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Micro-CT and histological investigation of the spatial pattern of feto-placental vascular density

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1 **Micro-CT and Histological Investigation of the Spatial Pattern of**  
2 **Feto-placental Vascular Density**

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## 46 1. ABSTRACT

### 47 Introduction

48 There are considerable variations in villous morphology within a normal placenta. However,  
49 whether there is a reproducible spatial pattern of variation in villous vascular density is not  
50 known. Micro-CT provides three-dimensional volume imaging with spatial resolution down to  
51 the micrometer scale. In this study, we applied Micro-CT and histological analysis to  
52 investigate the degree of heterogeneity of vascularisation within the placenta.

53

### 54 Method

55 Ten term placentas were collected at elective caesarean section, perfused with contrast  
56 agent and imaged whole with Micro-CT. Eight full depth tissue blocks were then taken from  
57 each placenta and imaged. Sections were taken for histological analysis. Data was analysed  
58 to investigate vascular fill, and vascular density in relation to location from cord insertion to  
59 placental edge at each scale.

60

### 61 Results

62 Whole placental imaging revealed no spatially consistent difference in villous vessel density  
63 within the main placental tissue, although there was a great degree of heterogeneity. Both  
64 block imaging and histological analysis found a large degree of heterogeneity of vascular  
65 density within placentas, but no strong correlation between villous vascular density and  
66 block location ( $r_s=0.066$ ,  $p=0.7$  block imaging,  $r_s=0.06$ ,  $p=0.6$  histological analysis).

67

### 68 Discussion

69 This work presents a novel method for imaging the human placenta vascular tree using  
70 multiscale Micro-CT imaging. It demonstrates that there is a large degree of variation in  
71 vascular density throughout normal term human placentas. The three-dimensional data  
72 created by this technique could be used, with more advanced computer analysis, to further  
73 investigate the structure of the vascular tree.

# 1           **Micro-CT and Histological Investigation of the Spatial** 2           **Pattern of Feto-placental Vascular Density**

3

## 4   **1. INTRODUCTION**

5   Fetal blood arrives at the placenta via two umbilical arteries, is transported  
6   across the placental surface via chorionic arteries, then passes deep into the  
7   placenta via stem arteries. From these dense vascular trees arise forming  
8   complex, multi-branching vascular beds<sup>1</sup>, bringing fetal blood in close proximity  
9   with maternal blood, allowing exchange<sup>2</sup>. Important obstetric pathologies,  
10   including pre-eclampsia and fetal growth restriction, are associated with changes  
11   in the villous vascularisation of the placenta<sup>3,4,5,6</sup>. Improving our understanding of  
12   normal placental vascularisation and the changes seen in pathology may improve  
13   our understanding of these diseases, and our ability to diagnose and treat them.  
14   There are considerable variations in villous morphology within a normal  
15   placenta<sup>7</sup>. However, whether there is a relationship between variation in villous  
16   vascular density and tissue location within the placenta in regard to umbilical cord  
17   insertion and placental edge is unclear. Histological analysis by Fox et al  
18   investigating the number of hypovascular or avascular villi, their measure of feto-  
19   placental vascularisation, in relation to tissue location within the placenta and  
20   found no statistically significant relationship in normal placentas<sup>7</sup>. However, they  
21   did show an increasing number with distance from cord insertion (156 centrally vs  
22   222 peripherally)<sup>7</sup>, suggesting there may be reduced vascular density in the  
23   placental periphery. Mayhew et al<sup>8</sup> did not reproduce this, finding no difference in  
24   villous vascular density with tissue location in relation to cord insertion and  
25   placental edge.

26

27 Micro-Computed Tomography (Micro-CT) provides three-dimensional volume  
28 imaging with spatial resolution down to the micrometre scale, although  
29 magnification is at the cost of field of view. It has the advantage of being non-  
30 destructive allowing further tissue analysis with other imaging or histological  
31 techniques. Micro-CT has already been shown to be effective in investigating the  
32 fetoplacental circulation of mouse placentas, demonstrating the growing  
33 complexity of the vascular tree with increasing gestational age<sup>9</sup>, and the effect of  
34 polycyclic aromatic hydrocarbons on the branching structure and tortuosity of the  
35 tree<sup>10</sup>. In human placenta, the technique has been used to measure placental  
36 vascular density in small blocks of tissue<sup>11</sup>, and demonstrate reduced vascular  
37 density in fetal growth restriction compared to normally grown controls<sup>12</sup>. Imaging  
38 of the whole human placenta, using a corrosion technique, has also been  
39 investigated<sup>13</sup>, finding a significantly smaller number of chorionic artery  
40 branches<sup>13</sup> and longer venous and shorted arterial vasculature in fetal growth  
41 restriction compared to normal placentas<sup>14</sup>. Standard Computed Tomography  
42 angiography has been used to investigate the microvasculature of the placenta,  
43 finding no difference in macrovascular volume between normal and FGR  
44 placenta, despite a reduction in placental size<sup>15</sup>.

45 Recently, we optimised a technique for placental perfusion and Micro-CT imaging  
46 without corrosion, followed by histological analysis of perfused tissue<sup>16</sup>, which  
47 has the advantage of providing both multiscale Micro-CT and traditional histology  
48 in the same placenta. Multiscale imaging allows the whole placenta to be imaged  
49 at lower magnification, to get an overview of the vascular structure, and then  
50 blocks can be imaged at higher magnification to visualise the vascular tree down  
51 to, although not including, the terminal villi. This approach also has the benefit of  
52 allowing assessment of vascular fill with the perfusion medium.

53

54 In this study, we apply this novel imaging method to investigate the degree of  
55 heterogeneity of vascularisation within the placenta.

