Cell free hemoglobin in the feto-placental circulation: A novel cause of fetal growth restriction?

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Short Title: free circulating fetal hemoglobin

Non-standard abbreviations

A1M – alpha 1 microglobulin

EBB – Earle's Bicarbonate Buffer

DKK - Dickkopf-related protein

DEA-NONOate – diethyl NONOate

eNOS – endothelial nitric oxide synthase

EPO - erythropoietin

FABP1 – fatty acid binding protein 1

FGR – fetal growth restriction

fHbF – free fetal hemoglobin

fHbF:HO-1 – free fetal hemoglobin : heme oxygenase-1 ratio

FIHP – fetal-side inflow hydrostatic pressure

HbF – free hemoglobin

HBSS – Hank's buffered saline solution

HO – heme oxygenase

HO-1 – heme oxygenase 1

HPAECs – human chorionic plate artery endothelial cells

IBC – individualised birthweight centile

IL1α – interleukin-1 alpha

L-NNA - Nω-nitro-L-arginine

NO –nitric oxide

TBM – tissue basement membrane

TNFα – tumor necrosis factor alpha

Abstract

Cell free hemoglobin (fHb) impairs vascular function and blood flow in adult cardiovascular disease. In this study we investigated the hypothesis that fetal fHb (fHbF) compromises vascular integrity and function in the feto-placental circulation, contributing to the increased vascular resistance associated with fetal growth restriction (FGR). Women with normal and FGR pregnancies were recruited and their placentas collected freshly post-partum. FGR fetal capillaries showed evidence of erythrocyte vascular packing and extravasation. Fetal cord blood fHbF was increased in FGR compared with normal pregnancies ($P<0.05$), whilst elevation of its ratio with HO-1 suggests failure of expected catabolic compensation, as occurs in adults. During *ex vivo* placental perfusion, pathophysiological fHbF concentrations significantly increased fetal-side microcirculatory resistance ($P<0.05$). fHbF sequestered nitric oxide in acute and chronic exposure models ($P<0.001$), whilst fHbF-primed placental endothelial cells developed a pro-inflammatory phenotype, demonstrable by activation of NFκB pathway, generation of IL-1α, TNFα (both $P<0.05$), uncontrolled angiogenesis and disruption of endothelial cell flow-alignment. Elevated fHbF contributes to increased feto-placental vascular resistance and impaired endothelial protection. This unrecognised mechanism for fetal compromise offers a novel insight into FGR, and potentially unexplained associated poorer fetal outcomes such as fetal demise/ stillbirth.

Keywords: endothelium, placenta, vascular compromise, blood flow resistance.

Introduction

Fetal growth restriction (FGR) is the failure of a fetus to meet its genetically determined growth potential.(1) Whilst many cases lack an identifiable cause, FGR is strongly associated with fetal demise, and accompanies 50% of unexplained stillbirths.(2) Positive correlations between low birthweight, increased risk of adult cardiovascular disease and stroke have been documented, with growing evidence of the latent consequences of adverse prenatal programming.(3, 4)

In healthy pregnancy, umbilical resistance, measured antenatally by umbilical artery Doppler velocimetry, correlates accurately with postpartum fetal-side *ex vivo* placental perfusion resistance.(5) The placental villous microcirculation is an important locus of vascular resistance and reduced feto-placental blood flow threatens fetal growth and survival.(5, 6) Ordinarily, the upward adjustment of placental blood flow across gestation is critical in meeting growth demands, and for development. However, in FGR these feto-placental adaptations can fail, restricting the feto-placental circulation and compromising placental function. In its severest form, antenatal umbilical arterial Doppler velocimetry reveals atypical waveforms, such as absent or reversed end-diastolic flow.(7-9)

Under conditions of altered placental blood flow, fetal hemoglobin (HbF) is thought to be upregulated in an attempt to compensate for chronic hypoxia.(10, 11) This excess may be transferred to the maternal circulation in pre-eclampsia, a disease associated with FGR, where placentally-derived cell free fetal hemoglobin (fHbF) has also been reported.(12-14) Whilst the direct impact of fHbF on the fetal circulation remains unclear, it is proposed that erythrocyte behaviour is disturbed in FGR, with elevations of nucleated red cells defined in the placenta in as many as 89% of cases, even those devoid of Doppler anomalies.(15, 16) Although difficult to substantiate in the fetus, perpetuated erythrocytes are known to rupture under modest elevations in vascular shear stress; conditions which, without doubt are present in FGR cases, and manifest by flow abnormalities on antenatal Doppler (17). Under these circumstances, free hemoglobin release is inevitable, but under normal homeostasis remains finely regulated.

In the absence of innervation and autonomic regulation, the feto-placental unit is reliant upon humoral mechanisms for buffering feto-placental blood pressure.(18) Within this context, nitric oxide (NO) is critical for vasodilation and maintaining blood flow.(5, 18, 19) Whilst encapsulated in red cells, the NO scavenging capacity of hemoglobin is significantly reduced, by at least 1,000-fold.(20, 21) However, in its extracellular (free) form, sequestration of NO by oxyhemoglobin occurs instantaneously. In the feto-placental circulation, compromised induction of endogenous heme-oxygenase (HO-1), as a primary heme-scavenging enzyme, could exacerbate this situation.(22) Upregulation of HO-1 in the term placenta is expected from previous work demonstrating a protoporphyrin-induced increase in HO-1 expression, most notably in the stem villous

vasculature. Attenuation of this pathway is already associated with increased TNFα-induced cytotoxicity.(23)

We therefore hypothesise that cell free fetal hemoglobin is upregulated in the fetal circulation in growth restricted pregnancy, causing endothelial activation and vasoconstriction in the human feto-placental circulation in this disease.

Materials and Methods

Ethics: Tissues were acquired following informed written consent from women attending St Mary's Hospital, Manchester (all subjects), UK. Ethical approvals were obtained from the Licensing committee: North West Research Ethics Committee (reference numbers: 08/H1010/55, 08/H1010/55 (+5), 11/NW/0650). All experiments were performed in accordance with relevant guidelines and regulations.

Research Volunteers: Placentas were obtained from normal singleton pregnancies. To define groups, an individualized birthweight centile (IBC) was calculated according to the GROW algorithm (Perinatal Institute, Birmingham, West Midlands, UK). Groups were normal or FGR. FGR diagnosis was based on the Delphi-consensus definition as solitary parameter of IBC below the 3rd centile for gestation.(24)

Reagents: Unless otherwise stated, reagents were supplied by Sigma-Aldrich (Poole, Dorset, UK). fHbF was extracted from whole umbilical cord blood and purified as described previously.(25)

Feto-placental vascular appearance in FGR: An archive of stored transmission electron micrographs (TEMs) of freshly fixed placental tissue from FGR (N=15) were analyzed for endothelial morphology and erythrocyte distribution. For this analysis, specimens of placenta had been obtained immediately after delivery and a small piece of villous tissue excised from beneath the centre of the basal plate. This was diced into small fragments with a razor blade on dental wax and fixed for 4 hours at room temperature in 2-5 % glutaraldehyde in 0.1 M cacodylate buffer pH 7.4. The tissue was then rinsed three times over 24 hours in buffer containing 3 mM calcium chloride. Post-fixation was carried out in 1% osmium tetroxide (TAAB Laboratories Equipment Ltd, Aldermaston, Berkshire, UK) in 0.05M sodium cacodylate buffer pH 7.4 for 1 hour at 4°C followed by a buffer rinsing step. The tissue was then dehydrated in an ascending alcohol series, treated twice with propylene oxide (TAAB Laboratories Equipment Ltd; 15 mins each) then left in a 1:1 mix of propylene oxide and TAAB epoxy resin for one hour at room temperature. It was then left overnight on a

rotator at 4°C in a mixture of 1:3 propylene oxide and epoxy resin and the following day given three changes of fresh resin at 45°C for one hour each before being embedded in gelatin capsules and polymerised for 72 hours at 60°C. Semithin sections, 0.5µm thick, were cut on a ultramicrotome (LKB, Mount Holly, NJ, USA), stained with 1% toluidine blue in 1% borax, after which ultrathin sections were prepared with a glass knife, mounted on copper grids and stained with uranyl acetate and lead citrate. These were examined in an AEI EM6B electron microscope at an accelerating volage of 60kV and appropriate areas photographed.

