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Anemia in late pregnancy induces an adaptive response in fetoplacental vascularization

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ABSTRACT

Introduction: Anemia during pregnancy may compromise fetal and newborn's health, however, little is known about how and when the fetoplacental vascularization is most vulnerable to anemia.

Methods: Using systematic and isotropic uniform random sampling, placental samples were collected from 189 placentas in a cohort study of Tanzanian women whose hemoglobin concentration was measured throughout pregnancy. Fetoplacental vessels and villi were defined as exerting either a transport or diffusion function. The vascularization patterns for transport and diffusion vessels and villi were assessed by stereology. Blood vessel length, surface area and diffusion distance as well as placental villi volume were calculated.

Results: Anemia from a gestational age of 23 weeks was significantly associated with increased fetoplacental vascularization in vessels and villi compared to women who were non-anemic throughout pregnancy. Transport surface vessel area: 0.31 m^2 [95% CI: 0.18–0.55], P = 0.01; Transport villi volume 19.8 cm^3 [95% CI: 6.37–33.2], P = 0.004, Transport vessel diameter 7.23 μm [95% CI: 1.23–13.3], P = 0.02. Diffusion vessel surface: 3.23 m^2 [95% CI: 1.55–4.91], P < 0.001 and diffusion villi volume: 29.8 cm^3 [95% CI: 10.0–49.5], P = 0.003). Finally, all the measured transport vessel and villi significantly parameters and diffusion vessel surface, vessel diameter and diffusion distance were associated with birth weight.

Discussion: Increased fetoplacental vascularization related to anemia from a gestational age of 23 weeks in pregnancy together with the association between fetoplacental vascularity and birth weight suggest that the timing of anemia determines the effect on fetoplacental vascularization and underlines the clinical relevance for proper development of fetoplacental vasculature.

1. Introduction

Globally, approximately 38.2 million pregnant women suffered from anemia in 2011 [1]. The causes of anemia are multifactorial including iron deficiency, malnutrition and infectious diseases [2,3]. Maternal anemia has been associated with preterm birth, reduced birth weight and impaired placental development reflected in an increased placental volume and an altered vascularity [4–7].

Throughout pregnancy the fetoplacental vasculature is developed and remodeled to accommodate the increasing demands from the growing fetus [8,9]. The vascular system in the placenta is developed through different processes. Vasculogenesis forms new vessels from precursor cells in early pregnancy. This is followed by angiogenesis, a process where new vessels are created of the preexisting vessels,

Abbreviations: GA, gestational age; Hb, Hemoglobin; H&E, Hematoxylin & eosin stain; mRDT, malaria rapid diagnostic test

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expanding the vascular network. Angiogenesis comprise both branching angiogenesis forming a branched web of capillaries and the non-branching angiogenesis where existing capillaries are expanded [8–10]. It has been suggested that a physiological adaptive response to low oxygen tension in anemic women could be an increased placental volume and vascular surface area, representing an increased branching angiogenesis [11]. Additionally, both timing and level of anemia could influence the way fetoplacental vascularization is affected. The relationship between hemoglobin concentration (Hb) and birth weight seems to be u-shaped [6] and poor placental development may partly influence this phenomenon where moderate-severe anemia in the first half of pregnancy could be detrimental [6,12,13]. Mild anemia in later pregnancy can reflect a drop in Hb due to the physiological plasma expansion observed in normal pregnancies, and has been associated with reduced risk of low birth weight [14].

In a prospective cohort study in Tanzania, we compared the effects of maternal anemia at different timepoints during pregnancy on fetoplacental vascularization patterns and birth weight using design-unbiased stereology to obtain three-dimensional information about volume, surface and length of the fetoplacental vascular network.

2. Methods

Among 538 pregnant women enrolled in the longitudinal study "Foetal exposure and epidemiological transition: the role of anaemia in early life for non-communicable diseases in later life" (FOETALforNCD) 409 placentas were collected. 209 placentas had the fetoplacental vascularization assessed with stereology blinded to clinical data.

The FOETALforNCD study was conducted in Korogwe and Handeni Districts, Northeastern Tanzania from July 2014 until December 2016 and consisted of two cohorts. A cohort of women enrolled before pregnancy and followed until delivery, and a cohort of women enrolled in early pregnancy. The inclusion criteria for the pre-conceptional cohort were age 18–40 years, a negative urine pregnancy test (HCG®, Vista Care Company, China), no unsuccessful conceiving ≥ two consecutive years, no modern contraceptive methods except condoms, no baby less than nine months, living in an accessible area, and willing to attend antenatal care and give birth at Korogwe District Hospital (KDH). Inclusion criteria for the pregnancy cohort was a pregnancy ≤14 weeks of gestation and a hemoglobin (Hb) concentration ensuring a 1:1 balanced inclusion of women with (Hb < 11 g/dL) and without (Hb ≥ 11 g/dL) anemia.

Gestational age was estimated with *trans*-abdominal ultrasound (5-2 MHz abdominal probe, Sonosite TITAN® and Sonosite Turbo®, US High resolution, Sonosite, Bothell, USA) using crown rump length in the 1st trimester [15] and head circumference in the 2nd trimester [16].