56

## 57 **2. METHOD**

### 58 2.1 Tissue Preparation

#### 59 *Placental Perfusion*

60 Experimental procedures were approved by Bloomsbury National Research  
61 Ethics Service Committee (REC Reference number 133888). Women  
62 undergoing elective term caesarean section following uncomplicated pregnancy  
63 at University College Hospital NHS Foundation Trust gave written consent.  
64 Placentas were taken directly from labour ward to the laboratory. In-depth  
65 discussion and justification of the perfusion process has previously been  
66 published<sup>16</sup>. In short, an umbilical artery was cannulated using a 22-gauge  
67 cannula, flushed with 0.9% sodium chloride with 5IU heparin/ml and sutured in  
68 place. An exit vent (approx. 1mm) was created in the umbilical vein, and the  
69 umbilical cord was clamped distally.

70 The placenta was perfused with 0.9% sodium chloride with 5IU heparin/ml, using  
71 gentle manual pressure, until the fluid exiting from the vent in the umbilical vein  
72 became pink and free from blood clots. 20ml Microfil (Flow Tech, Carver, MA), a  
73 lead based contrast agent developed for microcirculation perfusion, was then  
74 perfused through the umbilical artery cannula using gentle manual pressure until  
75 all chorionic arteries were filled, and Microfil could be seen in some of the  
76 chorionic veins. The umbilical cord was then clamped proximal to the point of  
77 cannulation, and the placenta was left at room temperature for 90 minutes to  
78 allow Microfil to set, as per manufacturer instructions.

79 A high-resolution photograph was then taken of the chorionic surface of the  
80 placenta next to a paper tape measure for scale, using a digital low-distortion

81 single-lens reflex camera. The placenta was then placed flat in 500-750ml 4%  
82 formalin for 48 hours to fix.

83

#### 84 *Micro-CT Image Acquisition*

85 The placenta was removed from formalin, wiped dry, and placed in a vacuum  
86 sealer roll (Andrew James Vacuum Sealer Rolls) and vacuum sealed. The  
87 placenta was then mounted in a custom-made foam block and placed upright on  
88 the stage in the micro-CT scanner (XTH225 ST Micro-CT, Nikon Metrology,  
89 Tring, UK). The placenta was imaged with a Molybdenum target at 80kV energy,  
90 88 $\mu$ A current, 1000ms exposure time, one frame per projection, 3141 projections  
91 over 360-degree rotation, with an isotropic voxel size of 116.5  $\mu$ m. The imaging  
92 time was 53 minutes 6 seconds.

93

94 The placenta was then cut into 2cm strips as per standard histological technique.  
95 Areas of placenta that appeared well perfused were identified and full thickness  
96 blocks of 1.5-2cm by 1.5-2cm were taken. The location from which blocks were  
97 taken was recorded using a digital photograph. Eight blocks were taken from  
98 each placenta. Each was wrapped in parafilm and mounted in a custom-made  
99 acrylic tube, resting on a plastic stand, and imaged using a Molybdenum target,  
100 50kV energy, 199 $\mu$ A current, 1 frame per projection, 1000ms exposure time,  
101 3141 projections over 360-degree rotation, with an isotropic voxel size of 13.5  
102  $\mu$ m. Each block took 53 minutes and 6 seconds to image.

103 The blocks were then placed in 30ml 4% formalin in preparation for histological  
104 analysis. The image volumes were reconstructed using a modified Feldkamp  
105 filtered back projection algorithm with proprietary software (CTPro3D; Nikon  
106 Metrology). Surface renderings of the volumes were then examined in VG



107 Studio MAX 2.2 (Volume Graphics, Germany) to check imaging quality (Figure  
108 1).

109

#### 110 *Histological Slide Preparation*

111 Two 10 $\mu$ m full thickness sections were taken from each block and stained with  
112 haematoxylin and eosin (H&E). For each slide, 6 micrographs at x100  
113 magnification were taken, three in the upper half of the tissue, close to the  
114 chorionic plate, and three in the lower half of the tissue, close to the basal plate.

#### 115 2.2 Image Analysis

##### 116 *Describing Tissue location in relation to cord Insertion and Placental Edge*

117 The high-resolution photograph of each placental chorionic plate surface was  
118 loaded into FIJI (ImageJ Version 2.0.0-rc-54/1.51f<sup>17</sup>), and the scale was set.  
119 The distance from the cord insertion to the centre of the site from which each  
120 block was taken, and the distance from cord insertion to placental edge through  
121 the site from which the block was taken were measured. The normalised location  
122 of the block was defined as the first distance divided by the second, multiplied by  
123 100.

##### 124 *Analysis of Whole Placental Micro-CT Imaging*

125 All whole placenta imaging analysis was performed in MATLAB (R2016b,  
126 MathWorks, 2016) using custom-designed algorithms. In order to analyse the  
127 data within MATLAB the whole placenta volume data was saved as a stack of  
128 TIFF files (266 to 492 files of 1682-2155 by 1475-2001 pixels in size). The  
129 placenta was always orientated within the stack so that each TIFF image sliced  
130 through the placenta parallel to the chorionic plate, and the distance from  
131 chorionic to basal plate increased through the stack of TIFF files.

132 Reading the whole placenta dataset at once was computationally prohibitive. In  
133 order to make analysis feasible on any computer, the volumes were divided into  
134 100 (10 x 10) three-dimensional cubes, allowing smaller chunks of data to be  
135 processed. The cubes were labelled with their position in the volume and could  
136 then be re-combined.

137 In order to perform analysis that was relevant to placental structure, the axis of  
138 the placenta was defined. A graphical user interface was created which allowed  
139 the user to open a two-dimensional maximum intensity projection of the whole  
140 placenta stack and manually set the point of cord insertion. To define the  
141 placental edge, placenta masks were drawn. To allow analysis by distance from  
142 cord insertion, distance maps were created. The pixel distance from cord  
143 insertion to placenta edge was measured for each placenta through 360 degree,  
144 and then normalised from 0 to 100.

