Definition and quantification of 3-dimensional imaging targets to phenotype pre-eclampsia subtypes: an exploratory study

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Abstract

Objective: Pre-eclampsia is a severe placenta related complication of pregnancy and aetiological knowledge, with limited early diagnostic and therapeutic options. Phenotyping of native placental three-dimensional (3D) morphology offers a novel approach to improve our understanding of the functional and structural placental abnormalities underlying this clinical syndrome. The aim of this project was to develop a 3D placental imaging protocol using multiphoton microscopy (MPM) and demonstrate quantifiable imaging targets for phenotyping 3D features of pre-eclampsia. Design: Exploratory pilot study. Setting: Single centre, MUMC. Sample: Formalin fixed placental biopsies from: term control (n=3), pre-eclampsia (n=3), preterm birth (n=2), 2nd trimester placenta (n=1), and intra-uterine growth restriction cases without pre-eclampsia (n=2). Methods: Placental slabs were visualised with MPM. Collagen and cytoplasm (based on inherent signal), and fluorescently stained nuclei and blood vessels, enabled the visualization of villous tissue with subcellular resolution. Segmentation based on pixel classification, deep learning, and clustering algorithms were used to generate quantifiable features. Main outcome measures: Trophoblast arrangement, 3D-villous tree structure, syncytial knots, fibrosis, and 3D-vascular networks were identified as imaging targets. Villous morphology, vascular fraction, vascular network (i.e., branchpoint density and diameter), nuclear density, and knot fraction were quantified to describe placental phenotypes. Results: Pre-eclamptic placentas had disorganized trophoblast arrangement, decreased vascular fraction, and altered vessel diameters, compared to control placentas. The developed 3D-methodology indicated that placental vasculature, syncytial knotting, and villous growth are altered in pre-eclampsia. Conclusion: Our preliminary data demonstrate the potential of the developed quantification method for phenotyping pre-eclampsia, to improve future disease stratification.

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Short running title: Phenotyping pre-eclampsia based on 3D-imaging

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Design: Exploratory pilot study.

Setting: Single centre, MUMC.

Sample: Formalin fixed placental biopsies from: term control (n=3), pre-eclampsia (n=3), preterm birth (n=2), 2ndtrimester placenta (n=1), and intra-uterine growth restriction cases without pre-eclampsia (n=2).

Methods: Placental slabs were visualised with MPM. Collagen and cytoplasm (based on inherent signal), and fluorescently stained nuclei and blood vessels, enabled the visualization of villous tissue with subcellular resolution. Segmentation based on pixel classification, deep learning, and clustering algorithms were used to generate quantifiable features.

Main outcome measures: Trophoblast arrangement, 3D-villous tree structure, syncytial knots, fibrosis, and 3D-vascular networks were identified as imaging targets. Villous morphology, vascular fraction, vascular network (i.e., branchpoint density and diameter), nuclear density, and knot fraction were quantified to describe placental phenotypes.

Results: Pre-eclamptic placentas had disorganized trophoblast arrangement, decreased vascular fraction, and altered vessel diameters, compared to control placentas. The developed 3D-methodology indicated that placental vasculature, syncytial knotting, and villous growth are altered in pre-eclampsia.

Conclusion: Our preliminary data demonstrate the potential of the developed quantification method for phenotyping pre-eclampsia, to improve future disease stratification.

Keywords: Placenta, multiphoton microscopy, pre-eclampsia, placental syndromes, stratification.

Tweetable abstract of 110 characters

Subcellular 3D imaging of placenta provides a potential key to improve disease stratification of the pre-eclamptic syndrome.

Introduction

Pre-eclampsia (PE) is a systemic vascular Fdisorder affecting 4-5% of pregnant women globally[1]. PE is a leading cause of maternal and foetal morbidity and mortality, and confers a life- long risk to maternal and infant's cardiovascular health[2-5]. Classically, PE manifests as new-onset hypertension and proteinuria after 20 weeks of gestation[6]. Nevertheless, symptoms, severity, time of onset, and outcome can vary significantly and early diagnosis remains challenging [6, 7].

The wide range of clinical phenotypes makes PE more fitting to be a syndrome instead of a single disease. Especially the distinction early- and late-onset PE (EO-PE and LO-PE) is frequently applied and both are likely to have distinct aetiologies[8, 9]. Nonetheless, the exact definition of EO-PE and LO-PE is controversial.