Measurement of Plasma fHbF and HO-1: A Vacutainer system with 21 gauge needle attachment was utilized as the standard collection method. Umbilical vein blood was collected into a citrate tube without perturbations of flow during filling and the method standardised for collection from the cord vein insertion point, within 15 minutes of birth. This was followed by immediate centrifugation in the laboratory (within 10 minutes of collection at 1500 x g x 10 minutes at ambient room temperature). This method was determined following a stringent optimisation process to prevent iatrogenic hemolysis, including: different anticoagulants (EDTA, or citrate), the operating technique for venipuncture (closed-vacuum versus 21G needle and syringe technique), the venipuncture site and time lapsed prior to centrifugation. A citrate collection method was associated with negligible artefactual hemolysis (median increased 0.05589 ug/ml + IQR 0.0687) and temporal standing for 20 minutes resulted in a modest increase in detectable fHbF (median 0.1909 ug/ml + IQR 0.2230). Early collection into a citrate tube with immediate processing resulted in a low hemolysis with tight variance.

fHbF was measured using a sandwich-ELISA(26) (Lund University, Lund, Sweden) in venous cord plasma of normal and FGR pregnancies. Blood was taken at the cord insertion point and processed following the optimized method. Ninety six-well microtiter plates were coated with mouse anti-HbF antibodies (4µg/ml in PBS) overnight at ambient temperature. In the second step, wells were blocked for two hours using blocking buffer (1% BSA in PBS), followed by an incubation with HbF calibrator or the samples (normal n=12; FGR n=5 for 2 hours at ambient temperature). In the third step, HRP-conjugated mouse anti-HbF antibodies (diluted 1:5000) were added and incubated for 2 hours at ambient temperature. Finally, a ready-to-use 3,3′,5,5′-tetramethylbenzidine (Life Technologies, Stockholm, Sweden) substrate solution was added, reaction was stopped after 20 minutes using 1.0 M HCl and the absorbance was read at 450nm using a Wallac 1420 Multilabel Counter (Perkin Elmer Life Sciences, Waltham, MA, USA). Where sufficient plasma was available, plasma HO-1 expression was measured by immunoassay (Enzo Life Sciences, Exeter, Devon, UK). HO-1 was used as denominator for matched fHbF concentrations (fHbF:HO-1 ratio), subject to plasma availability (n=12).

fHbF Assay Development: Human γ-chains were prepared by dissociation of purified HbF with p-mercuribenzoate and acidic precipitation(27, 28). The absolute purity of HbF (from contamination with HbA), and of γ-chains (from contamination with α- and β-chains), was determined by non-denaturing PAGE followed by blotting with purified anti-human γ-chain (26, 29). Mouse antibodies to human γ-chains, specific for HbF, were produced and purified by AgriSera AB (Vännäs, Sweden). Anti-HbF antibodies were then conjugated with horseradish peroxidase (Lightning-Link HRP, Innova Biosciences, Cambridge, Cambridgeshire, UK) to provide a quantitative indirect reporter.

Additional biochemical validation of altered fHbF fetal plasma status in FGR: In testing the hypothesis that fHbF is elevated in FGR, additional assays were performed on the same fetal plasma samples to validate expressional or hemolytic changes that might have occurred *in vivo* linked to changes in plasma fHbF status, including erythropoietin (EPO), hemopexin, haptoglobin and α-1 microglobulin (A1M). Measurement of lactate dehydrogenase (LDH) was also performed as a validation against artefactual hemolysis and undertaken according to manufacturer's instruction. All kits were from Abcam (Cambridge, Cambridgeshire, UK).

Effect of fHbF on feto-placental vascular resistance tested by *ex vivo* human dual placental perfusion: Normal placentas (≥37 weeks) were perfused in open circuit using *ex vivo* dual circuit perfusion, as previously described in an adaptation of the model developed by Schneider *et al*.(30, 31) Briefly, fetal-side inflow hydrostatic pressure (FIHP) was monitored using a Medex pressure transducer (Digitimer, Welwyn Garden City, Hertfordshire, UK) and recorded using a Nanologger (Gaeltec Nanologger, Isle of Skye, Inner Hebrides, UK) following perfusion with Earle's Bicarbonate Buffer (EBB). Fetal-side flow rate was determined following assurance that NO-evoked, flow-mediated vasodilation was effective, by incrementally adjusting the flow rate and observing temporal flow mediated vasodilation. Minimum acceptable fetal-side venous return was ≥80% of arterial inflow. Maternal-side perfusion at 14mL/min was started when steady-state FIHP was reached and delivered evenly into the intervillous space by five cannulae.

fHbF was introduced from a syringe driver (Precidor model, Infors AG, Basel, Switzerland) into the fetal inflow-line through a pre-pump injection port, obtaining FIHP responses to a pathophysiological dose-range of fHbF found in fetal plasma (0.01-1.0mg/mL). Following completion, the fetal-circuit was equilibrated with 100μmol/L Nω-nitro-L-arginine (L-NNA), to inhibit endothelial nitric oxide synthase (eNOS) and the dose response to fHbF repeated. For each dose of fHbF and vehicle, FIHP within the *ex vivo* dual perfused

placenta was compared to its previous steady-state, with alterations in resistance reported as an absolute change. As proof of concept, NO sequestration was corroborated *in vitro* using a published method, 0.3mg/mL fHbF was reacted with 120µmol/L diethyl-NONATE (DEA-NONOate), as further proof of NO catabolism with fHbF in the feto-placental circulation and a spectrophotometric survey conducted.

Effect of fHbF on shear-stress evoked endothelial NO synthesis: Human placental arterial endothelial cells (HPAECs) from normal pregnancies were isolated, cultured and characterized using an established method.(32) Confluent HPAECs were subjected to 20 dynes/cm^2 shear stress for 48 hours (Ibidi, Munich, Bavaria, Germany). Nitrite, the stable breakdown product of NO, was measured in the flow-conditioned medium using the Griess reaction (Thermo Fisher, Loughborough, Leicestershire, UK). 100 µl of 0.8% (w/v) VCl3 was added to each well of a 96 well clear plate as a nitrite reduction step. Vanadium trichloride (VCl_3) was selected as the chemical reductant according to a previously published method (33). Method validation was performed in our laboratory using serial dilutions of silver nitrate to yield a satisfactory linear plot with the linear regression equation y = 0.103+0.002x (R2 = 0.98; p=<0.0001). Greiss reaction was then performed according to the standard manufacturer's method. Lysates were used to analyze proteome dysregulation (see below).

Effects of fHbF on *in-situ* vascular morphology: Chorionic artery plate sections from placentas from normal pregnancy were exposed to 0.6mg/mL fHbF, and cultured at 37^{o}C for 48 hours. Control vessels were exposed to HBSS vehicle. Vessels were fixed in sodium cacodylate buffer (0.1M) pH 7.4 and the lumen exposed by longitudinal incision. Specimens were processed for scanning electron microscopy (SEM).

Effect of fHbF on HPAEC angiogenesis: HPAEC growth on Matrigel, a placental-derived basement membrane matrix (Corning, Bedford, Bedfordshire, UK) was utilised to assess angiogenesis. HPAECs (2.8 x 10^4 cells/mL) were observed after 24 hours' incubation with 0.3mg/mL fHbF or PBS as control. HPAECs assembled into capillary-like tubule networks on this matrix. Tubule formation was quantified by microscopy using systematic examination of seven fields per view. Image Pro 7 software (MediaCybernetics Inc, Silver Spring, MD, USA) was used to analyse the tubule formation in the presence of fHbF (0.3mg/mL) or PBS vehicle. These values were then multiplied together, which was used as a net measure of angiogenesis.

Proteomic endothelial 'cell stress' array and inflammatory pathway investigation: Flow-conditioned HPAEC cells were lysed (see above) and harvested into ice-cold RIPA buffer. Standardization was performed for equivalent protein loading (mic**r**o-BCA kit; Thermo Fisher). Cell stress array blotting was

undertaken according to manufacturer's instructions (R&D Systems, Abingdon, Oxfordshire, UK). A proteomic profile was obtained from fHbF-exposed and unexposed cells.