145 The greyscale threshold for placental tissue and Microfil filled vessels were then  
146 defined for every placenta data set. This was done in FIJI by determining the  
147 mid-point between the greyscale peaks for air and placenta as the threshold for  
148 placenta, and the point midway between the greyscale peaks for tissue and  
149 Microfil as the threshold for Microfil. This threshold was then used in MATLAB to  
150 segment the placental tissue and vascular tree of each placenta.

151 Once the vessels had been segmented, a vascular skeleton was created.

152 Vessels were eroded from both sides in an iterative manner until only the  
153 centreline remained, this centreline was defined as the vascular tree skeleton.

154 After skeletonisation, the radius of the vessel for every voxel along the skeleton  
155 was measured, as the distance from the skeletonised midline of every vessel to  
156 the boundary of the thresholded vessel.

157 Once vessel radius was known, vessels with a radius larger than 6-voxels  
158 (equivalent to approximately 700 $\mu$ m) were excluded from further analysis, as they  
159 were thought to mostly represent chorionic, not villous, vessels.

160 *Analysis of Placental Block Micro-CT Imaging*

161 To calculate the villous vascular density of each block of placental tissue, the  
162 reconstructed block volume was loaded into VG StudioMAX 2.2 (Volume  
163 Graphics, Germany). An area of interest was drawn over the bottom third of the  
164 tissue (the location for the villous vascular tree). Volumes were thresholded  
165 using the grey-scale histogram, with the threshold set at a point midway between  
166 the intensity peaks for air and tissue to segment the placenta and contrast filled  
167 vessels, and halfway between the intensity peaks for tissue and Microfil to  
168 segment the vessels perfused with Microfil. The volume of the placental tissue  
169 and of Microfil was then measured automatically, and the vascular density  
170 calculated as the volume of vessel divided by the volume of placental tissue and  
171 vessel, presented as a percentage.

172 *Histological Analysis*

173 A validated<sup>18</sup>, automated pipeline, created in FIJI (ImageJ Version 2.0.0-rc-  
174 54/1.51f<sup>17</sup>) was used to analyse the histological sections as shown in Figure 2.  
175 The Trainable Weka Segmentation plugin (Version 3.1.2)<sup>19</sup> was used to segment  
176 image features on the micrographs into three classes; perfused vessels and  
177 background (Microfil and white space (Microfil shrinks during histological  
178 processing so does not fill the whole lumen<sup>10</sup>)), un-perfused vessels (vessels  
179 containing red cells) and villous tissue (Figure 2).

180 The output images were thresholded to select the three classes defined above.  
181 The “Analyse Particle” tool was used to measure the cross-sectional area of the  
182 perfused and unperfused vessel lumens. This applies restrictions in terms of the  
183 minimum and maximal area of the particle and the circularity, and outputs a list of  
184 the area measurements for each particle within the limits. The tool was set to  
185 include particles with an area between 60-10,00,000 $\mu\text{m}^2$  and circularity 0.20-

186 1.00, to exclude non-vessels incorrectly segmented, and the background. For the  
187 villi, the whole of the segmented cross-sectional area was measured (Figure 2).  
188 The automated system output CSV files listing the perfused and un-perfused  
189 vessel lumen area and the villi area. These were input into a database, with one  
190 spreadsheet for each placenta (Microsoft Excel for Mac, Version 15.29, 2016).  
191 The vascular fill and vascular density were then calculated for each block.  
192 Finally, a manual check was performed, by comparing each micrograph against  
193 the calculated vascular fill and density. This was to guard against limitations  
194 within the automated analysis pathway causing erroneous results.

195

#### 196 *Statistical Analysis*

197 Data is presented as mean  $\pm$  SD. Statistical analysis was performed in SPSS  
198 Statistics (IBM version 23) and MATLAB. Group comparison was done using the  
199 Kruskal-Wallis H test, with post-hoc pairwise comparison of statistically significant  
200 results using Dunn's procedure with a Bonferroni correction for multiple  
201 comparisons. Correlation was done using Spearman Rank Correlation as the test  
202 for normality was not fulfilled. Statistical significance was set at 95%.

203

### 204 **3. RESULTS**

205 Ten placentas delivered by elective caesarean section after 38 weeks'  
206 gestational age from uncomplicated pregnancies, with neonatal birth weight  
207 greater than the tenth centile (UK-WHO Growth Charts), were investigated (see  
208 Table 1). All women included in the study were non-smokers and did not take  
209 recreational drugs; all had an epidural for their Caesarean section (CS) birth  
210 which was a primary elective CS in 4 women and a repeat elective CS in 6  
211 women; they did not receive any antibiotics, magnesium sulphate or oxygen  
212 resuscitation and their blood pressures remained <140/90 throughout delivery.

213

## 214 3.1 Vascular Fill From Histology

215

216 Vascular fill was assessed as perfusion may not fill every vessel evenly with

217 contrast agent, and unfilled vessels will not be visualised in imaging. To ensure

218 results are representative of vascularity, not perfusion, it is essential to ensure

219 vessels are filled, and exclude inadequately perfused tissue.

220 Placentas were imaged at two different resolutions, visualising different sized

221 vessels. In order to investigate the vascular fill relevant to each resolution, we

222 calculated it in vessels with a cross-sectional area  $> 10,000\mu\text{m}^2$  which was

223 relevant to whole placental imaging, (n=960 micrographs; 6 micrographs/slide, 2

224 slides/block, 8 blocks/placenta), and then in vessels with an area  $> 200\mu\text{m}^2$ 

225 which was relevant to block placental imaging (n=480 micrographs; 3

226 micrographs/slide, 2 slides/block, 8 blocks/placenta); only the micrographs of

227 tissue close to the basal plate were used for the analysis of vessels with an

228 area  $> 200\mu\text{m}^2$  as this was the area analysed in imaging. The results are shown

229 in Table 2.