The current stratification only focusses on time of onset of clinical symptoms, gestational age at time of birth, or a combination of these, without referring to underlying causes[6, 9, 10].

Quantifying altered placental morphology in different clinical presentations of PE is key to distinguish the clinical syndrome into distinct pathological phenotypes. Currently, 2D histological evaluation is the gold standard for placental investigation[11-13]. Yet, none of the histological findings are specific for PE, i.e., are also observed in other placental syndromes like intra-uterine growth restriction (IUGR)[14-16].

More advanced imaging methods such as confocal microscopy have been used for imaging terminal villous vasculature[17-20]. However, the villous tree is built up of stem villi (of which the diameter can be up to 3000 μm), intermediate villi, and terminal villi[21].

Multiphoton microscopy (MPM) is a tomographic (3D imaging) technique with subcellular resolution. It is based on multiphoton excited fluorescence and features deep tissue penetration (up to 1 μm)[22]. Therefore, thick tissue slabs (containing different components of the villous tree) can be visualized, closely to their native form. Moreover, MPM is efficient in exciting autofluorescence signal, suitable for label free imaging of imaging cytoplasm and connective tissue even in vivo[23].

MPM has been applied to investigate placental membrane architecture[24, 25]. But, to our knowledge, phenotype quantification of the villous tree and vasculature of healthy and PE placentas with MPM has not been described yet. The aim of this study was to develop a MPM placental imaging protocol and to demonstrate quantifiable imaging targets. Quantification of these targets could aid in a more unequivocal characterization of different PE subtypes and improve disease stratification.

Methods

Tissue collection and study population

11 placental biopsies were collected from Maastricht University Medical Centre, the Netherlands, between November 2020 and May 2021. 3 placental biopsies from uncomplicated pregnancies and 1 PE sample were collected directly after birth. 6 Placental samples (n=2 PE) were collected from the pathology department and received on formalin. Collection and usage of the placentas was approved by the Medical Ethics Committee Academic Hospital Maastricht and Maastricht University (METC, 16-4-047). Clinical characteristics of all placental samples are summarized in Table S1.

Tissue dissection and fixation

Placental tissue (˜20 cm^3) was sectioned from a central region of the chorion, stored for 24h at 4°C and frequently washed with phosphate buffered saline (PBS) to remove blood. Subsequently it was fixed (1h) in 4% paraformaldehyde (PFA) and stored in PBS at 4°C.

Nuclear and vascular fluorescent dyes

Placental tissue slabs (˜10mm^3) were fluorescently labelled to visualize nuclei and vascular structures. Tissue was permeabilized in 0,1% triton, 1% BSA (20 min), and incubated with 10μg/ml Ulex Europaeus Agglutinin I (UEA I), DyLight® 594 (Vector laboratories, CA, USA, cat#DL-1067-1, em_{max}=618nm) for 1-3h, in the dark, at room temperature. After washing with TBS, nuclei were labelled with 5μM SYTO 13 green (Invitrogen, cat#S7575, em_{max}= 509nm) for 30 minutes in the dark at room temperature.

Mounting and MPM imaging

For imaging, samples were mounted on a 50 mm glass bottom petri dish (MatTek, Ashland, MA, USA) filled with PBS. MPM was performed with a Leica TCS SP 5 (Leica Microsystems, Wetzlar, Germany)

multiphoton microscope. Excitation was at 780 nm. Fluorescence was collected with a 20x Leica HCX APO L, NA 1.0 water immersion objective or a 25x NA1.05 Olympus objective. Alternatively, the Leica Stellaris 8 Dive (Leica Microsystems, Wetzlar, Germany) employing similar settings was used.

Autofluorescence and second harmonic generation (SHG) from unstained samples was detected by an external detector with bandpass filter 460-525 nm, and a forward detector with 380-420 nm bandpass filter, correspondingly.

Signal of stained samples was detected using 4 channels. SHG was collected as described before. Internal detectors with detection bandwidth 444-496 nm, 508-559 nm, and 586-650 nm recorded autofluorescence, nuclear stain, and vascular stain respectively.

All images were acquired in 8-bit, with bidirectional scanning mode and at least 1024x1024 pixels. Z-step size ranged from 1.5 µm to 5µm. Image stitching was performed with the Leica Stellaris Dive microscope equipped with motorized stage.