Information and clinical history was collected upon enrolment. The women were scheduled for five antenatal visits at GA 11-14, 20-22, 26-28, 32-34, and 37–39 weeks. At each contact, medical examination, including screening for malaria and anemia, was performed. Venous blood was collected in EDTA tubes (6 mL, BD, Franklin Lakes, USA), kept at 2–8 °C until processing at KDH within 2 h of collection. Hb was measured using a Sysmex KX-21 N haematological analyzer (Sysmex Corporation Kobe, Japan). Iron and folic acid supplementation were offered throughout pregnancy (combination tablet of 200 mg ferrous sulfate (~43 mg elemental iron) and 400 μg folate (Ferrolic–LF®, Laboratory and Allied LTD, Kenya)). Anemia in pregnancy was defined as Hb < 11 g/dL and treated with either 2-3 combination tablets of iron-folic acid per day or with Hemovit® multivitamin syrup (200 mg Ferrous sulfate, 0.5 mg B6, 50 μg B_{12}, 1.5 mg Folic acid and 2.33 mg zinc sulfate per 5 mL, Shelys Pharmaceuticals, Tanzania) 10 mL 2-3 times daily. Malaria was diagnosed by malaria rapid diagnostic test (ParaHIT®, Span Diagnostics, India) or CareStart (Access Bio, USA) followed by blood slide microscopy [17]. mRDT positive cases and selected blood samples were also tested by PCR [18]. Malaria were treated with Artemether-Lumefantrine (Lumartem 20mg/120 mg, CIPLA LTD, India) in the 2nd and 3rd trimester and with quinine (Quinine tablet 300 mg, S Kant Health care LTD, Gujarat, India or Quinine injection 600 mg/2 mL, NCPC International Corporation, China) in the 1st trimester. HIV-negative women were given intermittent preventive treatment for malaria with Sulfadoxine-Pyrimethamine (1500 mg/75 mg) from 16 weeks of gestation, at least one month apart. Hypertension was defined as systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg (r-champion® N, Rudolf Riester, Jungingen, Germany) on ≥ two occasions, and pre-eclampsia as hypertension with proteinuria observed after a GA of 20 weeks. Diabetes was diagnosed with an oral glucose tolerance test in the 3rd trimester [19] (HemoCue Glucose 201 analyzer, HemoCue AB, Angelholm, Sweden). At birth, birth weight (precision 5–10 g, 140 M107600, ADE, Germany), mode and place of delivery were noted and the newborn was assessed for congenital malformations.

2.1. Sampling

The FOETALforNCD study investigated the effect on both anemia and malaria on placental development and the sample size for stereology was based on convenience sampling. The majority of studies in placental stereology have been based on 30-100 samples (1:1 ratio cases and controls), and this study therefore aimed for 200 placentas: 50 with anemia (without malaria), 100 with malaria (with or without anemia), and 50 controls (without malaria and without anemia). Placentas from singleton pregnancies without congenital malformation and complete data on Hb were included. After validation of the data, the final sample size included 189 placentas (Table 1) [20]: 52 placentas with anemia (without malaria), 73 placentas with both anemia and malaria, 19 placentas with only malaria and finally 45 controls without neither malaria nor anemia (Fig. 1).

The placentas were processed immediately after delivery if birth took place at KDH and as soon as possible after home delivery, and processing time was documented. The umbilical cord was clamped after delivery of the newborn. After placental delivery, membranes and cord were removed and the placenta was weighed (precision 1 g, Digital scale 852, SECA, UK) and photo-documented.

Ten full depth placental tissue blocks were collected using systematic uniform random sampling (Supplementary Figure 1) to ensure an equal probability of the entire placenta to be sampled [21,22]. The placenta was cut in slices of 2 cm and positioned to form a long line. The sampling distance between the blocks was 1:10 of the line length, and the sampling starting point was chosen following a random number printed on a specially designed matrix. Samples from membrane and cord were also collected. Samples were fixated in 10% formalin until further processing in Denmark.

Sections for paraffin embedding were collected by isotropic uniform random sampling (Supplementary Figure 2). The principle of Mattfeldt's orientator was used to ensure a random 3D orientation of the sections [23]. An isotropic cut surface was generated, and the tissue was processed and embedded in paraffin (Vacuum Infiltration Processor, Sakura Proshop). Sections of 4 μm were cut and immunohistochemically stained with CD34 using Dako PT Link & Dako Autostainer Link48, Agilent, USA. H&E was used as a nuclear and background histochemical stain.

2.2. Stereological examination

Five slides of CD34-stained sections from each of the 189 placentas were sampled systematically and analyzed by stereology using an Olympus BX50 light microscope with a 20× lens (NA 0.70), LBD filter, Olympus DP70 digital camera and new CAST version 7.2.0.3197 software (Visiopharm, Hoersholm, Denmark) (Supplementary Figure 3).

To facilitate stereological analysis the vessels and villi were categorized as transport and diffusion vessels and villi based on their function. Stem villi and immature intermediate villi are both leading

Table 1a
Clinical characteristics of pregnancies for placentas studied, Control placentas: Hb ≥ 11 g/dL (n = 64).

	N		n (%)	Median (range)/Mean (± SD)
Gravidity	64			4 (1-7)
Parity	64			3 (0-6)
Gestational age (weeks)	64			40 (± 2.7)
Maternal age (years)	64			28 (17-40)
Race	64	Black	64 (100)	
Ethnicity	64	Tanzania	64 (100)	
Tribe	64	Samba	22 (34.4)	
		Zigua	25 (39.0)	
		Pare	3 (4.6)	
		Other	14 (21.9)	
Prenatal Medication	64	Iron	63 (98.4)	
		IPTp	63 (98.4)	
		Folic acid	63 (98.4)	
		Hemovit	4 (6.3)	
		Antimalarial	17 (26.6)	
		Anti-helminthic	61 (95.3)	
		Anti-Hypertensive[a]	7 (10.9)	
Drugs	64	Cigarettes	0	
		Alcohol	0	
		Other	0	
Previous prenatal admission	64		ND[b]	
Blood pressure ≥ 140/90 mmHg	64		7 (10.9)	
Screened for diabetes	64		64 (100)	
Antibiotics during labor	64	None	61 (95.3)	
		Penicillin	1 (1.6)	
		Other	2 (3.1)	
Beta strep status	64	Unknown	64 (100)	
Antenatal steriods	64		0	
Magnesium Sulfate	64		1 (1.6)	
Anesthesia	64	Epidural	0	
		Narcotics	0	
		General	2 (3.1)	
		None	62 (96.9)	
Cervical ripening agent	64	Prostaglandin E_1	0	
		Prostaglandin E_2	0	
		Mechnical	0	
		Misoprostol	8 (12.5)	
Delivery mode	64	Vaginal delivery	62 (96.9)	
		C-section, repeat, before labour	1 (1.6)	
		C-section, repeat, during labour	0	
		C-section, primary, before labour	1 (1.6)	
		C-section, primary during labour	0	
Oxygen given at delivery	64		0	
Birth weight (grams)	64			2895 (± 635)
Placenta weight (grams)	64			420 (± 109)
Sex of newborn	64	Female	35 (54.6)	
		Male	29 (45.3)	
Processing time (min)[c]	55			55 (± 35)

Notes: a) Nifedipine or/and methyldopa for women with hypertensive disorder during pregnancy by the clinicians discretion. b) Previous prenatal admissionsvaried including admission to observe for malaria, reduced movement and suspected labor. c) Time from delivery of the placenta until end of processing.
Abbreviations: C-section – cesarean section. IPTp – intermittent preventive treatment in pregnancy for malaria. IQR – interquartile range. ND – not determined. SD – standard deviation.