230

231 The fill of vessels greater than  $10,000\mu\text{m}^2$  was generally very good, with 65 of 80

232 blocks having 100% fill. The lowest mean vascular fill was 77% for placenta 4. No

233 placentas were excluded from further analysis due to poor fill. The fill of vessels

234 with an area greater than  $200\mu\text{m}^2$  was less good, with 42 out of 80 blocks having235 vascular fill  $< 75\%$ . To ensure that vascular density calculations reflected vascular

236 density rather than vascular fill, these blocks were excluded from further analysis,

237 leaving 38 blocks, spread between the ten placentas. There was no statistically

238 significant correlation between block location and vascular fill ( $r_s -0.009$ ,  $p=0.9$ ),

239 suggesting fill was not worse in peripheral compared to central placental tissue.

240

241 3.2 Vascular density with Normalised Distance from cord insertion

242

243 *Whole Placental Imaging*

244 At the magnification achievable with Micro-CT for whole placental imaging, mean  
245 vascular density for the 10 placentas was 0.5% (SD±0.5, range 0.3% to 1%). To  
246 investigate the relationship between villous vessel density with the distance from  
247 the umbilical cord insertion, vascular density maps were drawn for each placenta.  
248 This showed how the villous vascular density varied throughout the placental  
249 volume (Figure 3). Using the normalised placenta distance maps the mean  
250 vascular density for each of the 100 regions from the site of the umbilical cord  
251 insertion to the placental edge was calculated and plotted (Figure 3). There was  
252 no spatially consistent difference in villous vessel density within the main  
253 placental tissue, although there was a great degree of heterogeneity, as shown  
254 by the large error bars on the combined graph (Figure 3). However, there was a  
255 tendency towards reduced vascular density in the peripheral 20% of the placenta,  
256 as shown by the downward trend of the combined mean (Figure 3).

257

258 *Block Placental Imaging*

259 At the magnification used for block imaging, mean vascular density for the 38  
260 included blocks was 4% (SD±2%, range 1-13%). To investigate the variation in  
261 villous vascular density between placentas, a box plot was drawn (Figure 4).  
262 Villous vascular density within one placenta commonly varied by up to 4%,  
263 representing a 100-150% increase in vascular density between blocks. There  
264 was no significant difference in the mean ranks of villous vascular density  
265 between placentas ( $\chi^2 = 13.06$ ,  $p=0.2$ ). To investigate if there was a difference in  
266 villous vascular density at this resolution with distance from the umbilical cord  
267 insertion, villous vascular density was plotted against location from the umbilical

268 cord insertion to the placental edge for each included block (Figure 4). There  
269 was no correlation between villous vascular density and block location ( $r_s=0.066$ ,  
270  $p=0.7$ ).

271

#### 272 *Histology*

273 The mean villous vascular density measured with histological analysis over all 80  
274 blocks was 19% (SD  $\pm 5\%$ , range 8-38%). A boxplot was drawn to visualise the  
275 difference in vascular density between the placentas (Figure 4). The vascular  
276 density of blocks from one placenta often varied by 10%. There was a significant  
277 difference in the mean ranks of villous vascular density between placentas,  
278 ( $\chi^2 = 30.35$ ,  $p < 0.01$ ). Post-hoc pairwise comparison showed that the significant  
279 difference in vascular density were between placenta 1 (median vascular density  
280 12.6, IQR=3.8%) and placentas 2 (23.4, IQR=5.6%,  $p=0.01$ ), 3 (21.7, IQR=2.3%,  
281  $p=0.03$ ), 4 (23.2, IQR=10.4%,  $p=0.02$ ) and 8 (21.9, IQR=1.9%,  $p=0.03$ ). To  
282 investigate if there was a difference in villous vascular density on histological  
283 analysis with distance from the umbilical cord insertion, villous vascular density  
284 was plotted against location from the umbilical cord insertion to placental edge for  
285 each block (Figure 4). There was no correlation between villous vascular density  
286 and block location ( $r_s=0.06$ ,  $p=0.6$ ).

287

#### 288 **4. DISCUSSION**

289 This work investigated villous vascular density in normal, term human placenta,  
290 over three scales, in relation to placental tissue location.

291 Whole placental micro-CT imaging was performed with an isotropic voxel size of  
292 116.5 $\mu\text{m}$ . The advantage of whole placental imaging is that it captures data  
293 throughout the placental volume, so that spatial analysis is possible. The  
294 disadvantage is that the large field of view is at the cost of magnification, so only

295 the larger villous vessels are visible. The mean vascular density at this  
296 magnification was 0.5% (SD±0.51, range 0.3% to 1%). No consistent spatial  
297 pattern in vascular density through the placental tissue was observed, however  
298 there was a tendency towards reduced vascular density in the peripheral 20% of  
299 the tissue.

300 Block placental imaging benefits from higher magnification compared to whole  
301 placental imaging, at the cost of field of view. The increased magnification  
302 allowed visualisation of vessels in the villous tree, excluding only the terminal  
303 capillaries. The mean villous vascular density at this magnification (voxel size of  
304 13.5µm) was 4% (SD±2%, range 1-13%). This shows a large degree of variability  
305 in vascular density within and between normal term placentas. When vascular  
306 density was examined in relation to tissue location between the umbilical cord  
307 insertion and placental edge, no strong correlation was found ( $r_s=0.066$ ,  $p=0.7$ ,  
308 powered to detect a correlation coefficient of 0.5 or greater).

