area covered by TV, %	3.9±3.4	7.8±8.2	13.5±9.2	14.3±5.4	No	0.0009	<0.0001
Number FC/ TV	6.2±2.8	6.3±2.0	6.5±1.6	6.8±3.3	No	0.2926	0.4483
Number VSM/ TV	2.7±0.5	3.0±0.8	3.4±0.5	3.6±0.6	No	0.0013	0.0001
Total length VSM/ TV (μm)	42.4±15.2	47.8±19.3	54.0±16.3	71.6±20.3	No	0.0594	0.0004
Length DD/ FC (μm)	3.8±2.4	4.1±2.6	2.9±1.0	3.3±1.0	No	0.0653	0.1314
Circumferences TV (μm)	263.5±77.4	284.7±74.1	258.6±39.6	304.1±66.1	No	0.2919	0.1402
Area TV (μm2)	4008.8±2235.1	5133.7±2720.3	3768.4±987.7	4946.2±1014.0	No	0.1068	0.0302
Circumferences FC (μm)	423.8±215.7	454.1±168.1	480.1±326.3	432.8±78.5	No	0.3225	0.4255
Area FC/ TV (μm2)	1400.0±982.5	2141.7±1164.2	1652.4±464.7	2369.3±1009.6	No	0.0184	0.0042
Area FC/ TV, %	35.8±12.6	41.1±5.5	43.9±5.2	45.0±9.4	No	0.0549	0.0677
Circumference VSM/ TV, %	16.6±5.1	17.5±8.2	21.0±5.5	24.3±7.7	No	0.0115	0.0017

PE, Preeclampsia; HELLP, Hemolysis, Elevated Liver enzymes and Low Platelet; TV, Terminal villi; FC, Fetal capillary; VSM, Vasculo-syncytial membrane; DD, Diffusion distance; ND, Normal distributed. [1]p-value including the idiopathic preterm control, the chorioamnionitis (+/- funisitis) and the PE (+/- HELLP) groups, [2]p-value including all groups.

0-0.20 as "slight agreement", 0.21-0.40 as "fair agreement", 0.41-0.60 as "moderate agreement", 0.61-0.80 as "substantial agreement" and 0.81-1 as "perfect agreement" (Landis and Koch, 1977). In this rating scheme, the inter-observer variability of the present study is at the edge of "substantial agreement" to "perfect agreement".

In addition to the TV analysis, immature intermediate villi were counted as well (Fig. 3).

Statistical analyses

When the D'agrostino and Pearson omnibus normality test did not find a departure from a Gaussian