Table 1b
Clinical characteristics of pregnancies for placentas studied, Anemic placentas: Hb < 11 g/dL (n = 125).

	N		n (%)	Median (range)/Mean (± SD)
Gravidity	125			3 (1-8)
Parity	125			2 (0-7)
Gestational age (weeks)	125			40 (± 2.0)
Maternal age (years)	125			26 (14-55)
Race	125	Black	125 (100)	
Ethnicity	125	Tanzania	125 (100)	
Tribe	125	Samba	51 (40.8)	
		Zigua	42 (33.6)	
		Pare	16 (12.8)	
		Other	16 (12.8)	
Prenatal Medication	125	Iron	125 (100)	
		IPTp	122 (97.6)	
		Folic acid	125 (100)	
		Hemovit	54 (43.2)	
		Antimalarial	74 (59.2)	
		Anti-helminthic	97 (77.6)	
		Anti-Hypertensive[a]	5 (4.0)	
Drugs	125	Cigarettes	0	
		Alcohol	2 (1.6)	
		Other	0	
Previous prenatal admission	125		ND[b]	
Blood pressure ≥ 140/90 mmHg	125		5 (4.0)	
Screened for diabetes	125		125 (100)	
Antibiotics during labor	125	None	120 (96.0)	
		Penicillin	2 (1.6)	
		Other	1 (0.8)	
		Unknown	2 (1.6)	
Beta strep status	125	Unknown	125 (100)	
Antenatal steriods	125		0	
Magnesium Sulfate	125		1 (0.8)	
Anesthesia	125	Epidural	0	
		Narcotics	0	
		General	11	
		None	114	
Cervical ripening agent	125	Prostaglandin E_1	0	
		Prostaglandin E_2	0	
		Mechnical	0	
		Misoprostol	14 (11.2)	
Delivery mode	125	Vaginal delivery	114	
		C-section, repeat, before labour	3	
		C-section, repeat, during labour	4	
		C-section, primary, before labour	0	
		C-section, primary during labour	3	
		C-section, unknown if repat/labour	1	
Oxygen given at delivery	125		0	
Birth weight (grams)	125			2993 (± 526)
Placenta weight (grams)	125			470 (± 112)
Sex of newborn	125	Female	67 (53.6)	
		Male	58 (46.4)	
Proceesing time (min)[c]	125			61 (± 37)

Notes: a) Nifedipine or/and methyldopa for women with hypertensive disorder during pregnancy by the clinicians discretion. b) Previous prenatal admissionsvaried including admission to observe for malaria, reduced movement and suspected labor. c) Time from delivery of the placenta until end of processing.
Abbreviations: C-section – cesarean section. IPTp – intermittent preventive treatment in pregnancy for malaria. IQR – interquartile range. ND – not determined. SD – standard deviation.

the blood to the terminal villi and were defined as transport villi. The transport villi were recognized by a central positioned vessel surrounded by smooth muscle cells and connective tissue or a large vessels