309 Histological analysis was performed to allow visualisation of all villous vessels  
310 within the villous vascular tree, including terminal capillaries. The disadvantage of  
311 this method was that the vessels were only seen in two-dimensional cross  
312 section. Histological analysis of the villous vascular tree showed a mean vascular  
313 density of 19% (SD ±5%, range 8-38%), consistent with previous measures in the  
314 literature<sup>8,20</sup>. Again, there was no strong correlation between vascular density and  
315 tissue location with respect to the distance from the umbilical cord insertion to the  
316 placental edge ( $r_s=0.06$ ,  $p=0.6$ , powered to detect a correlation coefficient of 0.5  
317 or greater).

318

319 Variation in fetoplacental vascular density has been hypothesised to correspond  
320 to maternal perfusion, with evidence that the fetoplacental blood flow can be  
321 modulated to match maternal perfusion and therefore oxygenation<sup>21,22,23</sup>. This



322 would facilitate efficient exchange regardless of physiological changes in  
323 maternal blood supply, that could occur daily secondary to maternal position. The  
324 mechanism by which vasoconstriction may occur is not known, with proposed  
325 mechanisms including nitric oxide released by the villous vascular tree causing  
326 vasodilation in stem arteries supplying well oxygenated areas<sup>23</sup>, or inhibition of  
327 potassium channels causing vasoconstriction in the smooth muscle or small  
328 arterial walls in poorly oxygenated areas<sup>24</sup>. These theories may explain the large  
329 degree of heterogeneity in vascular density seen in this work. It is possible that  
330 more vascular dense areas represent areas of higher maternal perfusion and  
331 oxygenation in utero. In vivo techniques, such as oxygen sensitive MRI<sup>25,26</sup>, may  
332 help us correlate in vivo perfusion and ex vivo vascular density in the future.

333

334 Our study is limited by a few issues. Ideal imaging would be capable of capturing  
335 the entire three-dimensional structure of the placental vascular tree down to the  
336 level of the terminal villi. However no imaging technology currently exists capable  
337 of both the field of view and magnification that this requires. We attempted to  
338 overcome this limitation by imaging at different magnifications but with reduced  
339 sampling volumes, and used this imaging data to investigate repeating spatial  
340 patterns in vascular density. As with all perfusion work, accuracy of results relies  
341 on good vascular fill<sup>27</sup>. Attempts were made to limit the effect of poor perfusion by  
342 using an optimised perfusion technique<sup>16</sup>, by choosing well perfused tissue to  
343 image at higher resolution, and by examining tissues histologically and excluding  
344 poorly perfused tissue. By choosing well perfused tissue to image at higher  
345 magnification however, block sampling was therefore not random. This is a  
346 limitation of the work, as applying the findings of statistical analysis globally to  
347 tissue relies on the assumption that tissues were randomly sampled. To mitigate  
348 the impact of this sampling technique we ensured that the samples were taken  
349 from the whole placenta, from umbilical cord to placental edge in every case.

350

351 In this work vessels were separated from tissue using simple greyscale  
352 thresholding, and a size threshold applied to select only the villous (not chorionic)  
353 vessels. More advanced algorithms exist that may improve the segmentation,  
354 combining grey-scale thresholding and algorithms that grow the vascular tree  
355 based on proximity and similarity of grey-scale values and local vesselness  
356 properties<sup>28,29,30,31</sup>. This approach would optimise the number of voxels correctly  
357 identified as vessel and minimise the noise. At present the data produced is too  
358 large and complex for available software to analyse, so further technical work is  
359 needed to optimise the vascular tree segmentation.

360 The main advantage of micro-CT imaging is that it captures the three-  
361 dimensional structure of the vascular tree. Improved segmentation of vessels  
362 would allow more advanced, derived analyses such as skeletonisation, which has  
363 been used by Rennie et al to examine in detail the branching structure and  
364 tortuosity in mouse placenta<sup>10</sup>. This has been attempted in corrosion cast  
365 imaging with micro-CT by Junaid et al<sup>13</sup>. The software they used however was  
366 limited as it was not optimised for placental data and was not capable of locating  
367 the vascular tree spatially within the placenta. This makes the branching pattern  
368 difficult to understand or analyse in a meaningful way. Data obtained using the  
369 methodology described above could be used in the development of algorithms  
370 capable of analysing features of the vascular tree such as vessel width, tortuosity  
371 and branching structure in relation to placental features such as umbilical cord  
372 insertion, chorionic vessels and placental edge. This would be an exciting  
373 application, and an important step in understanding the human placental vascular  
374 tree and how it varies in health and important obstetric pathologies, such as fetal  
375 growth restriction and pre-eclampsia.

376

377 This work presents a novel method for imaging the human placenta vascular tree  
378 using multiscale Micro-CT imaging. It demonstrates that there is a large degree  
379 of variation in vascular density throughout normal term human placentas, but  
380 does not find a reproducible spatial pattern of vascularisation between placentas.  
381 The three-dimensional data created by this technique could be used with more  
382 advanced computer analysis, to further investigate the three dimensional spatial  
383 structure of the vascular tree, and so improve our understanding of variation in  
384 normality and disease.

385

386

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474

#### 475 Statements of Contribution

476 Rosalind Aughwane

477 I declare that I have contributed to the design, acquired the data and performed  
478 the analysis of this study, that I am the primary contributor to the manuscript, and  
479 that I have seen and approved the final version. I have no conflicts of interest to  
480 declare.

481

482 Claire Schaaf

483 I declare that I have contributed to the design, execution and analysis of this  
484 study and that I have seen and approved the final version. I have no conflicts of  
485 interest to declare.

486

487 Ciaran Hutchinson

488 I declare that I have contributed to the design, execution and analysis of this  
489 study and that I have seen and approved the final version. I have no conflicts of  
490 interest to declare.