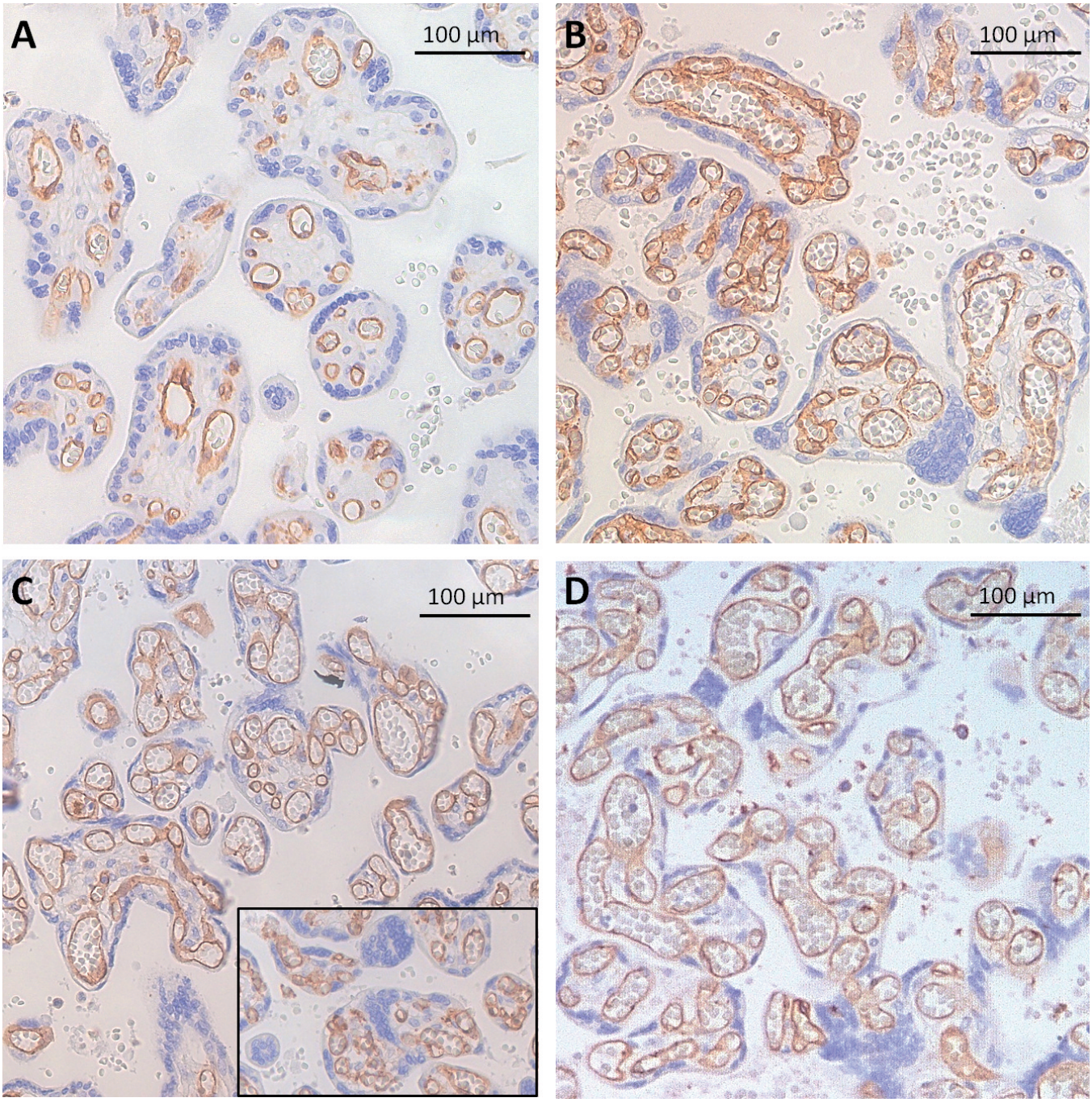

Fig. 2. Representative part of the high power field of each group. Idiopathic preterm control (**A**), Chorioamnionitis (+/- funisitis **(B)**, Preeclampsia (+/- HELLP) **(C)** and Non-complicated term control **(D)**.