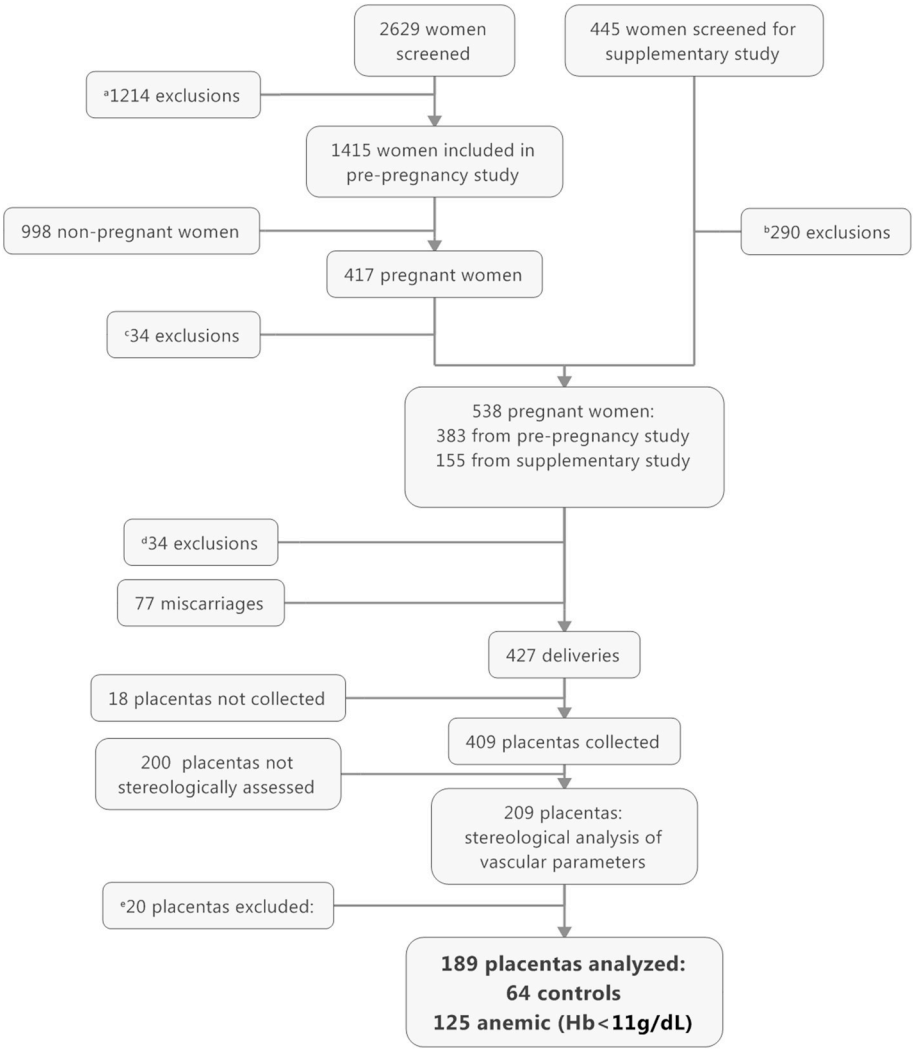

Fig. 1. Flow chart displaying the study overview and selection of the placentas for analysis.
189 analyzed placentas: 129 placentas from the pre-pregnancy study and 60 from the supplementary study. a) 1214 exclusions from the pre-pregnancy study: 51 living outside of study area, 322 on modern family planning, 313 not eligible by age, 93 having a child aged < 9 months, 116 infertile, 208 who were pregnant, 34 who did not consent and 77 for other reasons. b) 290 exclusions from the supplementary study: 119 with a GA > 14 weeks, 142 due to imbalance of Hb ratio and 29 for other reasons. c) 34 exclusions after confirmed pregnancy: 6 did not consent, 12 miscarried before inclusion to pregnancy study, and 16 had ongoing miscarriage when identified as pregnant. d) 34 exclusions from pregnancy study: 27 lost to follow-up and 7 withdrew consent. e) 20 exclusions of placentae: 6 with incomplete stereological data, 5 twins, 1 with congenital malformation and 8 with incomplete data on hemoglobin concentration. Hb: Hemoglobin g/dL.

surrounded by a fibrous reticular stroma. Mature intermediate and terminal villi both contain small vessels almost without any surrounding tissue and this is where the materno-fetal exchange occurs. Hence, these were categorized as diffusion vessels [10].

Framed areas with a step length of 2000 × 2000 μm was investigated using three probes to assess length and surface area of the fetoplacental vessels and the volume of the placental villi (Supplementary Figure 3). Intervillous space, septa, and inactive areas were assessed but not quantified.

Transport and diffusion vessel length was estimated using a 2D counting frame with an area of either 100,000 μm^2 or 5000 μm^2 (Supplementary Figure 3). Transport and diffusion vessel surface area was estimated using a line grid with a length per point of 14.6 μm. The volume of transport and diffusion villi was estimated using a point grid (Supplementary Figure 3). Six test points per field of view were used for counting the intervillous space, while transport and diffusion villi, inactive area and septa were counted with 24 test points.

The placental volume V(Pl) was estimated for each placenta by the formula:

$$V(PL)(cm3) = 1.05\frac{g}{cm^3}\cdot \text{placental weight (g)}$$

Transport and diffusion vessel profiles were counted to estimate the length of the vessels, defined as the statistical length of the central part of a vessel. The number of vessel profiles per reference volume (RV), the vessel length density, was calculated as follows:

$$L_V(*/Pl): = \frac{2\sum Q(*)}{\frac{a}{p}\cdot \sum P(Pl)}(\mu m^{-2})$$

$L_V(*/PL)$:: micro vessel length density in placenta, *: micro vessels, Pl: Placenta, $\sum Q(*)$: sum of micro vessel profiles in the counting frame, $\frac{a}{p}$: area per point, $P(Pl)$: control points,

The total vessel length for the transport and diffusion vessels:

$$L(*, Pl)\,(km) = L_V(*/Pl)\cdot V(Pl)$$

$L(*, Pl)$: total micro vessel length in placenta, $V(PL)$: total placental volume.

Intercepts of transport or diffusion vessels were counted to estimate the vessel surface area:

$$S_V(*/Pl): = \frac{2\sum I(*)}{\frac{l}{p}\cdot \sum P(Pl)}(\mu m^{-1})$$

$S_V(*/PL)$: micro vessel surface area density, $\sum I(*)$: sum of micro vessel intercepts, $\frac{l}{p}$: length per test point, $\sum P(Pl)$: *sum of*

The total vessel surface area for the transport and diffusion vessels:

$$S(*, Pl)(m2) = S_V(*/Pl)\cdot V(PL)$$

$S(*, Pl)$ total micro vessel surface area.

Volume density was estimated by counting transport or diffusion vessels presenting below a point:

$$V_V(\#/Pl): = \frac{\sum P(\#)}{\frac{nb}{\sum P(Pl)}},$$

V_V: volume density, #: villi, $\sum P(\#)$: the total sum of points, V(#, Pl): total villi volume in placenta, *nb*: number of points.

By multiplying by the corresponding placental volume the absolute villi volume was estimated:

$$V(\#, Pl)\,(cm^3) = V_V(\#/Pl) \cdot V(PL)$$

The diffusion villi diffusion distance (D_D), the radius of the cross-sectional area of the tissue around a vessel, assuming that each vessel has a cylindrical shape and supplies a tissue cylinder, was calculated with the following equation:

Diffusion villi diffusion distance (μm): $D_D = \sqrt{\frac{1}{\pi \cdot L_V(*/Dif)}}$

The number of diffusion vessel profiles per reference volume, the diffusion vessel length density, $L_V(*/Dif)$, was calculated as follows:

$$L_V(*/Dif): = \frac{2\sum Q(*)}{\frac{a}{p} \cdot \sum P(Dif)} (\mu m^{-2})$$

$P(Dif)$: total diffusion points.

Vessel diameter was calculated from the length and surface:

$$\text{Vessel diameter } D_v = \frac{S(*,Pl)}{\pi \cdot L(*,Pl)}$$

2.3. Histological examination

Up to 10 H&E stained slides from each placenta, the free membranes and the umbilical cord were histologically examined by a pathologist (LG). Slides were blinded and examined with a 4× lens (NA 0.2) and/or a 10× lens (NA 0.45) and five slides with a 40× lens (NA 0.95). Morphological abnormalities including acute inflammation in the cord, membranes and villi, chronic inflammation in decidua, signs of maternal and fetal vascular malperfusion, villitis of unknown etiology (VUE), chorangiosis, immature foci, fetal erythroblastosis, calcifications and change due to fetal death were assessed [24].