491

492 Alex Virasami

493 I declare that I have contributed to histological analysis in this study and that I  
494 have seen and approved the final version. I have no conflicts of interest to  
495 declare.

496

497 Maria Zuluaga Valencia

498 I declare that I have contributed to the automated FIJI analysis of histology, and  
499 that I have seen and approved the final version. I have no conflicts of interest to  
500 declare.

501

502 Neil Sebire

503 I declare that I have contributed to the design, execution and analysis of this  
504 study and that I have seen and approved the final version. I have no conflicts of  
505 interest to declare.

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508 I declare that I have contributed to the design, execution and analysis of this  
509 study and that I have seen and approved the final version. I have no conflicts of  
510 interest to declare.

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514 study and that I have seen and approved the final version. I have no conflicts of  
515 interest to declare.

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519 study and that I have seen and approved the final version. I have no conflicts of  
520 interest to declare.

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523 I declare that I have contributed to the design, execution and analysis of this  
524 study and that I have seen and approved the final version. I have no conflicts of  
525 interest to declare.

526

527 Anna L David

528 I declare that I have contributed to the design, execution and analysis of this  
529 study and that I have seen and approved the final version. I have no conflicts of  
530 interest to declare.  
531

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Table 1; Table showing the characteristics of the pregnancies and deliveries of the placentas included in this work.

Table 2; Table showing the vascular fill for vessels with an area  $> 10,000\mu\text{m}^2$  (vessels are within the visual resolution of whole placenta Micro-CT) and vessels with an area  $> 200\mu\text{m}^2$  (vessels within the visual resolution of the placental block Micro-CT).

Figure 1; Micro-CT imaging of a human placenta perfused with Microfil. Surface renderings made using VG StudioMAX 2.2 (Volume Graphics, Germany) were thresholded halfway between the grey scale intensities of tissue and Microfil. A and B; the whole placenta, imaged with an isotropic voxel size of  $116.5\mu\text{m}$ . C; a slice through the whole placenta, showing the geometric arrangement of chorionic and villous vessels. D and E; two blocks imaged with an isotropic voxel size of  $13.5\mu\text{m}$ . The complex vascular tree is clearly seen, with whole imaging showing chorionic and stem vessels, and block imaging showing the villous vascular tree down to the terminal capillaries.

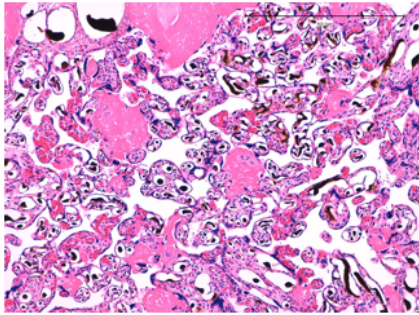
Figure 2; FIJI Histological Analysis Pipeline.

Figure 3; A and B; example of normalised distance maps radiating out from the umbilical cord insertion for placenta 3 (A) and 9 (B), C and D; example vascular density maps for the same placenta, E and F; graphs showing mean vascular density for each of the 100 regions from the umbilical cord insertion (0) to the placental edge (100), for each placenta. G; the combined mean vascular density with distance from the umbilical cord insertion with error bars showing standard deviation.

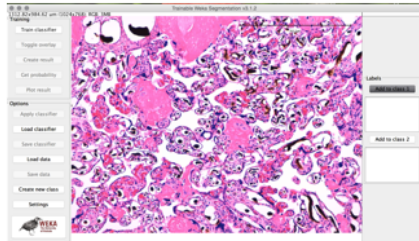
Figure 4: A and B: Box plots showing the spread of block vascular density (box shows 25<sup>th</sup> to 75<sup>th</sup> centile, with midline showing the median) between placentas, measured with block  $\mu\text{CT}$  (A) and histological analysis (B). C and D; Graphs showing correlation between block villous vascular density and normalised block location in relation to the umbilical cord insertion (0) and placental edge (100) measured with block  $\mu\text{CT}$  (C) and histological analysis (D).

Parameter	Clinical Characteristics of Pregnancies for Placentas Studied			
Gravidity	Median = 3	25-75% =2-3	Range 1-5	
Parity	Median = 1	25-75% = 1-2	Range 0-3	
Gestational age (weeks)	Average = 39	SD = 0.37	Range 38+1 – 39+4	
Maternal age (years)	Average = 36	SD = 4.6	Range 31-46	
Ethnicity	Black = 1	White = 4	Other = 3	Unknown = 2
Birth weight (grams)	Average = 3565	SD =414	Range 2730-4000	
Placental weight (grams)	Average = 673	SD = 58	Range 551-745	
Baby's gender	Female = 3	Male =7		

Placenta	Vessels with an area >10,000 $\mu\text{m}^2$			Vessels with an area >200 $\mu\text{m}^2$		
	Number of blocks with vascular fill 100%	Mean Vascular Fill over all blocks (%( $\pm$ SD))	Minimum Block Vascular Fill (%)	Number of blocks with vascular fill >75%	Mean Villous Vascular Fill over all blocks (% ( $\pm$ SD))	Minimum Block Villous Vascular Fill (%)
1	8	100 (0)	100	6	85 (21)	34
2	6	97 (8)	77	3	59 (30)	18
3	7	99 (3)	91	3	68 (26)	23
4	4	77 (40)	10	3	58 (34)	8
5	6	87 (31)	17	2	55 (20)	18
6	8	100 (0)	100	6	85 (18)	43
7	6	98 (5)	7	3	62 (23)	47
8	6	98 (3)	91	4	70 (25)	16
9	7	99 (2)	95	4	72 (19)	46
10	7	100 (1)	97	4	74 (21)	37
	N=65	95 (14)	17	N=38	69 (11)	8



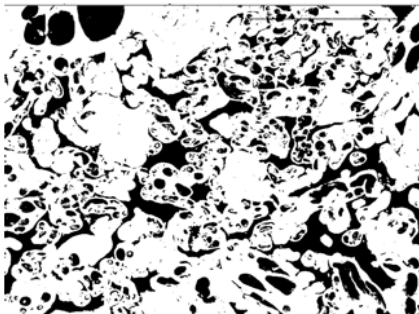
A TIFF file of the micrograph was opened in FIJI. As the files were large the image was reduced in size by half, this kept good resolution whilst allowing faster analysis. The scale was set ( $0.78\mu\text{m}=1$  pixel).