Image processing and quantitative evaluation

Segmentation and volume quantification of villous tissue and vessels

Images were pre-processed, analysed, and viewed with*FIJI* [26]. Pre-processing consisted of de-noising with a 3D median filter, sigma 1. Subsequently, placental villi (autofluorescence), vessels, and SHG signal were segmented with the FIJI Labkit plugin[27].

Volume and surface areas of vessels and villi were quantified with*3D ImageJ suite* [28] (Figure S9, S10). Binary images were imported into *3D ROImanager* [28] to obtain surface area and volume measurements. Villous vascular fraction was calculated by dividing the vascular- and villous volume and converted to percentage. The surface area to volume ratio (SA/Vol) was quantified as a measure for effective diffusion area.

Quantifying vascular network characteristics

Binarized vascular images (exported via FIJI to .nrrd files) were imported into *3Dslicer* [29, 30] to quantify vascular network characteristics. The *VMTK*module[31, 32] of 3D slicer was used to extract a network model from vasculature, and to provide automatic quantification. More detailed procedures are described in Supplementary Methods.

Nuclear segmentation and quantification

Stardist 3D employs a neuronal network to separate densely packed nuclei in 3D image stacks[33]. The model was trained with 5 manually annotated image stacks, and thereafter used to segment placental nuclei.

Quantitative analysis of predicted label maps was performed in FIJI with 3D ROI manager[28]. Nuclear density was defined as the number of nuclei relative to the villous volume (#nuclei/villous volume in mm^3).

Segmentation of syncytial knots was performed with *KnotMiner* , available at https://github.com/stegmaierj/KnotMiner.*KnotMiner* was built as an extension package for the MATLAB toolbox SciXMiner[34]. The volume of knots was normalised to the volume of the corresponding villus to determine knot fraction. Knot shape was described by elongation index (EI=intermediate principal axis/ longest principal axis) and flatness index (FI=Short principal axis/intermediate principal axis).

Statistical analysis

Data are given as mean ±SD. GraphPad prism 5.0 was used for statistical analysis and graph representation. ANOVA with Bonferroni post-test was used to assess significant differences in parameters that are normally distributed. In case data was assumed not normally distributed Kruskal Wallis with Dunn's post-test was employed. If normality could not be statistically determined (by Shapiro-Wilk test) because of too small sample size, the assumption of normality was made. P values of <0.05 were considered statistically significant.

Results

Imaging targets in placental tissue

1. Trophoblast and fibrotic regions: disorganised trophoblast in pre-eclamptic placenta

H&E stained placental chorion illustrated nuclear accumulations and the villous tree components (Figure 1A, 1B). As frequently reported in literature[13, 35], EO-PE placenta was characterised by increased syncytial knotting (Figure 1B) in comparison to term control tissue (Figure 1A). As depicted in Figure 1, MPM imaging of unstained tissue (Figures 1C,1D, Figure S1) and stained tissue (Figures 1E-1H) provided significantly more detailed information about the structural organisation of the trophoblast. Term control placenta was characterized by a structured trophoblastic layer (Figure 1C). Nuclei were closely arranged to each other, and cytoplasmic space was small (inset Figure 1C). Trophoblast of the EO-PE case was disorganised, and numerous nuclear accumulations were present (Figure 1D and Figure S1B & S2A). Nuclei were unevenly distributed with larger appearing cytoplasmic spaces between nuclei (inset Figure 1D). Additionally, excessive collagen accumulation, i.e., fibrosis, was especially observed in stem villi of EO-PE placenta (Figures S2A, S2B).

2. Nuclear 3D architecture: syncytial knots have distinct 3D organisations in PE

Distinct nuclear aggregate shapes on villi were identified (Figures 1E - H). The term knots is often used to describe all nuclear aggregates seen in villi. However, nuclear aggregates can be subdivided in (apoptotic) knots, bridges and sprouts. Knots, mostly found towards term, are condensed nuclear aggregates protruding slightly from the villous surface[36]. Bridges are aggregated nuclei that connect two villi and are thought to provide structural support. Sprouts, mainly common in young placentas, are the initiating points for new developing villi[37]. Sprouts (Figure 1F), bridges (Figure 1G), and knots (Figure1E and 1H, Figure S3) could be easily distinguished from one another with 3D information. Prominent nuclear accumulations in term placenta were frequently bridges. Although bridges can be distinguished on 2D slices (Figure 1A), their interpretation is imaging-depth dependent and they are easily misinterpreted as knots without additional 3D information (Figure S3).