2.4. Definition of exposure to anemia

Anemia was defined as Hb < 11 g/dL [1] and further subdivided into mild-moderate anemia (9.1–10.9 g/dL) and moderate-severe anemia (Hb ≤ 9 g/dL) [25] for sensitivity analyses.

To investigate how the timing of the anemic onset affected the fetoplacental vascular development, the women were divided into three groups: women who were only anemic before 23 weeks GA, women who were only anemic from 23 weeks GA, and women who were anemic before and after a GA of 23 weeks. Controls were women who were non-anemic (Hb ≥ 11 g/dL) throughout pregnancy.

2.5. Statistics

Statistical analyses were performed using Stata 13.1 (Stata corp Lakeway College Station, USA). All stereological indices (transport and diffusion vessel length, vessel surface, villi volume and diffusion vessel distance) had a parametric distribution and simple and multivariate linear regression modeling were used to assess the association between anemia (Hb < 11 g/dL) in the three GA intervals and each of the stereological indices. Non-anemic women were used as the reference group.

Potential confounders were identified using a conceptual framework approach [26] and tested with Students' t-test, one-way ANOVA, and simple linear regression or Spearman-Rank correlation where relevant. Variables with a p-value < 0.2 in univariate analyses were included in multivariate analysis (Supplementary Table 1, Supplementary Table 2). The final multivariate regression models were generated using backwards stepwise selection procedure and included all variables with a p-value < 0.1. A p-value < 0.05 was considered statistically significant. Malaria is a major cause of anemia and is associated with changes in the fetoplacental vascularization [27]. An interaction term between malaria and anemia was therefore generated and included in the multivariate models if statistical significant. Final models were tested for multicollinearity using the variance inflation factor (vif) and for omitted variables (ovtest).

Association between birth weight and the stereological indices were assessed by simple and multivariate linear regression including only live born singletons delivered after 28 weeks of gestation without congenital malformations and assessed within 24 h of delivery.

To ensure that all degrees of anemia could be analyzed as one group the vascular parameters for women experiencing anemia Hb ≤ 9 g/dL both before and after a GA of 23 weeks were compared to women with Hb 9.1–10.9 g/dL both before and after a GA of 23 weeks using Students' t-test.

Finally, to ensure that the effect of anemia was not explained by malaria, vascular parameters were compared using Students' t-test among women with Hb ≤ 9 g/dL before and after a GA of 23 weeks after stratifying by malaria infection. The number of women (n = 1) who were only anemic (Hb ≤ 9 g/dL) before 23 weeks gestation and the number of women (n = 2) who were only anemic (Hb ≤ 9 g/dL) from 23 weeks GA were too small to be included in the sensitivity analyses.

2.6. Ethics

FOETALforNCD was granted ethical clearance by the Medical Research Coordinating Committee of the National Institute for Medical Research, Tanzania (NIMR/HQ/R.8a/Vol. IX/1717). Procedures were conducted in accordance with Declaration of Helsinki [28] and Good Clinical and Laboratory Practice [29]. Trial registration: NCT02191683.

3. Results

125/189 women experienced anemia (Hb < 11 g/dL) at least one time during pregnancy, and among these 24 had moderate-severe anemia (Hb ≤ 9 g/dL). Sixty-four women, who were non-anemic throughout pregnancy, constituted the control group (Table 2).

3.1. Transport vessels and villi

The most pronounced effect of anemia was observed in women exposed to anemia from a GA of 23 weeks. Among these women, a statistically significant increase in transport vessel surface and transport villi volume, both with almost a third compared to controls, transport vessel diameter with an increase of more than 20% and a trend towards an increased transport vessel length of more than 20% were observed (Fig. 2, Table 3). These associations were statistically significant for vessel length, vessel surface and villi volume after adjusting for confounding factors (Table 3).

Anemia before 23 weeks of GA did not significantly affect the

Table 2
Distribution of anemia in the different gestational intervals.

	< 23w	≥23w	< 23w + ≥23w
Hb ≤ 9 g/dL	1	2	21
Hb 9.1–10.9 g/dL	12	22	39
Total anemia (Hb < 11 g/dL)	13	24	88[a]
Hb ≥ 11 g/dL (Control)			64

[a] Hb < 11 g/dL comprise 18 additional women who were not included in the anemia subgroups. They had Hb ≤ 9 g/dL in < 23w and Hb 9.1–10.9 g/dL in ≥23w or vice versa. Hb: Hemoglobin. < 23w: up to 23 weeks of gestational age. ≥23w: from 23 weeks of gestational age to delivery.