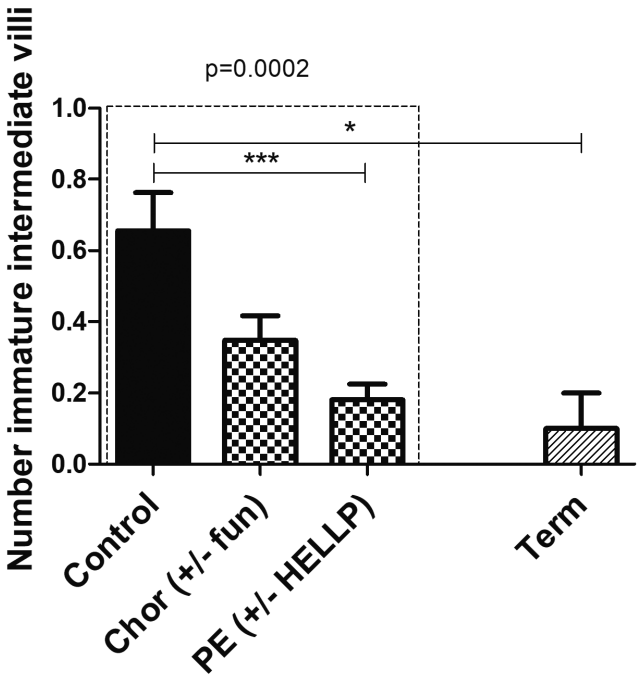

Fig. 3. Mean number of immature intermediate villi, fully located in the high power. Data are presented as median with SEM. Ns: p>0.05, *p≤0.05, ** p≤0.01, *** p≤0.001. P-values given include all 3 groups.

distribution, the ANOVA one-way analysis of the variance was used followed by the multiple comparison Tukey post-hoc test. If not, the Kruskal-Wallis test was used followed by the Dunn's multiple comparison post-hoc test using GraphPad Prism 6 for Windows, GraphPad Software, La Jolla California USA. A p-value <0.05 was considered as significantly different and was presented as follows: Ns: p >0.05, *p ≤0.05, **p ≤0.01, ***p ≤0.001. Inter-observer agreement was tested by the Intraclass Correlation Coefficient test. Correlates of PE and chorioamnionitis, including all markers for AVM and the clinical parameters used in our study were subjected to multivariate analysis. These correlates were weighted by means of binary stepwise forward logistic regression against each other and only those that demonstrated independence from each other were retained in the model using SPSS (IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp).

Results

Clinical characteristics of the patients

Basic parameters of the 4 test groups: the idiopathic preterm group (preterm control), the chorioamnionitis group, the PE group and the non-pathological term

Table 3. Comparison between placental weight in the cohorts and their expected weight.

Variable	Placental weight in cohort Mean ± SD	Expected placental weight Mean ± SD	p
Idiopathic preterm	286±117	276±27	0.6872
Chorioamnionitis (+/- funisitis)	296±70	258±32	0.0041
PE (+/- HELLP)	220±60	273±32	<0.0001

PE, Preeclampsia; HELLP, Hemolysis, Elevated Liver enzymes and Low Platelets.

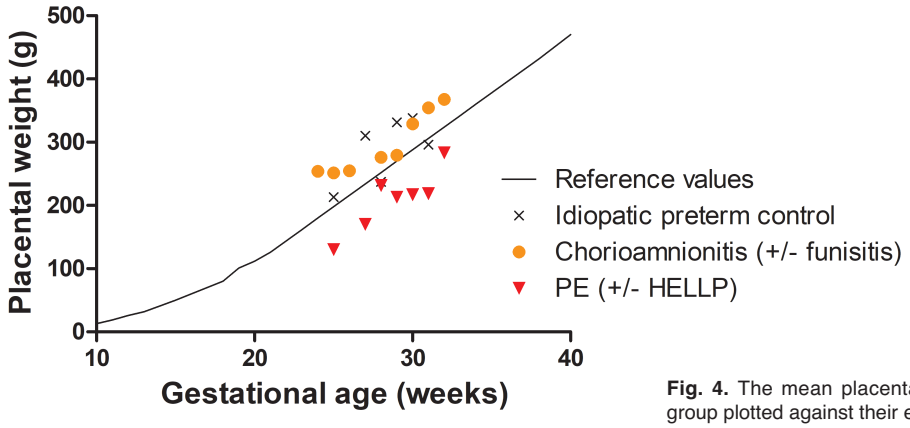

Fig. 4. The mean placental weight per gestational age for each preterm group plotted against their expected placental weight.

group (term control, gestational age >37 weeks) were tested for several clinical parameters. All data are presented as means and standard deviation (Table 1).