EO-PE placentas were characterized by presence of wave-like syncytial knots (WLKs) (Figure 1E). Although WLKs were most dominantly present on stem villi (Figure 1H), this pattern was also observed on intermediate villi (Figure 3A lower red arrow), and in villi from hypertensive pregnancy (Figure S1D). LO-PE placenta had numerous large, surface-extending knots (Figure 1H). Knots in term control placenta were frequently small, regional, mildly protruding (Figure 2E). Additionally, knots in term control placenta were observed in close relation with so-called vasculosyncytial membranes (Figure S3), which represent major areas of feto-maternal exchange[38].

Quantification of nuclei and their architecture: Model performance

Nuclei in placental chorion are arranged closely to each other, and have varying intensities (i.e.

knots are brighter, and deeper in tissue signal to noise ratio decreases). This makes intensity-based segmentation very challenging. The machine-based learning approach *Stardist* [33]was employed to segment nuclei. An overview, of the developed imaging methodology and quantification, is given in Figure S4.

Representative images of placental nuclei of term control and EO-PE are illustrated in Figure 2 and Figure S5. Knots appeared bright and very dense (2D images Figures 2A, 2E). Corresponding 2D images (Figures 2B, 2F) and 3D images (Figures 2C, 2G) segmented with *Stardist*demonstrate the labels of all individual nuclei identified. These images were used to calculate nuclear density. The classification of knots performed with *KnotMiner* is presented in Figures 2D and 2H.

Although non-significant, EO-PE placentas tended towards higher nuclear density compared to preterm control placenta (Figure 2I). Knot fraction (the villous volume occupied by knots) tended to be higher in EO-PE compared to preterm control placenta (Figure 2J). While significance of LO-PE could not be tested (single value), IUGR knot fraction differed non-significantly from term control. LO-PE and IUGR placenta

both tended towards increased knot fraction compared to term control placenta. Notably, preterm placenta did not have a lower knot fraction than term control placenta (Figure 2J).

Knot shape was on average slightly (non-significantly) flatter and (significantly) less elongated in term control- and preterm placenta (Figure 2K) compared to IUGR and EO-PE placenta, respectively. Individual knot shape quantification is represented in Figure S6.

3. 3D native villous tree organisation: altered surface-volume relations in pre-eclampsia

Villous 3D shapes were highly varying (Figures 3A-D). Depending on achievable imaging depth, linkages of terminal villi, intermediate villi, and stem villi, could be visualised. The investigated EO-PE placenta had large knob-like villous endings (Figure 3A) characteristic of PE placenta. These knob-terminals were not uncommon and frequently present on villous tips (Figure S7). Term control placenta had shorter but highly condensed villi (Figure 3B), while branching appeared excessive in an IUGR case (Figure 3C). Notable is that the LO-PE placenta had highly varying regions with limited branching (Figure 3D) and highly branched villi (Video S1). Segmentation procedures of intermediate and terminal villi are illustrated in Figures S8-S11.

Quantitative results indicated that villous surface area increases disproportionally to volume in investigated PE placentas (Figure 3E). Diffusion surface area and villous volume had a positive linear relation in term control placenta, whereas in PE a linear relation between surface area and volume was absent (Figures 3E, 3F).

The SA/vol ratio provides a measure for easiness of diffusive transport and branching is a biological mechanism to increase the SA/vol ratio. No significant difference was found between the SA/vol ratio of chorionic villi of control- and PE or IUGR placenta (Figure 3G). However, the villous SA/vol ratio tended to be highest in LO-PE placenta, which suggests increased villous branching compared to term placenta (Figure 3G). Notably, the villous SA/vol ratio was lowest in the term IUGR placenta (Figure 3G), while villi appeared highly branched (Figure 3C).

4. 3D placental microvasculature: a reduced vascular fraction in pre-eclampsia

For vasculature visualization, staining was performed with UEA-1 lectin[39], which is a direct and less complex method compared to immunostaining[17-20]. Segmentation was performed with Labkit (Figures S8, S11), and network extraction and quantification with VMTK (Figure S12). Vasculature of term placenta (Figure 4A) appeared highly torturous and branched, whereas LO-PE placenta illustrated a vascular network with longer segments and decreased branching (Figure 4B). Vessel diameters varied throughout a single placental vascular network (Figures S13-S13G). Diameters in term control placenta remained steady along a 10 μm equilibrium (Figure S13G). In the LO-PE case, diameters varied highly and could become very small (Figure S13F).