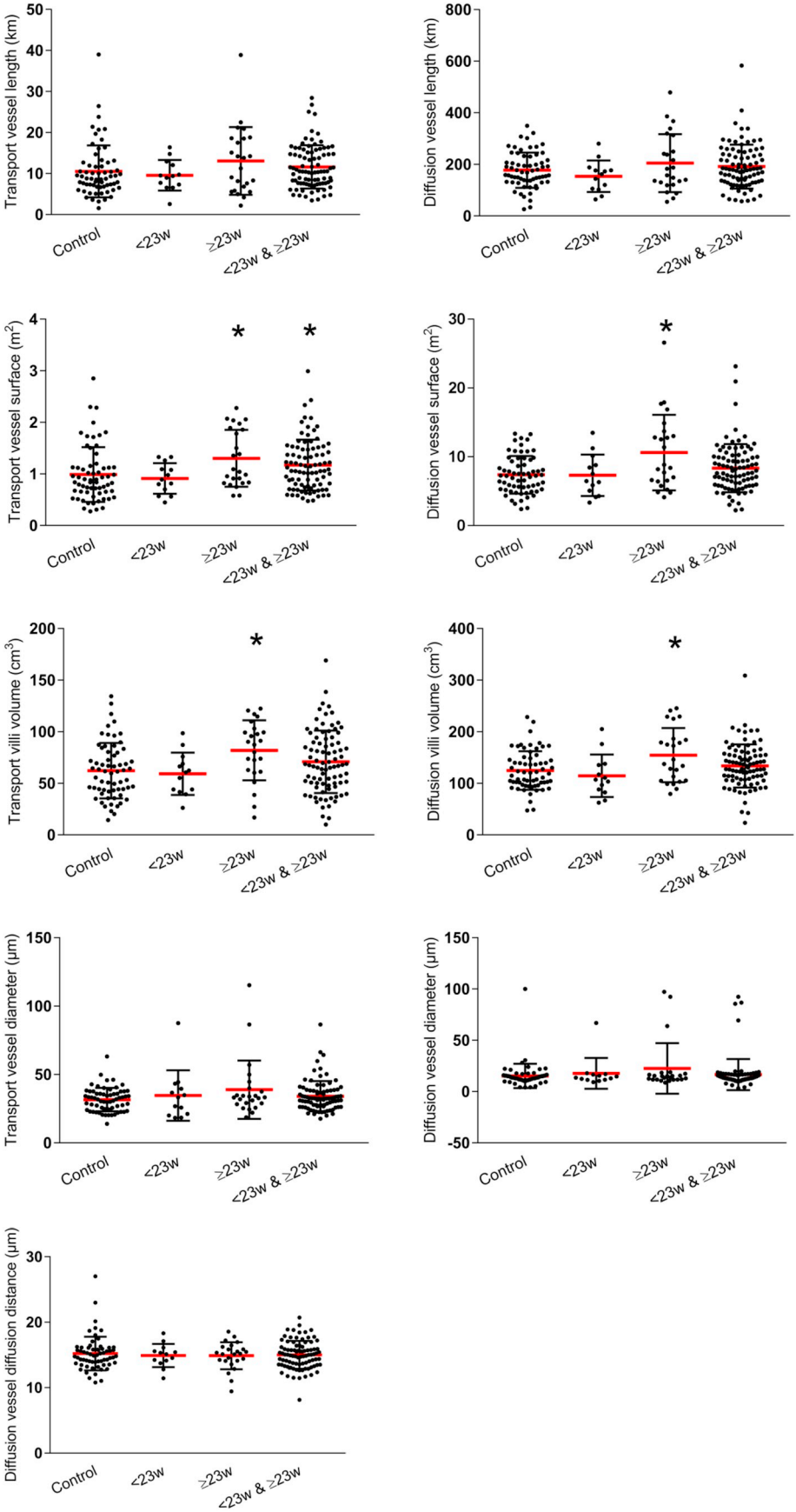

Fig. 2. Vascular parameters for transport and diffusion vessels and villi for women with Hb < 11 g/dL and controls. Mean (red line) with standard deviation (whiskers). *: P < 0.05 when compared to controls. Panels: 1: Transport vessel length (km). 2: Transport vessel surface (m^2) 3: Transport villi volume (cm^3). 4: Diffusion vessel length (km). 5: Diffusion vessel surface (m^2). 6: Diffusion villi volume (cm^3). 7: Transport vessel diameter (µm). 8: Diffussion vessel diameter (µm) 9: Diffusion vessel diffusion distance (µm).

Table 3
Transport Vessel and Villi. Effect of anemia (Hb < 11 g/dL) on vascular parameters before and after adjusting for confounding factors.

	Control (n = 64) (Reference)		Gestational age < 23 weeks (n = 13)			Gestational age ≥23 weeks (n = 24)			Gestational age < 23 weeks & ≥23weeks (n = 88)		
	Mean	SD	Effect	95%CI	P	Effect	95% CI	P	Effect	95% CI	P
Unadjusted											
Vessel length (km)	10.5	6.34	−0.95	−4.56, 2.66	0.60	2.54	−0.30, 5.38	0.08	1.09	−0.86, 3.04	0.27
Vessel surface (m^2)	0.99	0.53	−0.08	−0.38, 0.22	0.62	0.31	0.18, 0.55	0.01	0.18	0.02, 0.35	0.03
Vessel diameter (μm)	31.6	8.57	3.03	−4.59, 10.64	0.43	7.23	1.23, 13.3	0.02	2.48	−1.63, 6.59	0.24
Villi volume (cm^3)	62.2	26.8	−2.93	−20.0, 14.1	0.74	19.8	6.37, 33.2	0.004	8.64	−0.57, 17.8	0.07
Adjusted											
Vessel length (km)[a]			0.70	−5.83, 4.42	0.79	6.66	3.11, 10.21	< 0.001	3.99	0.64, 6.34	0.001
Vessel surface (m^2)[b]			0.01	−0.42, 0.44	0.95	0.49	0.17, 0.80	0.003	0.28	0.07, 0.49	0.01
Villi volume (cm^3)[c]			−3.74	−25.3, 17.8	0.73	29.8	14.8, 44.7	< 0.001	9.75	−0.68, 20.1	0.07

A multivariate linear regression model was generated for each vascular parameter as the outcome. The reference group in all models was placentas from women who were non-anemic throughout pregnancy (n = 64). [a]Adjusted R^2: 0.37, [b]Adjusted R^2: 0.29, [c]Adjusted R^2:0.35. The final models on the effect of anemia on vascular parameters were adjusted for the following confounders: **Transport vessel length**: malaria 0–14 weeks of GA, malaria after 28 weeks of GA, interaction between anemia and malaria 0–14 weeks of GA, interaction between anemia and malaria 15–22 weeks of GA, weight, paucigravidae, positive OGTT, placental processing time, tea consumption. **Transport vessel surface**:, interaction between anemia and malaria 0–14 weeks of GA, interaction between anemia and malaria 15–22 weeks of GA, interaction between anemia and malaria 28 weeks of GA to delivery, height, paucigravidae, positive OGTT, placental processing time **Transport villi volume**: malaria 0–14 weeks of GA, interaction between anemia and malaria 15–22 weeks of GA, interaction between anemia and malaria 23–27 weeks of GA, interaction between anemia and malaria 28 weeks of GA to delivery, height, positive OGTT. **Transport vessel diameter** was not significant in the adjusted model.

vascular transport parameters.