For assessment of NFκB and cytokine release, HPAECs were exposed to fHbF (0.07-1.2mg/mL) in separate static culture and cells were fixed in methanol and immunofluorescence stained for NF-κB p65 and DAPI. Cells were permibilised with 0.5% TritonX-100 (in PBS) for 30 minutes and washed with PBS before 0.01% sodium borohydride in TBS. This process was repeated three times before blocking with buffer (TBS/5% normal swine solution) and maintained for 1 hour at 21^{o}C. Cells were incubated overnight at 4^{o}C with NF-κB p65 1° antibody (Cell Signalling Technology, Leiden, The Netherlands) diluted 1:400 (TBS/1% BSA). Anti-Rabbit IgG (Dako, Stockport, Cheshire, UK) at 1:50 dilution was used as a negative control. Cells were assessed using fluorescence microscopy (Ziess, Cambridge, Cambridgeshire, UK) and NF-kB translocation assessed using a qualitative method with DAPI overlay, and blinded to fHbF exposure dose on a three tier scale (definite, intermediary or negative activation).

HPAEC cells exposed to fHbF under static culture conditions were lysed using radioimmunoprecipitation assay buffer for ELISA studies using DuoSet® kits (R&D Ssystems, MN, USA). A panel of NFkB-related inflammatory cytokines and triggers to the canonical route of NFkB activation (IL1α, IL1β, TNFα) were measured. ELISAs were performed on cell lysates, and undertaken according to the manufacturer's instruction. Protein quantity was measured using a Bio-Rad DC Protein Assay (Bio-Rad, Watford, Hertfordshire, UK), allowing adjustment for protein content (34). As a standardising method, results were analysed as a fold change from the control in each cell line.

Statistics: Data, tested for normality and analyzed using GraphPad Prism (La Jolla, CA, USA), are expressed as either mean +/- SE or median +/- range. Statistics are qualified throughout. P values <0.05 were considered significant.

Data availability: The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Results

Patient demographics: An extensive listing of separate pregnancy cohorts relating to each study group is given (**Table 1**).

Feto-placental vascular characteristics in FGR: Qualitatively, capillary avascularity was more common in FGR placentas, with erythrocyte vascular packing and extravasation restricted to FGR **(Figure 1)** leading us to investigate fHbF and HO-1 handling (see below).

fHbF is elevated in FGR cord blood: fHbF concentrations were significantly greater (**Figure2a**), whilst HO-1 concentrations were lower (**Figure 2b**), in fetal plasma from FGR compared to normal pregnancies (NS). The ratio of fHbF : HO-1 was significantly higher in FGR (**Figure2c**), with improved separation of the groups. Delivery mode had no significant effect on fHbF concentration (FGR group: Mann Whitney test P = 0.999; in the normal group the single normal vaginal delivery case had a fHbF plasma value of 0.73 μM, close to the median of 0.85 μM).

Additional biochemical validation of altered fHbF fetal plasma status in FGR: EPO and A1M levels were higher and hemopexin was lower in the cord plasma of FGR pregnancy (P<0.01, P=0.05 and P<0.01, respectively) compared to normal pregnancy; whilst haptoglobin levels were not different between the groups, (**Supplementary Figure 1**). LDH release was not different between groups: normals: 9.63 (7.22, 23.64; N=18); and FGR: 12.49 (5.39, 20.3; N=19); data shown as median (25^{th}, 75^{th} centiles); Mann-Whitney test: P= 0.663.

fHbF elevates feto-placental resistance through NO scavenging: fHbF caused a significant dose-dependent increase in FIHP, reflecting elevated downstream resistance in the placental villous microcirculation (**Figure3A & B**). Repeating the same dosing regimen in the presence of L-NNA (100μM) as an eNOS inhibitor, the starting baseline pressures were artificially elevated as expected, but peak elevations in FIHP were significantly attenuated, suggesting fHbF sequesters NO from the feto-placental circulation when it is present. As further corroboration in a separate experiment, we found the *in vitro* addition of DEA-NONOate, an NO donor, rapidly transformed oxyhemoglobin to methemoglobin (**Supplementary Figure 2**), demonstrating sequestration by fHbF.

fHbF reduces NO bioavailability in flow-conditioned endothelial cells: fHbF at FGR cord blood concentrations reduced total NO bioavailability (total NO_x) compared to vehicle, as determined by the Griess reaction (**Figure 3C)**.

fHbF disrupts endothelium laminar morphology: Exposure of placental chorionic plate arteries to 0.6mg/mL fHbF for 48 hours resulted in a dysmorphic luminal endothelial surface, with extensive cell-blebbing not observed in control arteries (**Figures 4B & 4A**, respectively). Whilst a dysregulated morphology could only be found in some zones of the chorionic plate artery endothelium exposed to fHbF, the PBS exposed control vessels were completely normal in appearance, as verified by independent observers blinded to the groups. Light microscopy of flow-conditioned HPAECs from normal pregnancies showed that 0.3mg/mL fHbF reduced cell density and disrupted normal cell morphology, preventing cell alignment with flow compared to controls (**Figure 4D & C**, respectively**).**

fHbF activates the feto-placental endothelium: fHbF promoted HPAEC angiogenesis on Matrigel; suggested by increased 'tubule' branching intensity (**Supplementary Figure 3**). This was consistent with the findings from a 'cell stress' proteome array for fHbF-exposed HPAECs. Within this array, several proteins associated with enhanced angiogenesis (DKK-4), fatty acid uptake, lipid peroxidation, vascular remodeling (FABP-1), and inflammation (FABP-1 and NFkappa-B) were up-regulated (fHbF 0.3mg/mL; **Table 2).** These effects were confirmed in an investigation of endothelial nuclear translocation of NFκB and cytokine release. 1.2 mg/mL fHbF (the high-end FGR fetal plasma level) evoked NFκB translocation to HPAEC nuclei within two and a half hours of exposure in static culture **(Figure5Aiii & iv.)**. This was not seen in the vehicle controls **(Figure 5A i.)** or at the median value of normal fetal plasma levels **(Figure 5A ii.).** Evidence of activation of NFκB targets was provided by a dose response increase in the release in interleukin-1α and TNFα (**Figure 5B & 5C respectively**) from HPAECs exposed to fHbF doses ≥0.2mg/mL.

Discussion

We found fHbF is elevated in the fetal circulation of FGR pregnancy, commensurate with modifications to levels of biochemical markers acting as endogenous heme handling agents. Elevations in fHbF levels in FGR plasma are likely to be inconsequential of hemolysis, since fetal plasma LDH levels were not different between groups. Such elevated levels of fHbF caused vasoconstriction in the *ex vivo* human placenta dual perfusion model, by sequestering NO. This was confirmed biochemically using NO donation to oxy-fHbF using spectrophotometry and in a HPAEC cell-flow model, where liberated levels of NO were reduced when exposed to fHbF. Furthermore, pathophysiological levels of fHbF evoked endothelial inflammation in HPAECs, confirming cell blebbing seen in *ex vivo* chorionic plate artery cultures. Severe reductions in feto-placental blood flow may threaten fetal growth and pregnancy viability.(35) Although the pathogenesis of FGR is unclear, feto-placental vascular compromise is implicated, manifesting as altered umbilical blood flow in the severest cases.

Elevated fHbF in the feto-placental circulation and heme handling mechanisms: HbF is a helical tetramer composed of a porphyrin ring and two attached alpha and two gamma globin subunits ($\alpha 2\gamma 2$) (17) encoded by alpha (Hb-α) and gamma (Hb-γ) globin genes; both expressed within the placental villi.(12) A twenty-fold upregulation in Hb-α in umbilical cord blood and amniotic fluid occurs in FGR pregnancies compared to normally grown equivalents, as well as increased fHbF mRNA in maternal blood, implying a strong hematological component to the disease.(10, 17) Indeed as a marker of chronic feto-placental hypoxia, increased hematocrit and erythropoiesis has already been reported by several authors but never before linked to hemolytic intravascular events. (11, 36, 37) Martenelli *et al.* have described how increased hematocrit in the cord of babies small for gestational age correlated with increased pulsatility index, as a measure of high feto-placental resistance.(37) In support of this notion, we have now demonstrated higher circulating concentrations of fHbF in cord blood in FGR and have provided convincing evidence of multiple deleterious effects of this fHbF within the feto-placental vasculature. Using an optimised method to minimise artefactual hemolysis, the median concentration of fHbF in fetal cord plasma was five times greater than that in the normal pregnancy group, suggesting a heme-generating insult. We found delivery mode had no effect on fetal plasma fHbF levels that might have been envisaged to arise through the mechanical effects of labour.