There were no significant differences in clinical characteristics found between all the preterm groups. In the term group gestational age, placental and birth weight were increased as expected (Table 1). Being small for gestational age in all groups did not correlate with chorioamnionitis (p=0.477) or PE (p=0.428) and was tested by logistic regression. In PE AVM has

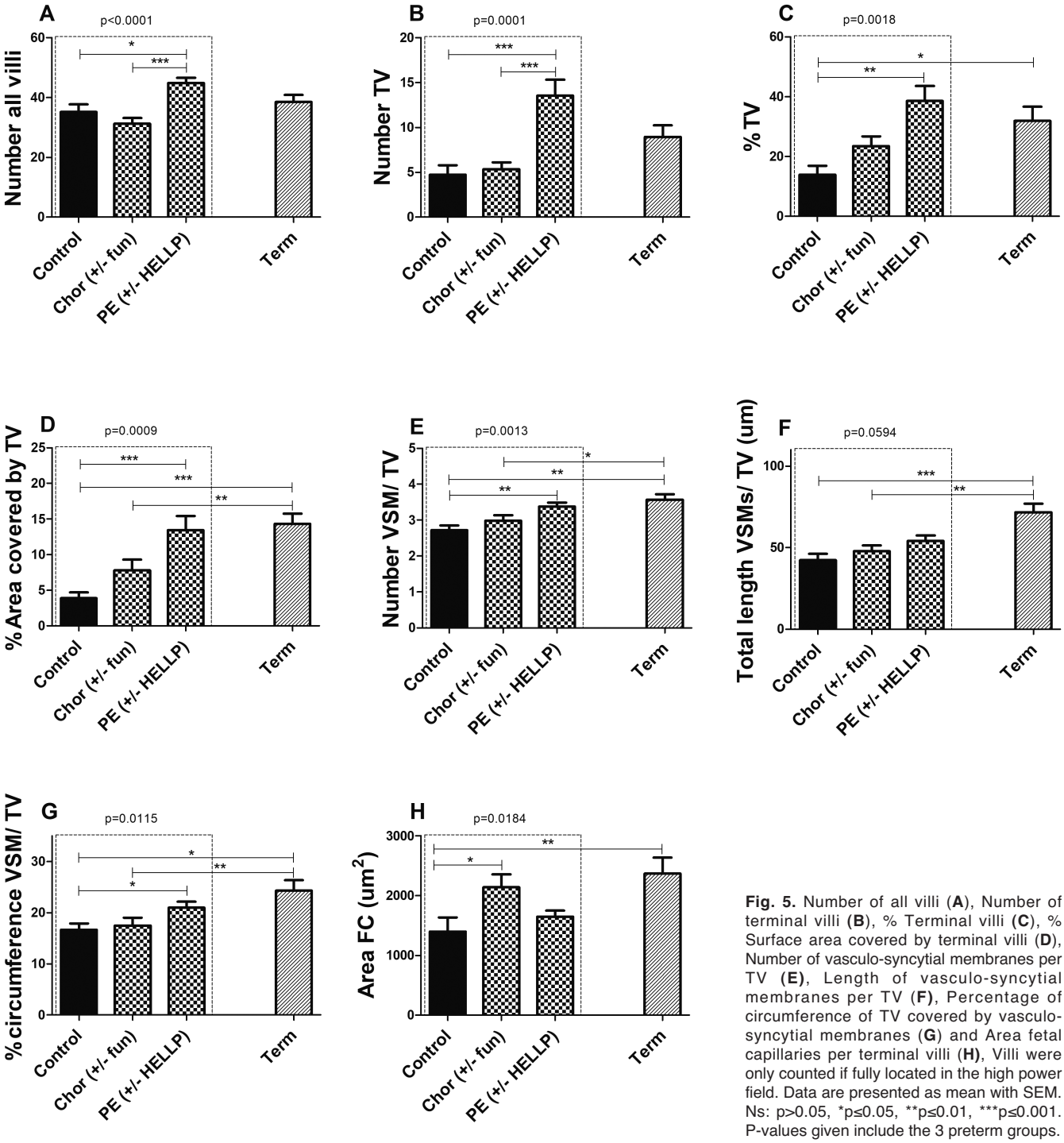

Fig. 5. Number of all villi (**A**), Number of terminal villi (**B**), % Terminal villi (**C**), % Surface area covered by terminal villi (**D**), Number of vasculo-syncytial membranes per TV (**E**), Length of vasculo-syncytial membranes per TV (**F**), Percentage of circumference of TV covered by vasculo-syncytial membranes (**G**) and Area fetal capillaries per terminal villi (**H**), Villi were only counted if fully located in the high power field. Data are presented as mean with SEM. Ns: p>0.05, *p≤0.05, **p≤0.01, ***p≤0.001. P-values given include the 3 preterm groups.

previously been shown. Therefore, the PE group served as internal validation of our scoring system.

Analysis of the placental weight adjusted for gestational age

The actual placental weight of the included placentas was compared to reference data of a control cohort (Benirschke and Driscoll, 1967; Castellucci, 2006). Placentas of the idiopathic preterm group did not show significant differences compared to the reference data of the control cohort. Reduced placental weight (19%, $p<0.001$) was found in the PE group, and increased placental weight (15%, $p=0.002$) in the chorioamnionitis group (Fig. 4 and Table 3).

Analysis of the parameters for histological villous maturation

Idiopathic preterm vs. fully maturated