Placentas from women experiencing anemia both before and from 23 weeks of GA showed an increased vessel surface area and there was a trend towards an increased villi volume (Fig. 2, Table 3). After adjusting for confounding factors both the increase in vessel surface and the increase in villi volume was statistically significant (Table 4).

3.2. Diffusion vessels and villi

Anemia from 23 weeks of GA was associated with an increased diffusion vessel surface of 44% and increased diffusion villi volume of more than 20% and there was a trend towards an increased vessel diameter on almost 50% (Fig. 2, Table 4). After adjusting for confounding factors vessel length and villi volume were statistically robust (Table 4).

Anemia before 23 weeks of GA or anemia both before and from 23 weeks of GA did not have a statistically significant impact on the characteristics of the diffusion vessel or villi (Fig. 2, Table 4).

3.3. Macroscopically and histological examination of placental pathology

The weight of anemic placentas was above the 90th percentile for 12 of the 125 placentas after correcting for GA [30]. 3/12 women experienced anemia from 23 weeks of GA and 9/88 experienced anemia both before and from 23 weeks of GA. Interestingly, among the control placentas only one had a weight above the 10th percentile and more than half (34/64) had a weight below the 10th percentile [30]. At the histological examination only few placentas from anemic mothers revealed abnormal morphology. Chorangiosis as observed in hypoxic conditions was not observed. Neither were edema, fetal erythroblastosis and immaturity. Five placentas had infarcts in more than 5% of the placental volume and in 13 placentas moderate to severe acute inflammation of the umbilical cord or the membranes were found. There were no other differences in histological changes between the anemic group and the controls and none of the histo-morphological changes observed were specifically associated with anemia. Control placentas had classical infarcts in more than 5% of the placenta in three placentas

Table 4
Diffusion Vessel and Villi. Effect of anemia (Hb < 11 g/dL) on vascular parameters before and after adjusting for confounding factors.

	Control (n = 64) (Reference)		Gestational age < 23 weeks (n = 13)			Gestational age ≥23 weeks (n = 24)			Gestational age < 23 weeks & ≥23weeks (n = 88)		
	Mean	SD	Effect	95% CI	P	Effect	95% CI	P	Effect	95% CI	P
Unadjusted											
Vessel length (km)	177	68.5	−23.7	−73.2, 25.8	0.35	27.1	−11.8, 66.1	0.17	13.9	−12.8, 40.6	0.31
Vessel surface (m^2)	7.38	2.73	−0.07	−2.21, 2.06	0.95	3.23	1.55, 4.91	< 0.001	0.95	−0.20, 2.10	0.11
Vessel diameter (μm)	15.3	11.8	2.40	−7.00, 11.8	0.62	7.29	−0.11, 14.7	0.053	1.21	−3.86, 6.30	0.64
Villi volume (cm^3)	124	37.5	−10.2	−35.3, 14.9	0.42	29.8	10.0, 49.5	0.003	8.85	−4.71, 22.4	0.20
Diffusion villi diffusion distance (μm)	15.22	2.57	−0.31	−1.67, 1.04	0.65	−0.34	−1.41, 0.72	0.53	−0.20	−0.94, 0.53	0.58
Adjusted											
Vessel length (km)[a]			−21.3	−89.0, 46.4	0.54	68.6	15.9, 121.4	0.01	20.3	−16.4, 57.1	0.28
Vessel surface (m^2)[b]			−1.00	−3.84, 1.84	0.49	1.79	−1.13, 3.71	0.07	0.31	−1.03, 1.66	0.64
Villi volume (cm^3)[c]			−28.3	−60.0, 3.48	0.08	35.2	6.98, 63.44	0.02	7.33	−11.3, 25.9	0.44

A multivariate linear regression model was generated for each vascular parameter as the outcome. The reference group in all models was placentas from women who were non-anemic throughout pregnancy (n = 64). [a] Adjusted R^2: 0.15, [b] Adjusted R^2: 0.26 [c] Adjusted R^2: 0.13 The final models on the effect of anemia on vascular parameters were adjusted for the following confounders: **Diffusion vessel length**: malaria 0–14 weeks of GA, interaction between anemia and malaria 0–14 weeks of GA, interaction between anemia and malaria 28 weeks of GA to delivery, age. **Diffusion vessel surface**: malaria 0–14 weeks of GA, paucigravidae, interaction between anemia and malaria 15–22 weeks of GA, interaction between anemia and malaria 23–27 weeks of GA, interaction between anemia and malaria 28 weeks of GA to delivery **Diffusion villi volume**: malaria after 28 weeks of GA, tea consumption, interaction between anemia and malaria 0–14 weeks of GA, interaction between anemia and malaria 23–27 weeks of GA. **Diffusion vessel diameter** and **Diffusion villi diffusion distance** were not significant in the adjusted models.

Table 5
Vascular parameters association with birth weight at delivery (n = 187).

		Adj. R^2	Coefficient (g)	95% CI	P[a]
Birth weight	Transport Vessel Length (km)	0.24	46.5	9.14, 83.8	0.02
	Transport Vessel Surface (m^2)		−448	−833, −64.0	0.02
	Transport Villi Volume (cm^3)		3.90	0.31, 7.48	0.03
	Transport Vessel Diameter (μm)		11.2	−0.48, 22.8	0.06
	Diffusion Vessel Surface (m^2)		52.6	25.4, 79.8	< 0.001
	Diffusion Villi Diffusion Distance (μm)		46.7	10.6, 82.7	0.01
	Diffusion Vessel Diameter (μm)		−10.2	−17.7, −2.67	0.01

[a] Multivariate models on the on the association between stereological parameters and birth weight. The model is adjusted for sex of the newborn and gravidity. CI: Confidence interval.

and in five placentas moderate-to-severe acute inflammation of the umbilical cord or the membranes were found. Three non-anemic placentas showed sections with malaria parasites and in three of the placentas small foci of sequelae from malaria infection were observed.