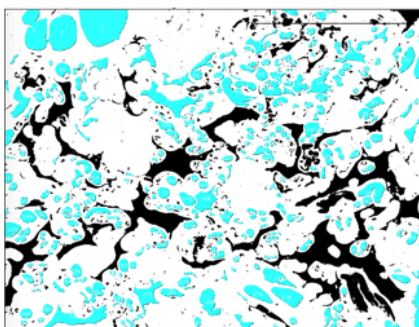
The image was loaded into the Trainable Weka Segmentation (Version 3.1.2) plugin for FIJI<sup>13</sup>. The trained classifier was loaded, and the tool was run to create a segmented result.



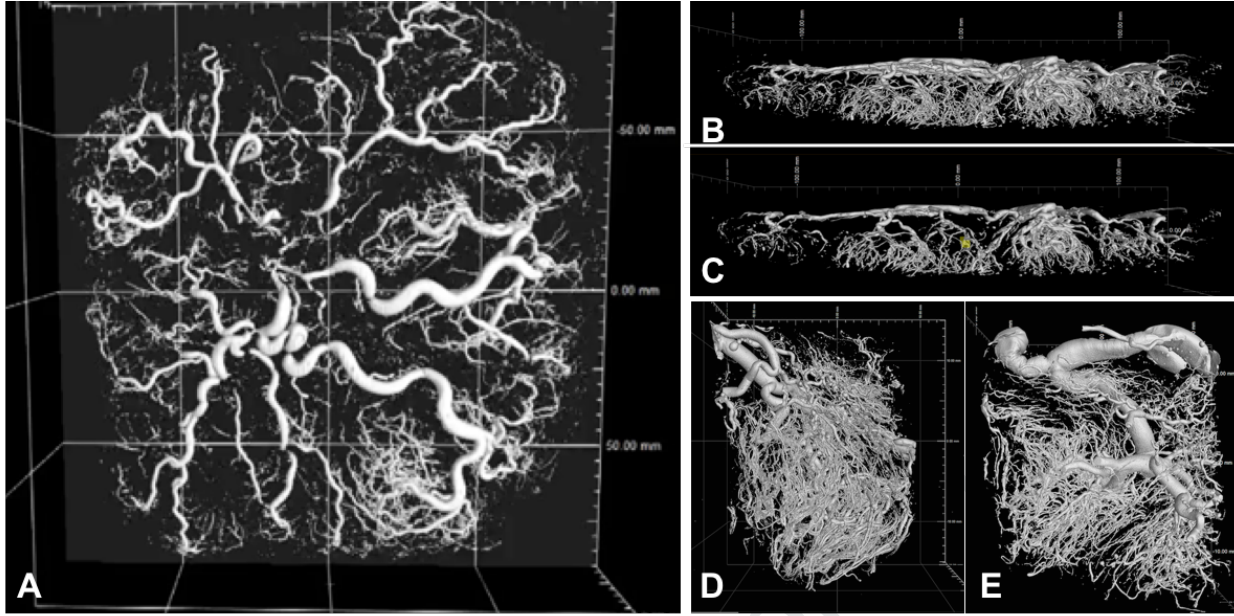
The resulting segmentation showed perfused vessels and background in green, un-perfused vessels in purple, and villi in red.