Vascular fraction measured the percentage of villous volume occupied by vascular structures. Our results demonstrate a significantly lower vascular fraction for the EO-PE placenta compared to (pre-)term control placenta (Figure 4C). Moreover, the vascular fraction in EO-PE was comparable to that of a 2nd trimester placenta (Figure 4C). LO-PE and EO-PE vascular fraction did not differ significantly although LO-PE tended towards higher vascular fraction than EO-PE. Vascular content in the LO-PE placenta was significantly lower than in term control placenta, when parametric statistical tests were used (Figure 4C).

Network properties were quantitatively investigated by examining branchpoint density (see Figure S12 for details on the procedure). Our preliminary data illustrated term placenta vascular networks, to have the lowest average branchpoint density (Figure 4D). Highest tortuosity (although not significant) was observed in the IUGR placenta without PE (Figure 4E).

Discussion

Main findings: Imaging targets and preliminary results

With MPM we could visualize 3D morphology of healthy and preeclamptic placenta but also quantify pathologic features. Quantification of those features was based on existing tools (*FIJI* , *VMTK* ,*Stardist*) and

an ad hoc developed tool (*KnotMiner*).

Disorganized trophoblast (Figures 1C-1D), knots with distinct 3D confirmations (Figure 1E-1H, Figure 2A), and long slender villi with knob-like endings (Figure 3A) were characteristic for PE placenta. PE placenta displayed increased syncytial knotting compared to controls (Figure 2J). The relationship between villous surface area and volume was also different in PE versus control placenta (Figures 3E-3F). Lastly, placental vasculature appeared to be maldeveloped in PE (Figures 4, S13). Differences between EO-PE and LO-PE support the concept that they have a different pathophysiology.

Strengths and limitations

Imaging of thick tissue (i.e., no need to make thin sections) allowed imaging of nearly intact 3D morphology of placental villi. This supported accurate identification of knots, bridges and sprouts, and allowed assessment of the intact 3D organization of the villous tree and intact microvascular networks. Classical histopathology is only able to give a single plane 2D representation of these complex 3D structures (Figures 1A,1B) and therefore MPM offers a better alternative. Imaging of even larger fields of view by image stitching (2 X 2,5 mm^2) is possible as demonstrated in Video S1, although it is more time consuming (3 hours).

MPM penetration depth of unprocessed placenta is limited to approximately 200 μm. Consequently, images of small (and frequently incomplete) networks cause risk for sampling bias. This could be addressed by optically clearing the tissue[40], a process that homogenizes the tissue's refraction index, to allow imaging down to some millimetres. Combining image stitching and optical clearing would increase the imaged volume many fold. Larger coverage would reduce sampling bias and improve image quantification. However, image acquisition time would increase correspondingly.

Limited sample size only allowed to generate preliminary results, but our goal was to demonstrate the method and its potential. Future efforts should focus on investigating regional variation within the same placenta (sampling bias) and variation between placentas with larger sample size. Additionally, obtaining a complete overview of clinical information (including doppler ultrasound findings[41]is crucial for future research, so that observations can be correlated accordingly.

Interpretation (in light of other evidence)

Trophoblast and knots

Hypoxia (and associated trophoblast damage) is central in current pathophysiological hypotheses of PE[42]. In PE, trophoblast turnover is no longer balanced. Apoptosis is increased[43], differentiation and fusion of the so-called cytotrophoblast to form the syncytium is decreased[44], while cytotrophoblast proliferation is reported to be increased or unaltered[45-47]. Altered trophoblast function provides a potential clarification of observed structural abnormalities in PE (Figure1C and 1D).

Moreover, placental hypoxia has been found to promote the aggregation of syncytial knots [48]. Increased syncytial knotting is a clear hallmark of pre-eclamptic placentas[13, 35]. Distinct 3D conformations (Figures 1E-1H) of knots are potentially related to different pathological mechanisms (i.e., being apoptotic or a mechanism to sequester effete nuclei and create regions for diffusional exchange). Especially WLKs were dominantly present on the investigated EO-PE placenta (Figure 1E). Nonetheless, wave-like apoptotic shedding is not specific for PE and has been described in stem villi from cases with severe IUGR, without PE[35, 49]. Quantification of knots (shape/volume) could potentially aid in subdivision of pathological, apoptotic, or non-pathological (including sprouts and bridges) knots accordingly. To our knowledge, different shapes of knots have been described[50], but quantitative data regarding relative incidences of alternative knot shapes is lacking. Current developed methodology provides an approach to quantitatively investigate knots and their shapes.