HO-1 enzyme, an inducible pathway of heme degradation, is present in the human placenta.(23, 38) Within cord blood plasma, we demonstrated that the expected vasculo-protective up regulation of HO-1 to protoporphryrins, reported elsewhere, fails to occur in FGR.(23) Furthermore, circulating HO-1 is known to reflect tissue contributions in similar vascular pathologies where free hemoglobins predominate.(39) The higher levels of fHbF and the absence of HO-1 up regulation in the cord blood of FGR pregnancies served to

exaggerate the fHbF : HO-1 ratio in FGR, implying uncoupling of HO-1 from excessive circulating fHbF. We suggest that a reduction in catabolic potential for fHbF could predispose the fetus to vascular compromise. By contrast in the normal group there was a tight clustering of the fHbF : HO-1 ratio within a finite low range, with downward correction of fHbF values at the upper range, suggesting an appropriate 'housekeeping' response of HO-1 to the prevailing intravascular hemoglobin in normal pregnancy. We therefore propose a novel use of a 'fHbF:HO-1 ratio' as a tool for assessing feto-placental heme-handling.

Murine models illuminate biological consequences of failed HO-1 induction. HO-1 heterozygotes (+/- HO-1) exhibit FGR, despite increased endothelial and inducible nitric oxide synthase.(38) Paradoxically, other human studies of FGR, including our own(5) show increased expression of NOS and increased NO metabolic products in the umbilical cord, consistent with the mouse model; but the failed compensatory response of HO-1 to excess fHbF and the fHbF:HO-1 mismatch we have now demonstrated in FGR pathology provides better insight to account for this apparent discrepancy.[5, 40, 41]

In validating increased fHbF levels in the FGR group as being a true *in vivo* event we measured the levels of other biochemical markers including some linked to cell free heme handling. The significant elevation in EPO levels in FGR is in keeping with placental hypoxia frequently observed in this disease; a process that may potentiate erythrogenesis. That said, our finding of no difference in LDH between groups indicates that a significant contribution of the fHbF plasma elevation in some FGR cases could arise through a mechanism of non-marrow hematopoeisis and independent of mechanical hemolysis. That embryonic tissue itself has an intrinsic capacity for α an γ globin production is a finding previously demonstrated in preeclampsia and other disciplines, and our data now supports this notion in FGR.(12, 42) Finding a reduction in fetal plasma hemopexin in FGR suggests that most of the circulating levels have been depleted through chaperoning fetal heme to the maternal liver. Fetal plasma hemopexin levels are normally lower than adult levels, consequential of a shorter half-life of fetal erythrocytes compared to adult erythrocytes and a greater need for heme detoxification of the fetal circulation (43). A further reduction of hemopexin in FGR fetal plasma suggests an even higher *in vivo* heme load in this disease. An elevation in fetal plasma A1M could be indicative of an attempt to compensate for higher heme levels in FGR, in keeping with similar observations in the maternal circulation in preeclampsia, where fHbF is also elevated (44).

fHbF evoked changes to blood flow resistance in the placenta: Within the *ex vivo* perfused human placenta, fHbF increased feto-placental blood flow resistance through an NO-dependent mechanism. The response was dose-dependent and confirmed through *in vitro* testing with an NO donor, with the most prominent effects observed at the pathophysiological levels of fHbF found in FGR cord plasma. Nevertheless, poor recoverability from vasoconstriction also suggests a secondary (NO-independent)

mechanism is at play. This non-recoverable vasoconstriction may be linked with the pronounced endothelial blebbing evoked by fHbF in chorionic plate vessels.

With loss of morphological conformity in flow-cultured HPAECs exposed to fHbF, alongside an 80% reduction in detectable NO, we propose that this atypical fHbF-evoked endothelial topography causes elevated feto-placental resistance and a decline in placental function. Increasing the length of the pathway in the placental transfer of hydrophilic nutrients is a separate consideration to any other additional dysregulation that might occur to the facilitated and active transport processes of nutrients across the syncytiotrophoblast. Here, we refer to the under-explored effects on the feto-placental endothelium.(45) Such dysregulated endothelial morphology is a feature of rhesus incompatibility of the new-born, another fHbF-laden vascular disease, associated with cytoplasmic blebs projecting from the endothelial cells of terminal villous capillaries with concomitant basement membrane thickening.(46)

This small cohort study of FGR cases was diagnosed on solitary Delphi-consensus of birthweight centile <3rd centile and provides preliminary evidence that fHbF is elevated in some cases, resulting in probable detrimental effects on fetoplacental vascular health and homeostasis *in vivo*.(24) Recruitment was inclusive of a wide scope of FGR aetiologies, but omitted suspected chromosomal-related disorders and cases of infection. Future studies aimed at investigating the direct association of elevated fHbF levels with dysregulated fetoplacental blood flow would benefit from the targeted recruitment of pregnancies with a spectrum of abnormal uterine and umbilical Dopplers, and those with additional objective evidence of fetal compromise indicated by abnormal middle cerebral artery and or Ductus venosus Dopplers and/or placental hypoxemia indicated by elevated cord blood erythropoietin levels and lactate

fHbF causes vascular inflammation in the placenta: Additionally, we noted numerous translational responses to fHbF, indicative of an altered endothelial proteome. In a cell stress array of HPAECs from a cell flow model and from time-lapse results of a further HPAEC static culture model, we found that nuclear translocation of NFκB occurred and its expression was-up regulated. NFκB is a known transcriptional regulator implicated in atherosclerosis.(47) Heme-activation of NFκB is known to occur during *in vitro* culture of human aortic smooth muscle cells,(48) but until now, it was unclear whether fHbF would invoke similar responses in the feto-placental vasculature. Our results suggest NFκB-dependent activation of cytokines occurs in fHbF exposed HPAECs. Both IL-1α and TNFα were significantly increased following fHbF exposure and NFκB nuclear translocation. To contextualize this, we suggest that a secondary NO-independent vasoconstriction observed in the perfused placenta might be explained by NFκB-dependent endothelial inflammation. Other notable directional responses in the cell stress array included a significant induction of the pro-angiogenic mediator DKK-4, an observation which paralleled increased tubule

formation for fHbF-exposed HPAECs grown on Matrigel. There was also an increase in FABP-1 synthesis; a mediator of lipid handling, and a transcriptional regulator previously implicated in FGR.(49, 50).

Hypothesised sequelae of events leading to poor pregnancy outcome: We propose a novel mechanism whereby unchecked fHbF within the human placenta, and potentially the fetus, establishes a vicious spiral of events beginning with NO sequestration. This significantly contributes to vasoconstriction and reduced vasodilatory paracrine signalling, promoting vaso-occlusion with localised erythrocyte extravasation. In the absence of adequate heme-handling capacity (HO-1), elevated fHbF levels provokes vascular inflammation, endothelial stress, proliferative placental angiogenesis and activation of a cytokine-dominated immune response. **Figure 6** illustrates this potential route to feto-placental compromise. With prolonged or severe hematologic dysfunction, the protective defences of the fetus become fully ablated with wholesale vascular collapse and fetal demise. Indeed, work from our laboratory shows prominent avascularity as a feature of peripheral lobules in FGR pregnancy (51). We therefore consider this mechanism a previously unrecognised pathway to FGR and potentially stillbirth. This knowledge will lead to future strategies to identify and treat failing pregnancies associated with incapacity to handle an elevated fetal heme load.

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Author contributions

Brownbill, Crocker, R. Jones, Myers, Hansson and Schneider incepted the ideas for study and supervised respective research groups and numerous PhD and Masters students and post docs in experimental design and training. Brook, Hoaksey, Gurung, Yoong, Sneyd, Baynes, and Gram carried out the experiments and developed assays, interpreted and promoted the work at various conferences. Bischof, Higgins and S. Jones provided biological material. C. Jones provided TEMs of FGR placentas. Greenwood, along with all supervisors provided results evaluation. Lang and Desoye trained Brownbill in the transfer of the cell technology to Manchester. All authors contributed to manuscript preparation.

Figure legends

Figure 1: TEM image from an FGR case exhibiting luminal packing and extravasation of fetal erythrocytes. Key: E=erythrocyte, CL=capillary lumen, VS=villous stroma and black arrowheads represent the demarcation between VS and tissue basement membrane.

Figure 2. The cell free fetal haemoglobin (fHbF) and heme oxygenase-1 (HO-1) status in the fetal plasma of normal and fetal growth restricted (FGR) pregnancy groups.