As expected, by comparing the fully maturated term group to the idiopathic preterm group, all markers for TV maturation were found to be increased. However, the number of all villi, number of TV and the number of syncytial knots (shedded or not shedded) were not increased significantly in the term group compared to the idiopathic preterm control (Figs. 5, 6 and Tables 2, 4).

All preterm groups

Comparing the subgroups PE and PE + HELLP, revealed no differences for all parameters included in this study (separate data not shown). The subgroups chorioamnionitis and chorioamnionitis + funisitis also did not show differences for all parameters included in this study (separate data not shown). Therefore, the samples were analyzed as one PE +/- HELLP group and chorioamnionitis +/- funisitis group. As it is considered that clinical and histological chorioamnionitis may show differences in villous maturity, we compared 6 samples diagnosed as clinical + histological chorioamnionitis to samples with chorioamniotic histological "only". There were no differences found for all parameters included in this study (data not shown). Therefore, all samples were analyzed as one chorioamnionitis group. Except for the

Table 4. Mean number AK, syncytial bridges, NA and STB per high power field.

Expressed in mean/view	Idiopathic preterm Mean ± SD	Chorioamnionitis +/- funisitis Mean ± SD	PE +/- HELLP Mean ± SD	Non-complicated Term Mean ± SD	ND	p^1	p^2
Number placentas	24	41	35	15	/	/	/
Number AK	0.7±0.9	0.3±0.3	1.7±1.3	0.7±0.3	No	<0.0001	<0.0001
Number syncytial bridges	5.3±2.4	3.5±1.4	8.0±2.4	8.3±2.6	Yes	<0.0001	<0.0001
Number STB	0.9±0.8	0.7±0.6	2.2±1.4	0.7±0.6	No	<0.0001	<0.0001

PE, Preeclampsia; HELLP, Hemolysis, Elevated Liver enzymes and Low Platelets; ND, Normal distributed; AK, Apoptotic knots; STB, Shed parts of syncytiotrophoblast. [1]p-value including the idiopathic preterm control, the chorioamnionitis (+/- funisitis) and the PE (+/- HELLP) groups, [2]p-value including all groups.