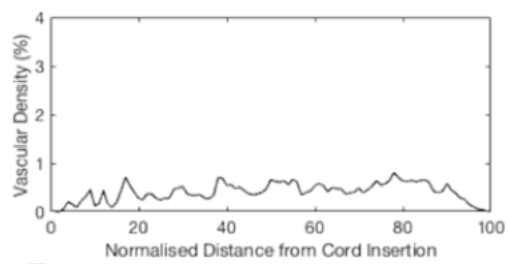
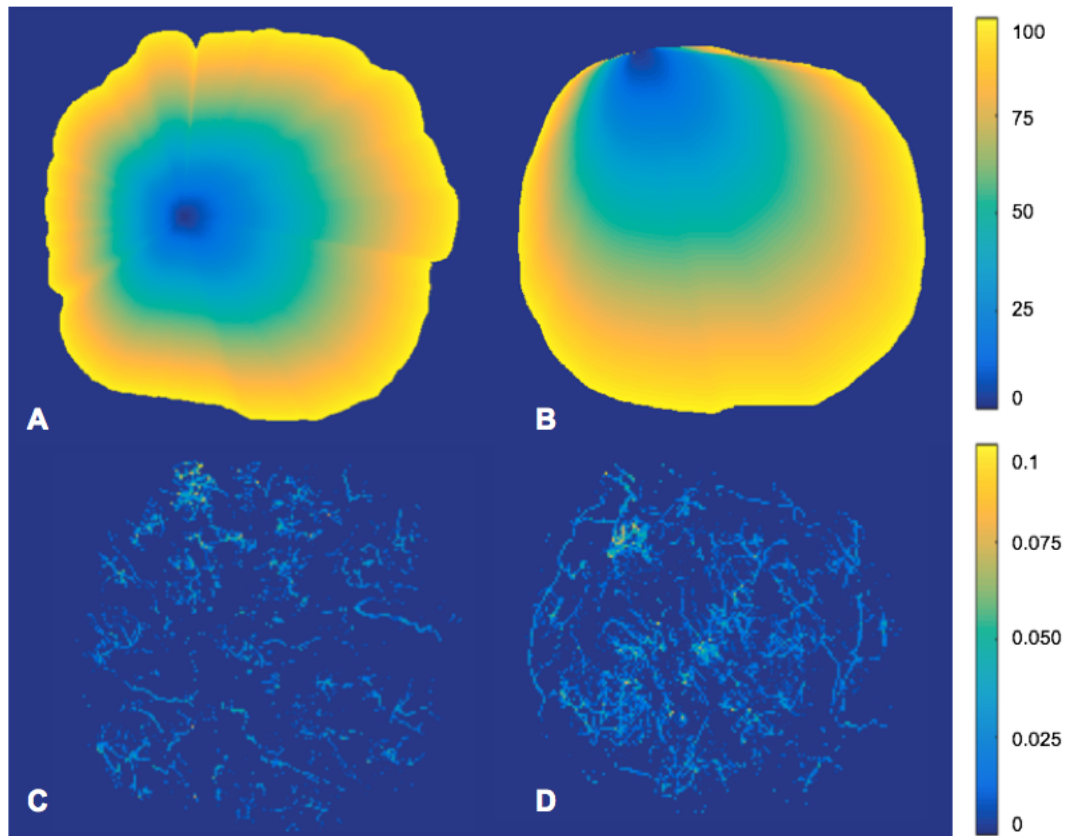
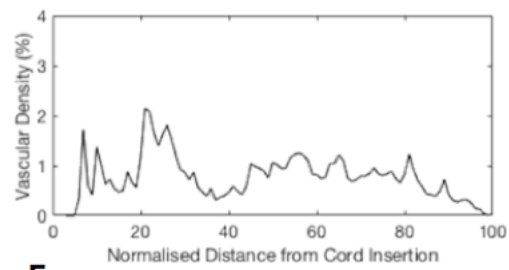
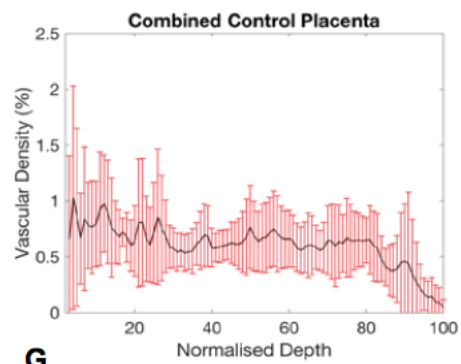
The images were thresholded to select perfused vessels and background (shown), un-perfused vessels, or villi.

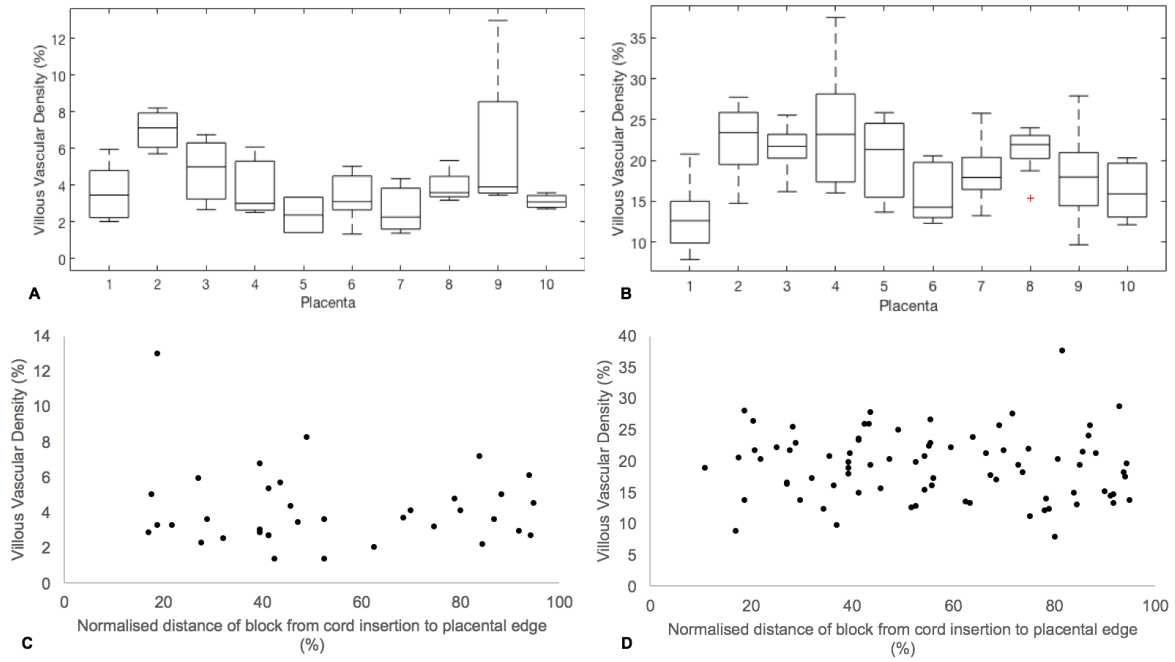


The Analyse Particle tool was used to measure area of the vessels. For vessels, the tool was set to include particles with a surface area between  $60-100000\mu\text{m}^2$ , and circularity 0.20-1.00. For the villi the whole of the segmented area was measured. The tool produces a table with a list of areas.



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Highlights:

- Micro-CT and histological investigation of vascular density in the placenta
- There is a large degree of variation in vascular density throughout placentas
- This imaging has potential for future spatial investigation of the 3D vascular tree

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Conflicts of interest; None.

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