Showing significant normal (●) and FGR (■) group differences in (a) fHbF fetal plasma levels (box and whiskers plot; medians (25^{th} & 75^{th} centile): 0.054 (0.038 & 0.094) & 0.222 (0.072 % 0.476) mg/mL; (N = 25 & 16, respectively; Mann-Whitney: *P = 0.013); (b) HO-1 fetal plasma levels (box and whiskers plot; medians (25^{th} & 75^{th} centile): 0.006 (0.003 & 0.011) & 0.004 (0.000 & 0.007) ng/ mL (N=14 & 8, respectively; Mann Whitney: NS); (c) fHbF : HO-1 ratio (box and whiskers plot; median (25 & 75^{th} centile): 9542 (680 & 16254) & 90493 (21056 & 521262), (N = 14 & 8, respectively; Mann-Whitney: ***P < 0.001).

Figure 3: NO sequestration by fHbF in acute and chronic *ex vivo* and *in vitro* models. (i) Effects on fetoplacental flow resistance in the *ex vivo* perfused placenta (n=8), shown as a representative FIHP trace (**Figure 3A**). A significant dose response in FIHP to fHbF occurred (**Figure 3B;** one-way ANOVA: P≤0.05; downward arrows: incremental doses of fHbF: control-EBB, 0.01, 0.03, 0.06, 0.1, 0.3, 0.6 and 1.0mg/mL, in the absence (Phase 1) and presence (Phase 2) of the eNOS inhibitor, L-NNA (100μmol/L); solid squares represent four minute delivery of fHbF). fHbF could not evoke significant peak elevations in resistance when NO production was absent (**Figure 3B;** ■; mean±SE; Kolmogorov-Smirnov: P = 0.8571) compared to when it was present (●; mean-SE; two-way ANOVA: $^{\#}$≤0.001; Dunnett's post-hoc analysis: 0.6mg/mL and 1.0mg/mL levels, *P≤0.05 both; Kolmogorov-Smirnov: P = 0.4286). (ii) fHbF reduced nitric oxide bioavailability in flow-cultured HPAECs after 24 and 48 hours (Figure 3C; ●) compared to vehicle (PBS)

control treated cells (Figure 3C; ■; n=6 cell lines for each group; one-way ANOVA P ≤0.01, Sidak's post-hoc test: *P≤0.05; ***P≤0.001; median and range shown).

Figure 4: fHbF evoked changes in endothelial morphology. SEM images of the luminal endothelial surfaces of human placental chorionic plate arteries: exposed *in situ* for 48 hours to HBSS control (**A**), or fHbF (0.6mg/mL; **B**); scale bar = 200 μm. Light microscopy of HPAECs grown under flow (20 dynes/cm^2; 48 hours) and exposed to either PBS control (**C**) or 0.3mg/mL fHbF; scale bar = 50 μm (**D**).

Figure 5: fHbF evoked endothelial activation. Representative images of fHbF induction of NFκB nuclear translocation (green fluorescent dye); confirmed by DAPI staining - **a**) in static HPAEC culture at 1 hour; doses selected were 0 (**i**; control; DAPI overlay shown); 0.07 (**ii**; median normal fetal plasma level), 0.2 (iii; median FGR fetal plasma level); and 1.2mg/mL (**iv**; high-end FGR plasma levels); n=4 normal HPAEC cell lines. fHbF induction of acute pro-inflammatory cytokine release into HPAEC static cultured conditioned medium at 24 hours for IL1α (**b;** one-way ANOVA: P = 0.0002; Bonferoni post-hoc test compared to control: **P > 0.01, ***P > 0.001; Kolmogorov-Smirnov: P > 0.100, all concentrations; N = 7) TNFα (**c**; Friedman: P = 0.0006; Dunn's multiple comparison test, compared to control: ***P > 0.001; N = 7).

Figure 6: Schematic of the 'fHbF vascular demise spectrum'. fHbF excess occurs as a response to feto-placental hypoxia and *de novo* production in the feto-placental unit[1] (12). This overwhelms primary vasculoprotective defences and heme-scavenging systems including haptoglobin, hemopexin, albumin and α1 microglobulin[2]. Nitric oxide (NO) sequestration occurs[3], causing increased vascular resistance[4], reduced feto-placental blood flow[5], placental microvascular congestion, and feto-placental hypertension[6]. Endothelial cells lose structural integrity[7]. Disturbed lipid handling may contribute to accelerated thrombosis and vascular occlusion[8]. As oxygen and nutrient delivery becomes compromised, fetal growth restriction (FGR) is a highly plausible clinical end point of vascular demise. Stillbirth may result when placental insufficiency passes an extreme threshold of diminished feto-placental perfusion[9].