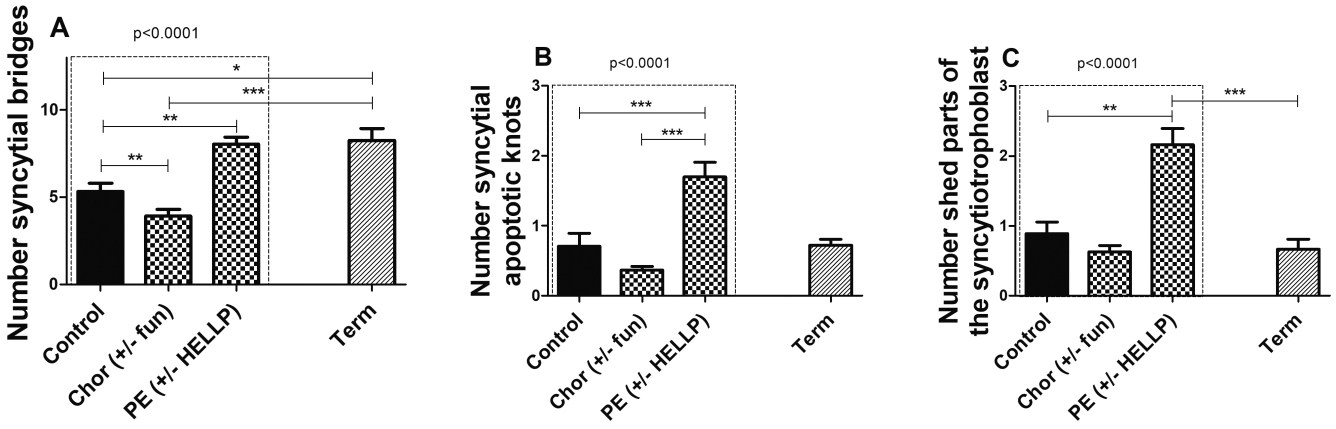

Fig. 6. Mean number of total Syncytial bridges **(A)**, Syncytial apoptotic knots **(B)** and Shed part of syncytiotrophoblasts (**C**), fully located in the high power field. Data are presented as mean with SEM. Ns: $p>0.05$, *$p\leq0.05$, **$p\leq0.01$, ***$p\leq0.001$. P-values given include the 3 preterm groups.

Table 5. Binary step forward logistic regression of significant parameters of histological villus maturation of preeclampsia (+/-HELLP) complicated placentas.

		Predicted Preeclampsia (+/-HELLP)				
Observed		No	Yes	Accuracy	Sensitivity	Specificity
Preeclampsia (+/-HELLP)	No	14	4	0.80	0.82	0.78
	Yes	4	18			
	Coefficient B	Adjusted p-value	Adjusted OR	95% CI		
Number all villi	0.118	0.013	1.125	1.026-1.234		
TV, %	0.086	0.007	1.089	1.024-1.160		

HELLP, Hemolysis, Elevated Liver enzymes and Low Platelet; TV, Terminal villi.

Table 6. Binary step forward logistic regression of significant parameters of histological villus maturation of chorioamnionitis (+/-funisitis) complicated placentas.

		Predicted Chorioamnionitis (+/-Funisitis)				
Observed		No	Yes	Accuracy	Sensitivity	Specificity
Chorioamnionitis (+/-Funisitis)	No	18	6	0.80	0.83	0.75
	Yes	7	34			
	Coefficient B	Adjusted p-value	Adjusted OR	95% CI		
Number syncytial bridges	0.372	0.010	1.450	1.092-1.925		
Number STB	0.983	0.010	2.672	1.266-5.643		

STB, Shed parts of syncytiotrophoblast.

length of the VSMs per TV, all parameters showed a significant difference between the 3 preterm groups (Figs. 5, 6 and Tables 2, 4).

Preeclampsia

When comparing the PE group to the preterm control group, the total number of villi was increased and there were more TV (absolute and relative to all villi) present. Furthermore, TV covered a larger area and showed more VSM covering an increased percentage of the TV circumference. The area covered by fetal capillaries was not changed. Numbers of syncytial knots and syncytial bridges were strongly increased. Numbers of all villi per view, as well as number, percentage of TV and the number of AK and shed parts of the syncytiotrophoblast were even higher in the PE group compared to the term control by 1, 34, 17, 57 and 69% respectively, however not statistically significant except for the number of shed parts of the syncytiotrophoblast (Figs. 5, 6).