Villous tree morphology: exchange surface and volume

Villous branching establishes an increased diffusional surface and the villous SA/Vol reflects easiness of nutrient exchange. Similar to our findings (Figure 3E), a disproportional relation between surface area and

volume has been reported in PE placentas based on a stereological approach[51]. However, some stereological estimates only observed altered exchange surface areas in IUGR cases, and not for PE alone[52, 53]. Stereology only provides estimates of 3D quantitative parameters while quantification of MPM images represents the intact 3D villi. 3D characterization by MPM will therefore aid in more accurate quantification of villous trees to confirm or reject abovementioned findings.

3D microvasculature: volume and network quantification

Terminal villous shapes are influenced by vascular development and abnormal vascular development is linked to PE[10]. A vascular casting study demonstrated a reduction of total vasculature in severe PE[54]. Moreover, higher frequency of avascular villi are described in EO-PE compared to term control[55], providing a potential explanation for reduced vascular fraction (Figure 4C). Moreover, vessel diameters are hypothesized to be influenced by placental hypoxia[10]. Therefore, vessel diameter patterns (Figures 4A, 4B) provide an interesting imaging target.

MPM enables investigation of intact 3D microvascular networks and network quantification. Besides branch-point density and tortuosity (Figures 4D, 4E), network quantification can provide numerous quantitative morphological descriptors (Figure S13). According to current hypotheses (Figure S14), LO-PE exhibits increased branching, i.e., predominant vascular development by branching angiogenesis. Conversely, EO-PE placental development is hypothesised to be characterized by predominance of non-branching angiogenesis[10, 56].

During normal development, non-branching angiogenesis is the dominant process of vascular growth later in gestation[57]. Since (EO-)PE pregnancies in our study group were terminated earlier than control pregnancies (Table S1), younger gestational age is a potential confounding variable. Hence, idiopathic preterm birth placenta, which mainly experienced branching angiogenesis, is a good additional control. Nevertheless, the vascular fraction from the idiopathic preterm control placenta and term placentas did not differ significantly in our preliminary data (Figure 4C). Differences (although non-significant) regarding network properties between term- and preterm placenta (Figures 4C, 4D) can be a result of developmental phases. This does indicate the importance of including age-matched control placenta to study vascular network properties in PE placenta with younger gestational ages.

The tortuous nature of the placental vessels makes them longer, increases their surface area and slows down blood flow to increase the time for diffusional exchange[18]. High tortuosity (as observed in a IUGR case (Figure 4E) could be an adaptational response for improving diffusion to compensate low villous SA/vol (Figure 3G). However, excessive tortuosity in vessels is also linked to pathological processes, like hypertension or weakened artery wall stiffness[58]. Vascular twisting would thus be an interesting feature to investigate further regarding placental disease.

Fibrosis

Finally, fibrosis, which is a prominent feature of PE[55], was identified as a quantitative marker (Figures S2 and S10). Since current analysis focused on intermediate and terminal villi only, fibrosis was not further analysed. Future work focussing on stem villi, where fibrosis was mostly observed (Figure S2), will include quantification of fibrosis.

Conclusion

Our results demonstrated MPM to be a suitable method to visualize placental tissue and the processing methodology developed can quantifiably differentiate placental morphologies. This methodology could be further utilised to phenotype PE subtypes, and ultimately aid in unravelling the multi-etiological nature of preeclampsia. In the future, stratification based on 3D placental morphology could aid in the search for early diagnostic markers and (preventative) treatment options to improve maternal and foetal outcomes.

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Disclosure of interest

All authors have no conflict of interest to declare.

Contribution of authorship

DK, SAN, and MVZ contributed to the design and conception of the study, and obtained funding. JP, DK and SH developed the imaging protocol for placental tissue. Sample preparation and imaging was performed by JP, and SH. The MATLAB extension *KnotMiner* was built by JS and DE. DE trained and employed Stardist for nuclei segmentation. Methods for image quantification and data analysis were established by SH and DK. SH and DK drafted the manuscript. JP, DE, JS, SAN, CS and MVZ read, contributed to manuscript revision, and approved the submitted version.

Details of ethical approval

The Medical Ethics Committee Academic Hospital Maastricht and Maastricht University (METC AZM/UMC) approved collection and usage of placental material on 17-08-2020, with reference number METC16-4-047, considering anonymous and retrospective data collection.

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