Figure 1

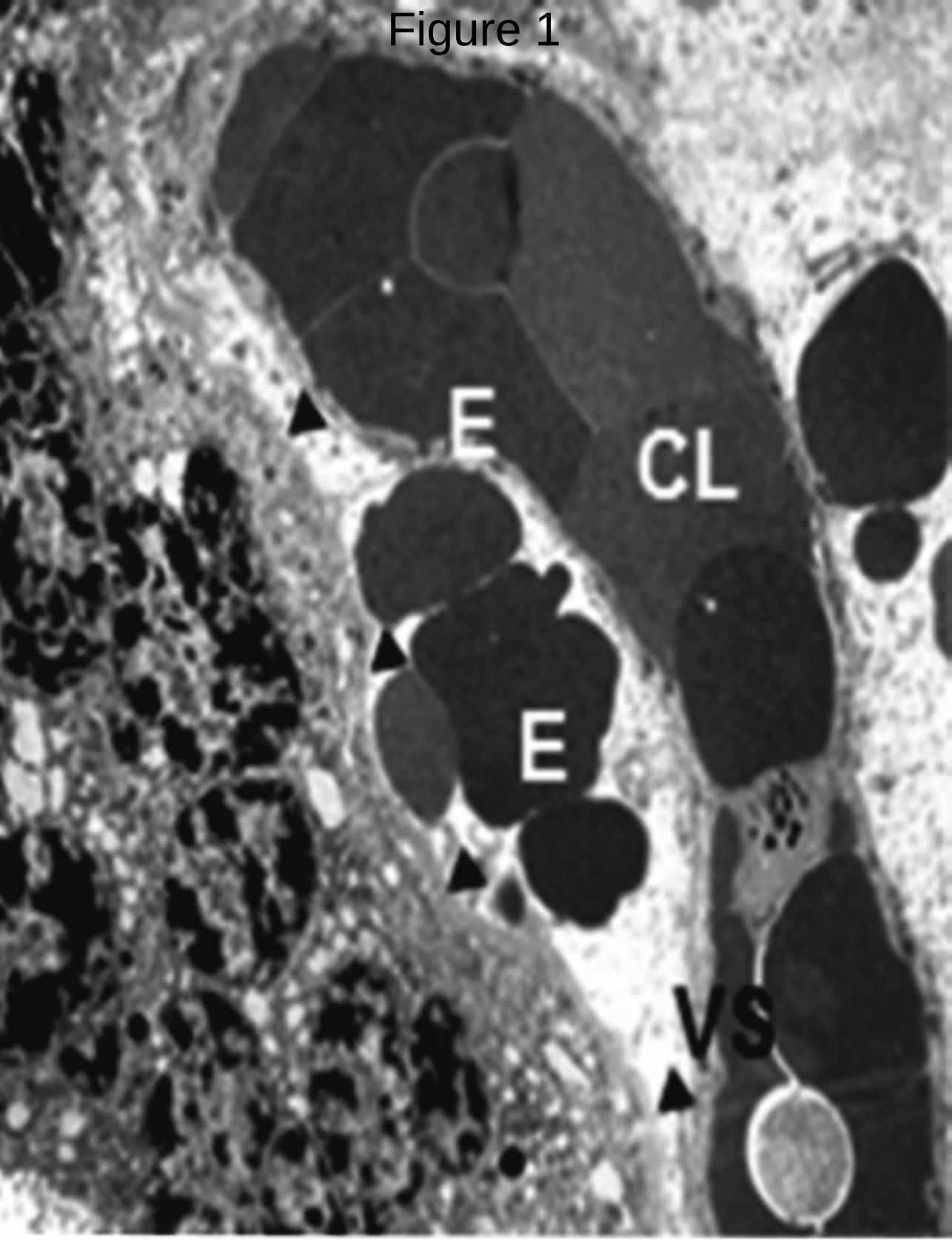


Figure 2

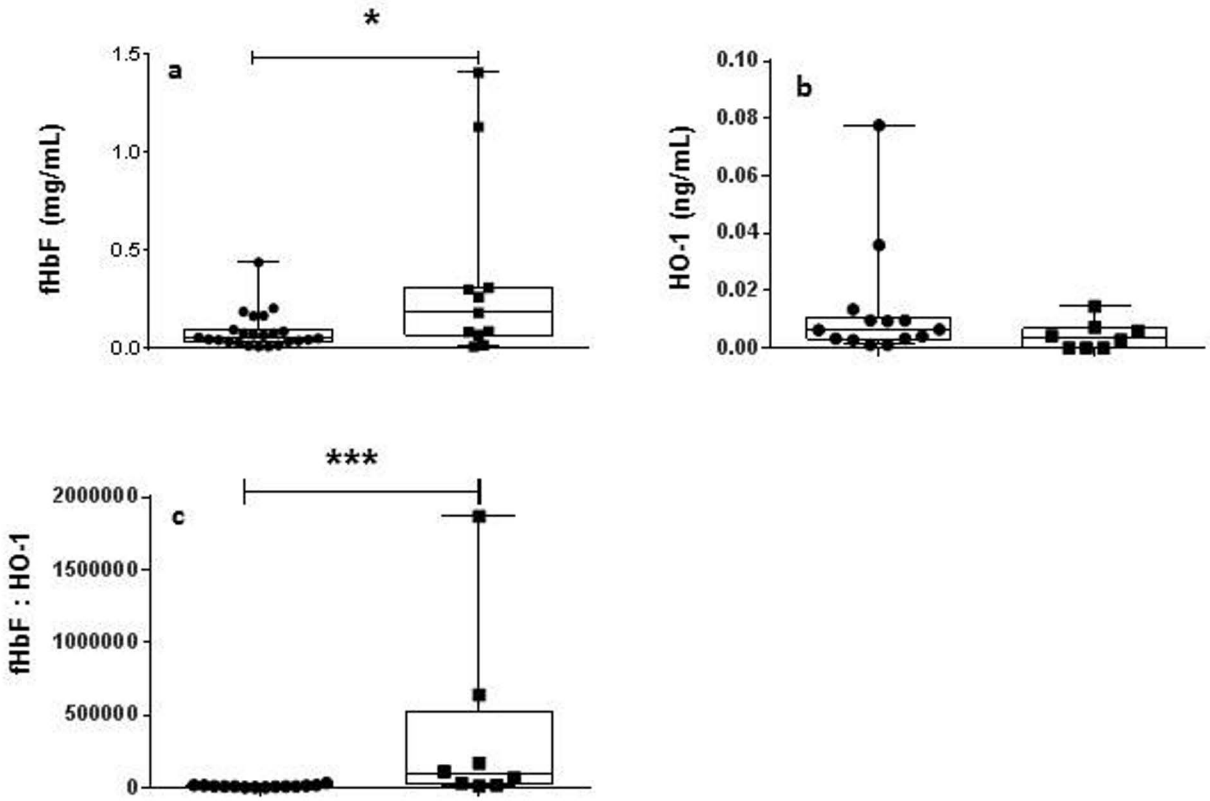

*
a
fHbF (mg/mL)
1.5
1.0
0.5
0.0
b
HO-1 (ng/mL)
0.10
0.08
0.06
0.04
0.02
0.00
***
c
fHbF : HO-1
2000000
1500000
1000000
500000
0

Figure 3

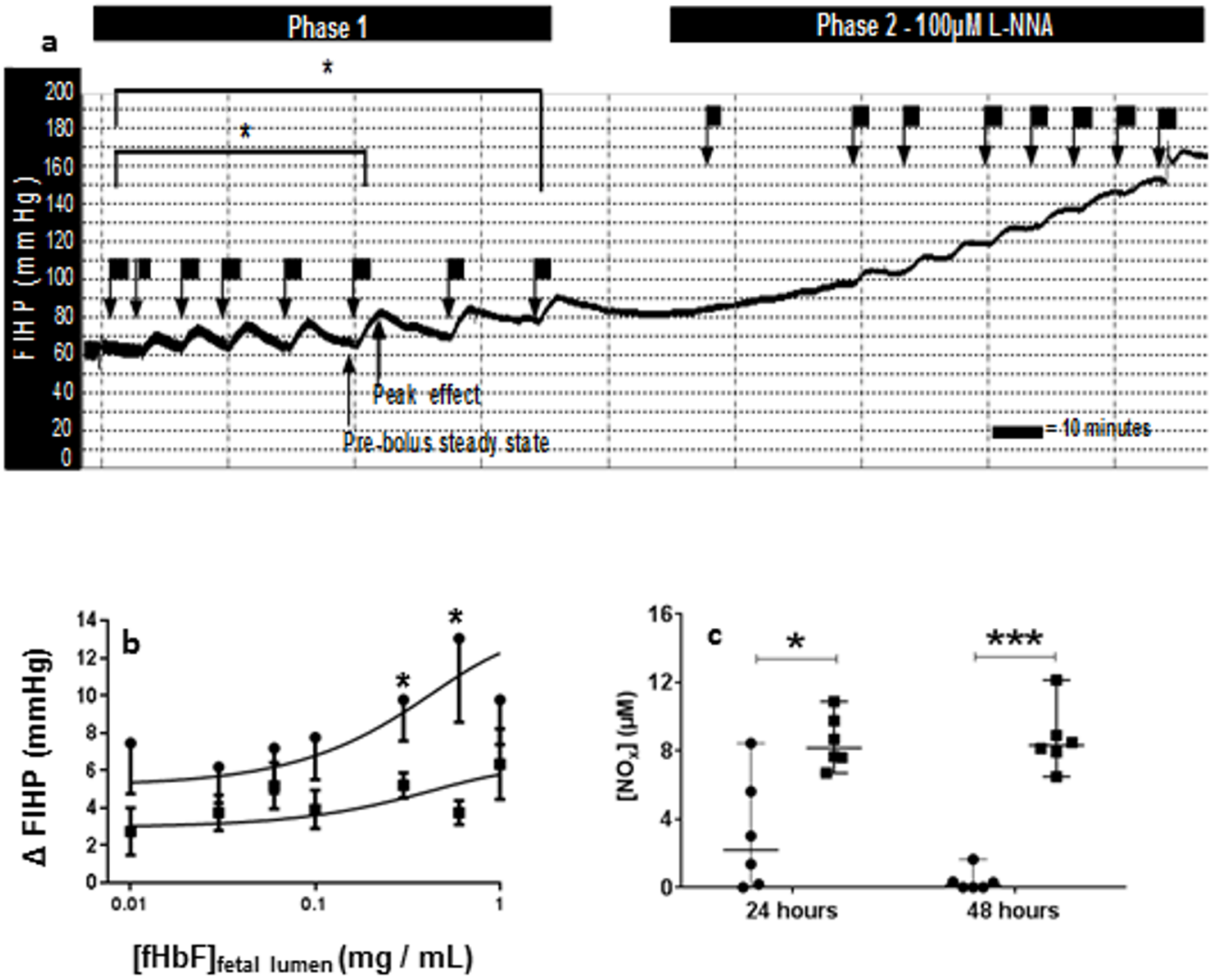

a
Phase 1
Phase 2 - 100μM L-NNA
FIHP (mmHg)
200
180
160
140
120
100
80
60
40
20
0
*
*
Peak effect
Pre-bolus steady state
= 10 minutes
b
Δ FIHP (mmHg)
14
12
10
8
6
4
2
0
*
*
0.01
0.1
1
[fHbF]fetal lumen (mg / mL)
c
[NO] (μM)
16
12
8
4
0
*
***
24 hours
48 hours