Chorioamnionitis

There was a significant increase in the area covered by fetal capillaries and a significant decrease in syncytial bridges (Figs. 5, 6).

Based on the binary step forward logistic regression analysis the significant parameters including the clinical parameters were weighted against each other. The number of all villi and the percentage of TV were found to be independent parameters in the prediction model of histological villous maturation (including only idiopathic preterm controls and cases of preeclampsia (+/-HELLP)) and were able to correctly predict 80% of the cases of PE within the cohort with a sensitivity of 0.82 and a specificity of 0.78. The number of syncytial bridges and shed parts of the syncytiotrophoblast were able to correctly predict 80% of the cases of chorioamnionitis within the cohort including only idiopathic preterm controls and cases of chorioamnionitis (+/-funisitis) with a sensitivity of 0.83 and specificity of 0.75 (Tables 5, 6).

Discussion

By using our placental parameters, we could show that:

1. While the number of all villi and TV, as well as syncytial knotting were not significantly different between term and preterm placentas, all other parameters accounting for histological villous maturation were increased in term placentas compared to the preterm

control. This may suggest that in preterm samples (30±2 weeks) branching of the placenta should be nearly completed, and histological villous maturation is then further increasing placental efficiency.
2. Concerning the PE group, all maturation markers were significantly increased compared to the preterm control group. The number of shed parts of the syncytiotrophoblast were even higher compared to the term control. This confirms that the PE group shows AVM in order to increase placental efficiency to compensate for compromised blood supply.
3. No differences between clinical and histological chorioamnionitis were found for all parameters included in this study. Furthermore, the chorioamnionitis group, including both subgroups, did not show any sign of AVM. However, the area covered by fetal capillaries was significantly increased. This may suggest that chorioamniotic placentas try to adapt to increase exchange capacity by dilation of fetal capillaries in response to the acute infection instead of developing histological AVM. This was supported by the fact that syncytial bridging was significantly decreased compared to the control group (Naeye et al., 1983a; Mihu et al., 2009; Taki et al., 2012).

The number of villi, TV, percentage TV and the total length of VSM progressively increases towards the third trimester of pregnancy to ensure optimal gas exchange and nutritional supply between mother and fetus (Mihu et al., 2009; Ruiz-Quinonez et al., 2014). Inadequate levels of VSM and TV in the placenta can be associated with a higher incidence of neonatal asphyxia and fetal distress (Fox, 1967; Sankar et al., 2012).

Our data show that in cases complicated with PE, several maturation markers for villous characteristics are closer to the term than to the idiopathic preterm group, or even exceeded that of the term group, suggesting placental hyper maturation.

The increase in the number of villi, TV and VSM/TV in the PE group suggests an early effort to increase the efficiency of the placenta for the diffusion of gases and nutritional compounds by AVM and increased branching of the villi in PE (Kaufmann et al., 1985). These data illustrate how a placenta, complicated with PE may compensate for the compromised blood supply, initiated by impaired remodeling of the spiral arteries (Burton et al., 2009). In line with our findings, earlier studies linked AVM (diagnosed as increased numbers of placental villi), to hypoxic conditions in utero, maternal vascular malperfusion, placental insufficiency and severe case of fetal growth restriction (Garcia-Honduvilla et al., 2018; Turowski and Vogel, 2018; Yaguchi et al., 2018). Recently, increased vascularization was described in PE placentas (Eddy et al., 2018). This may increase efficiency of the placenta and is in line with the increase of VSM we found in our PE group.

In addition, we found a decrease in weight of placentas complicated with PE. This suggests that the switch from placental growth to an increase in efficiency via maturation takes place earlier in gestation. This may be realized via earlier differentiation of mature intermediate villi and TV from immature intermediate villi. Indeed, we found a significantly reduced number of immature intermediate villi in PE placentas (Fig. 3). The resulting lower number of large stem villi might also contribute to the lower placental volume (Castellucci et al., 1990).