3.4. Fetoplacental vascularity and birth weight

To investigate the association between fetoplacental vasculature at delivery and clinically relevant pregnancy outcomes, the association between birth weight and the vascular parameters was evaluated. In univariate analyses, all vascular parameters were positively associated with birth weight, except vessel diameter (Supplementary Table 3). In a multivariate regression model adjusted for sex of the newborn and gravidity transport vessel length, transport vessel surface, transport villi volume and diffusion parameters vessel surface, diffusion villi diffusion distance and vessel diameter were significantly associated with birth weight (Table 5).

3.5. Sensitivity analysis

The vascular parameters were compared for women experiencing anemia of Hb ≤ 9 g/dL with women experiencing anemia of Hb 9.1-10-9 g/dL both before and from 23 weeks of GA. The two anemic groups showed the same trend in the vascular parameters and except for transport vessel surface the two Hb groups did not have statistical significantly different vascular parameters (Supplementary Table 4).

Malaria was common during pregnancy among women with moderate-severe anemia (12/24 of the women). To ensure that the observed association between vascularity and anemia was not modified by malaria, the vascular parameters were compared for women experiencing anemia Hb ≤ 9 g/dL with and without malaria. There were no observed differences in the vascular parameters of the two groups (Supplementary Table 5).

4. Discussion

This study investigated how anemia during pregnancy affected fetoplacental vascularization to determine the most vulnerable timepoints during pregnancy. Our results suggest that timing of the anemic event dictates the impact on the placental development and that especially anemia during the second half of pregnancy induces an adaptive response by affecting the fetoplacental vascularization.

Preplacental hypoxia due to anemia can induce vascularization [7] and increased placental volume and size have been observed after anemia [11]. Using stereological assessment, hyper-vascularization has also been detected in iron-deficient pregnancies in studies on 26 and 60 placentas [4,11]. In agreement with this, we observed a positive association between late pregnancy anemia and vascularity. Furthermore, as reported by others we found almost 10% of the placentas with anemia had a weight above the 90th percentile whereas more than 50% of the control placentas had a weight below the 10th percentile. The lower weight among the controls also indicates that the placentas from our cohort were smaller than placentas from caucasian women [30]. However, the increased placental volume and size do not necessarily represent improved functional capacity of the placenta [31].

Some studies have observed an increased risk of low birth weight and preterm delivery after 3rd trimester anemia whereas others have not [6]. Mild anemia in the 3rd trimester could reflect physiological plasma expansion causing a woman with Hb ≥ 11 g/dL in early pregnancy reaching an Hb concentration meeting the criteria of anemia in late pregnancy. Limited reduction of Hb concentration from early to late pregnancy has been associated with reduced birth weight and lower placental weight [14]. The increased vascularization observed after anemia in late pregnancy in this study could reflect a healthy pregnancy with proper plasma expansion and hence sufficient vascularization [10]. The importance of proper placental development was also reflected in the positive association between birth weight and the vascular parameters.

We did not observe an association between anemia before 23 weeks of gestation and fetoplacental vascularization. Twenty-three percent (3/13) of the women were anemic at multiple visits conducted before 23 weeks of gestation. This should have ensured sufficient time for anemia to impact fetoplacental vascular architecture. Some of the women could have resolved their anemia before influence occurred. The vascular parameters did not differ between the 88 anemic in the first and the second part of pregnancy and the 24 only anemic during the second part of pregnancy. This indicate that anemia during the first half of pregnancy might have limited impact on the development of the fetoplacental vasculature. The analyses should however be repeated in larger datasets.

Women with all degrees of anemia were investigated as one group. Previous studies on anemia and birth weight indicate that the degree of anemia matters [25]. The number of women with Hb ≤ 9 g/dL only before or only from 23 weeks of gestation was limited and did not allow for meaningful analyses. However, the sensitivity analyses did not show a systematic difference in vascular parameters among women with Hb ≤ 9 g/dL as compared to those with Hb 9.1–10.9 g/dL both before and after 23 weeks of gestation. We therefore believe that it was reasonable to group all degrees of anemia in the presented analyses with vascularization as the outcome.

Iron and folic acid supplementation during pregnancy has beneficial effects on maternal and newborn's health [32]. Supplementation might have attenuated the ability of the study to detect the effect of anemia on fetoplacental vascularization since some of the women with iron deficiency anemia would have resolved their anemia. However, anemia has multifactorial causes [2,3] and supplementation may not have influenced anemia due to other causes. Furthermore, > 50% of the women had iron deficiency in later pregnancy despite supplementation (not shown). This may explain our high proportion of women with anemia in late pregnancy. All women received prenatal supplementation, so a potential effect on vascularization would be present in both control and anemia groups. We therefore do not believe that supplementation would have substantially influenced our results.

Malaria is a major risk factor for anemia [2] and might as well influence placental blood flow [33–35]. Malaria was evaluated as a

potential confounding factor with an interaction term in the multivariate analyses, but the association between anemia and fetoplacental vascularization was consistently observed. Furthermore, we observed no vascular differences in among women with and without malaria in the sensitivity analyses. Despite the small sample size this suggests that anemia exerted an effect on vascularization not caused by malaria. The specific effect of malaria on fetoplacental vascularization is addressed elsewhere [27].

This study had several strengths. Firstly, biopsies were randomly collected by two separate sampling strategies. Placental deformation, typically shrinkage [36], is caused during processing, formalin storage, dehydration and paraffin embedding. However, multivariate analyses were adjusted for placental processing time and tissues were collected and treated equally for all placentas and we assume equal shrinkage between the groups during paraffin embedding. Furthermore, all stereological and histological assessments were blinded to ensure no bias by information on exposure to anemia. A larger study with stronger statistical power is needed to investigate the influence of Hb ≤ 9 g/dL on fetoplacental vascular development.

In conclusion, we show that anemia during the second half of pregnancy significantly affects the fetoplacental vascular development, which is positive correlated with birth weight.

Declarations of interest

None.

Conflicts of interest

The authors report no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.placenta.2019.03.009.

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