Figure 4

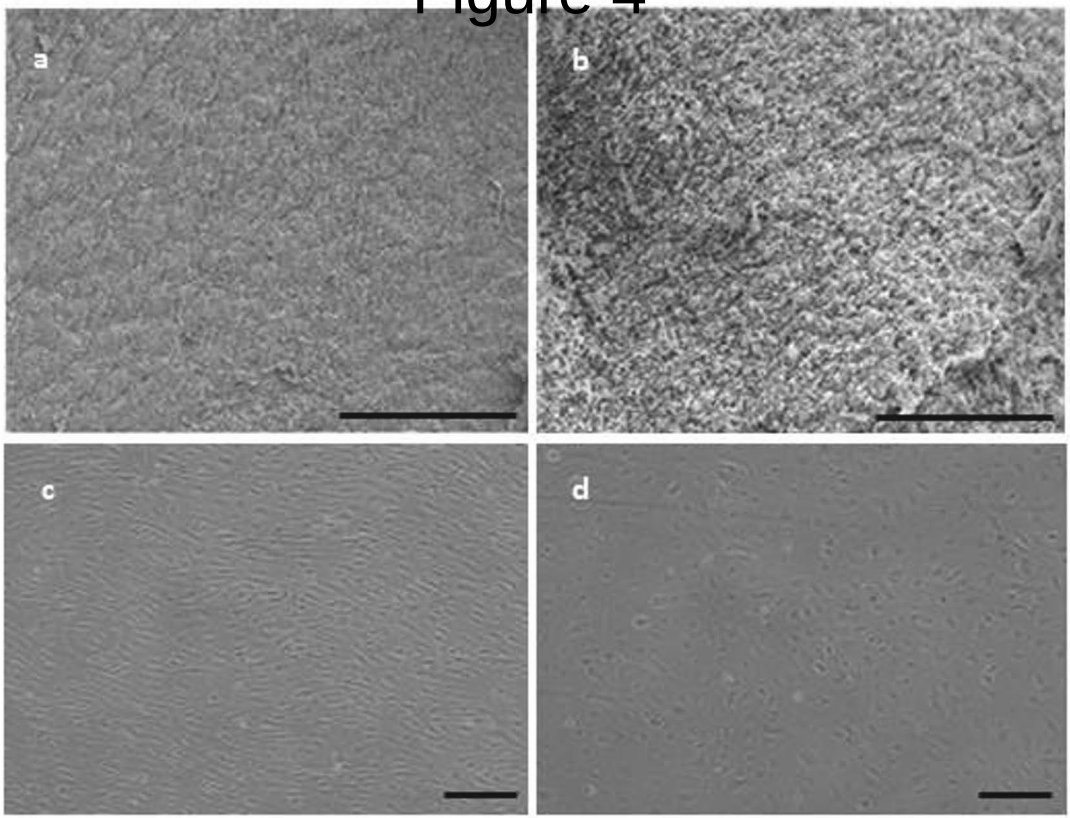


Figure 5

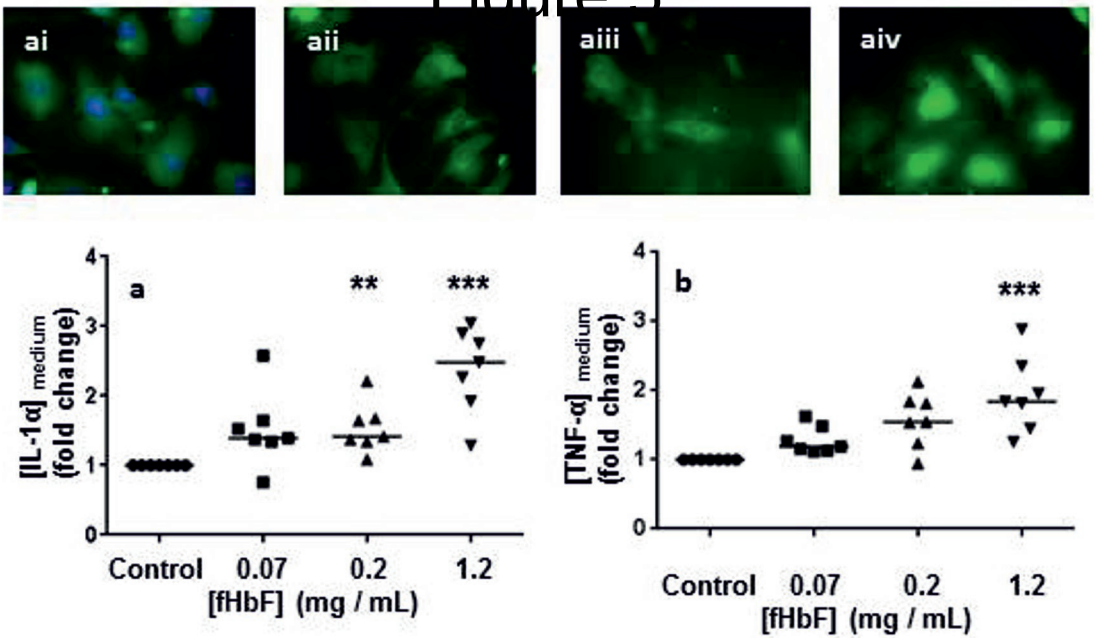

ai
aii
aiii
aiv
a
b
**
***
***
[IL-1α] medium (fold change)
[TNF-α] medium (fold change)
0
1
2
3
4
Control
0.07
0.2
1.2
[fHbF] (mg / mL)

Figure 6

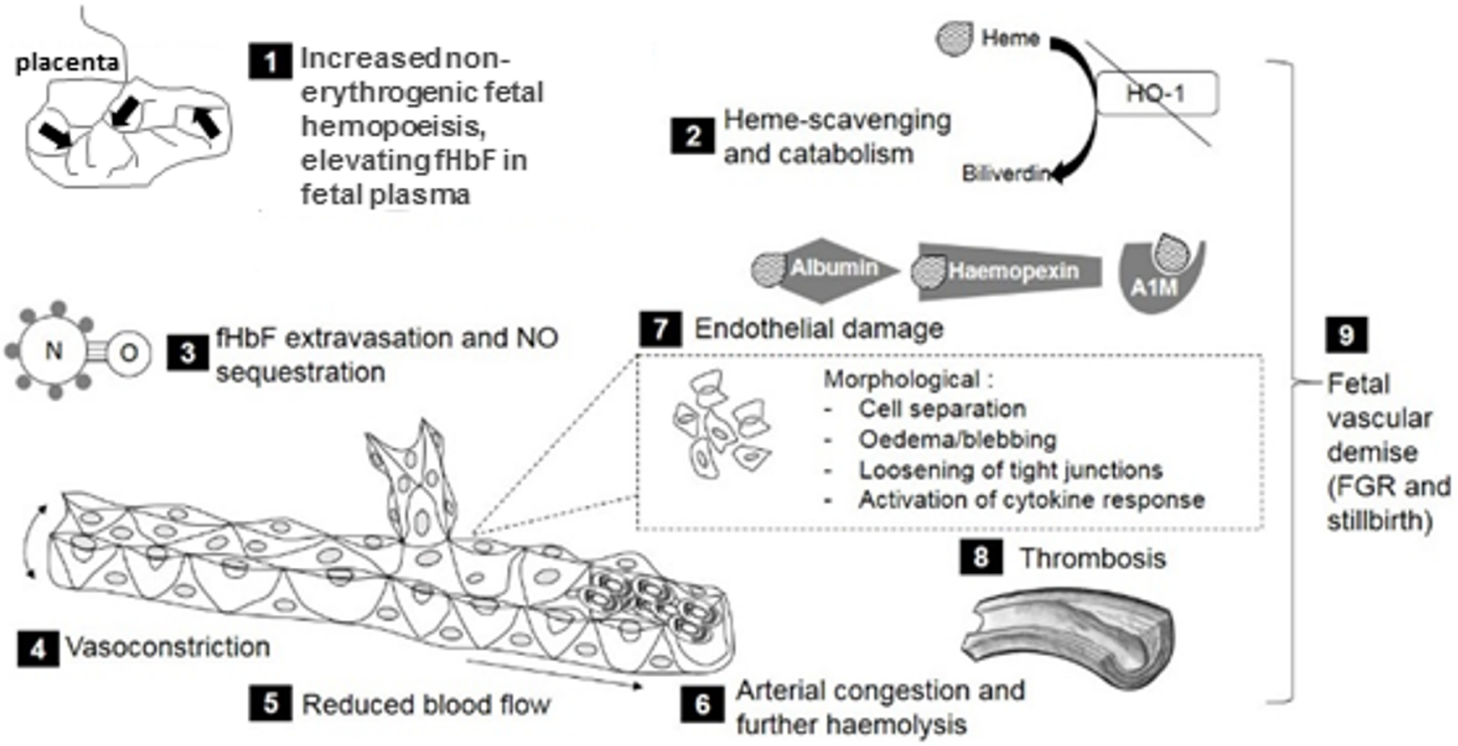

placenta
1 Increased non-erythrogenic fetal hemopoeisis, elevating fHbF in fetal plasma
Heme
HO-1
Biliverdin
2 Heme-scavenging and catabolism
Albumin
Haemopexin
A1M
N
O
3 fHbF extravasation and NO sequestration
7 Endothelial damage
Morphological :
- Cell separation
- Oedema/blebbing
- Loosening of tight junctions
- Activation of cytokine response
8 Thrombosis
9 Fetal vascular demise (FGR and stillbirth)
4 Vasoconstriction
5 Reduced blood flow
6 Arterial congestion and further haemolysis