Syncytial knotting and syncytial bridging were found to be increased in the PE group. This may indicate a reaction of the placenta to the known placental hypoxic stress in PE. In agreement with our data, hypoxia as a result of venous insufficiency, was already found to be responsible for increased numbers of syncytial knots and syncytial bridges and the changes in the remodeling state of placental villi (Garcia-Honduvilla et al., 2018; Ortega et al., 2018). This also may be an explanation for the higher amount of cellular debris around the villi found earlier (Huppertz et al., 2003; Myatt and Cui, 2004; Cindrova-Davies et al., 2007; Burton et al., 2009; Cindrova-Davies, 2009; Sharp et al., 2010; Arad et al., 2017).

In agreement with recent findings (Christians and Grynspan, 2019), placentas complicated with chorioamnionitis did not show such an AVM, but the area of fetal capillaries was increased per TV. Thus, placental inflammation is not reflected in placental maturation. Probably, increasing the volume of the fetal capillaries and thus the exchange volume in the placenta, may be an acute reaction to an acute infection. This may also explain the increased placental weight which might be due to extensive villous edema and fibrous connective tissue formation in response the placental inflammation, and/or to the increase in villous macrophages (Hofbauer cells) (Naeye et al., 1983b; Pankuch et al., 1984; Tang et al., 2011). These acute changes may explain why in most cases chorioamnionitis is not detected before birth and does not give rise to maternal symptoms and does not impair fetal growth but may induce preterm labour. Probably, the clinical symptoms are only elicited from bacteria reaching the maternal circulation in a very advanced stage.

The gold standard of placental analysis would be a three-dimensional (3D) reconstruction of the placental villous tree. The study showed that the current gold standard using 2D-histologic identification of villous types is still valid but needs to be used with caution (Haeussner et al., 2015). This underlines the need for adequate markers for 2D cross section analysis. Furthermore, the interpretation of the morphology of villous maturation is subjective. Recent studies suggest that the interpretation of maturation disorders might be improved and objectified by immunohistochemistry and molecular analysis (Turowski and Vogel, 2018).

By looking at AVM, we identified a unique combination of 11 different parameters, which may assist in the analysis of villous maturation in order to diagnose placentas complicated by PE, chorioamnionitis or preterm birth. In our scoring model, we minimized the

subjective steps by strict criteria and the implemented software. Moreover, the wide combination of detailed morphological parameters for placental development allowed us to:
a) show which histological adaptations take place in the placenta over gestation
b) identify independent parameters needed for the analysis of histological villous maturation for different preterm pregnancy-related complications
c) link changes to the pathophysiology of the pathological placenta

The number of all villi and the percentage of TV were identified as independent parameters and entered the model, which was able to correctly predict 80% of the PE cases. However, when examining complicated placentas with a well-defined clinical complication, we would recommend to analyze all 11 parameters that proved AVM in the PE group, because they are useful to judge maturity in a born placenta in detail and may provide pathologists with a tool to histologically underpin the clinical diagnosis of PE. As the recurrence risk of PE and chorioamnionitis is high (Laibl et al., 2006; Dypvik et al., 2017; Roberts et al., 2017), it is useful to increase the knowledge of specific placental aberrations/adaptations to prevent recurrence of the pathology during a next pregnancy or develop a better/earlier clinical management.

Sources of funding. Revolving Fund Erasmus MC Rotterdam. Sizzle Vanterpool was supported by the AXA grant. Maastricht. This study was also funded by the NUTRIM Graduate Program. The study sponsors were not involved in the study design, data collection and analysis, interpretation of the results or manuscript preparation.
Conflict of interest. The authors have no conflict of interest to declare.

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Accepted January 27, 2020