Table 1. Research volunteer demographics

A: fHbF cord blood ELISA:

group	normal (n=24)	FGR (n=13)	significance (P)
maternal age (years)	32 (26-38)	29 (23-33)	NS(0.213)[a]
mode of delivery:			
NVD	1	5	
CS	23	8	
parity	1.00 (0.25-1.00)	0 (0.00-1.00)	NS (0.091)[a]
GA (weeks)	39.1 (39.0-39.4)	38.5 (35.7-39.0)	0.028[a]
BW (g)	3400 (3191-3718)	2420 (1868-2580)	≤0.0001[a]
BMI	27(24-32)	25 (22-30)	
IBC	49.5 (30.7-72.2)	1.7 (0.5-2.5)	≤0.0001[a]

B: other experimental data (all normal pregnancies):

study:	fHbF *ex vivo* perfusion	HPAEC Griess	SEM analysis	HBAS	HPAEC proteome array	HPAEC NFkB ELISA	HPAEC ELISA
	(N=8)	(N=8)	(N=3)	(N=5)	(N=3)	(N=4)	(N=7)
maternal age (yrs)	32 (24-37)	33 (26-43)	29 (24-39)	26 (23-33)	31 (25-33)	29 (26-33)	29 (23-38)
mode of delivery:							
NVD	2(25%)	1(12.5%)	0(0%)	1(20%)	1(33.3%)	0(0%)	0(0%)
CS	6(75%)	7(87.5%)	3(100%)	4(80%)	2(66.6%)	4(100%)	7(100%)
parity	0(0-3)	0(0-2)	1(1-2)	1(0-2)	1(0-1)	2(1-3)	1(0-3)
lobule weight (g)	61 (27-89)	-	-	-	-	-	-
GA (weeks)	40.0	39.1	39.0	39.4	39.6	39.6	38.1

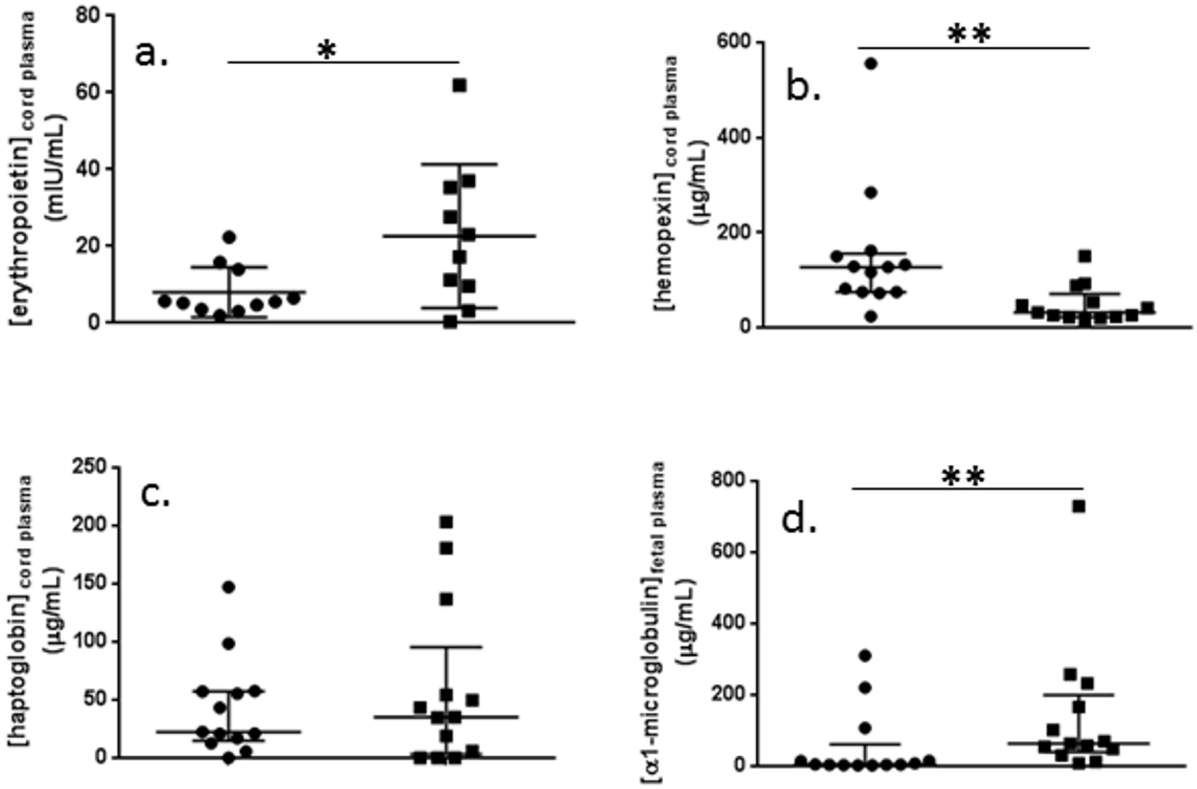
	(38.0-42.1)	(38.1-39.7)	(37.3-39.1)	(38.1-40.0)	(39.0-40.0)	(39.0-40.0)	(35.8-39.8)
BW (g)	3320	3390	3094	3800	3740	3480	3530
	(2946-3960)	(2620-3935)	(3010-3484)	(3145-3935)	(3145-3800)	(2890-3834)	(2420-3840)
IBC	44	65	73	85	82	59	74
	(27-86)	(22-88)	(53-81)	(26-88)	(26-85)	(26-89)	(17-99)

Data is presented as median (IQR). Data are presented as median (IQR) and comparative analyses undertaken by Mann Whitney U test [a]. FGR; fetal growth restriction; NVD: normal vaginal delivery; CS: caesarean section; GA: gestational age; BW: birthweight; IBC: individualized birthweight centile; SEM: scanning electron microscopy; HBAS: fHbF branching angiogenesis study; HPAEC: human placental arterial endothelial cells; SEM: scanning electron microscopy. There was some overlap of cases for HPAEC work where three cases represented in the HPAEC proteome array group also occurred in the angiogenesis (HBAS) arm, and one of these same cases also occurred in the HPAEC NFkB ELISA panel. All of the NFkB ELISA cases are also represented in the HPAEC cytokine ELISA work due to overlap between products of the same HPAEC experiments utilised for different methods.

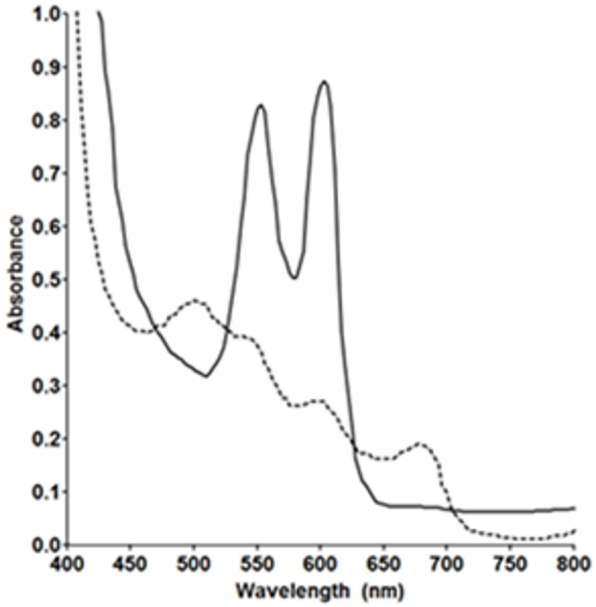
Table 2. The effect of fHbF (4.7μmol/L) on the stress-array protein phenotype in HPAECs under flow-cultured conditions.

protein	% increase from vehicle (*>50%, **>100%)	roles / associations	references
FABP1	**	coronary atherosclerotic burden	[44]
DKK-4	*	Pro-angiogenic, evidence in colonic cancer	[45]
Hif-1alpha	*	Pro-angiogenic; vascular dysfunction in ischemia-reperfusion injury by reducing bio-available NO	[46]
NFkappaB1	*	Pro-apoptotic; represses bcl-2 transcription; promotes inflammatory cytokines; dissociates tight-junctional complexes – represses occludin expression	[39, 47]

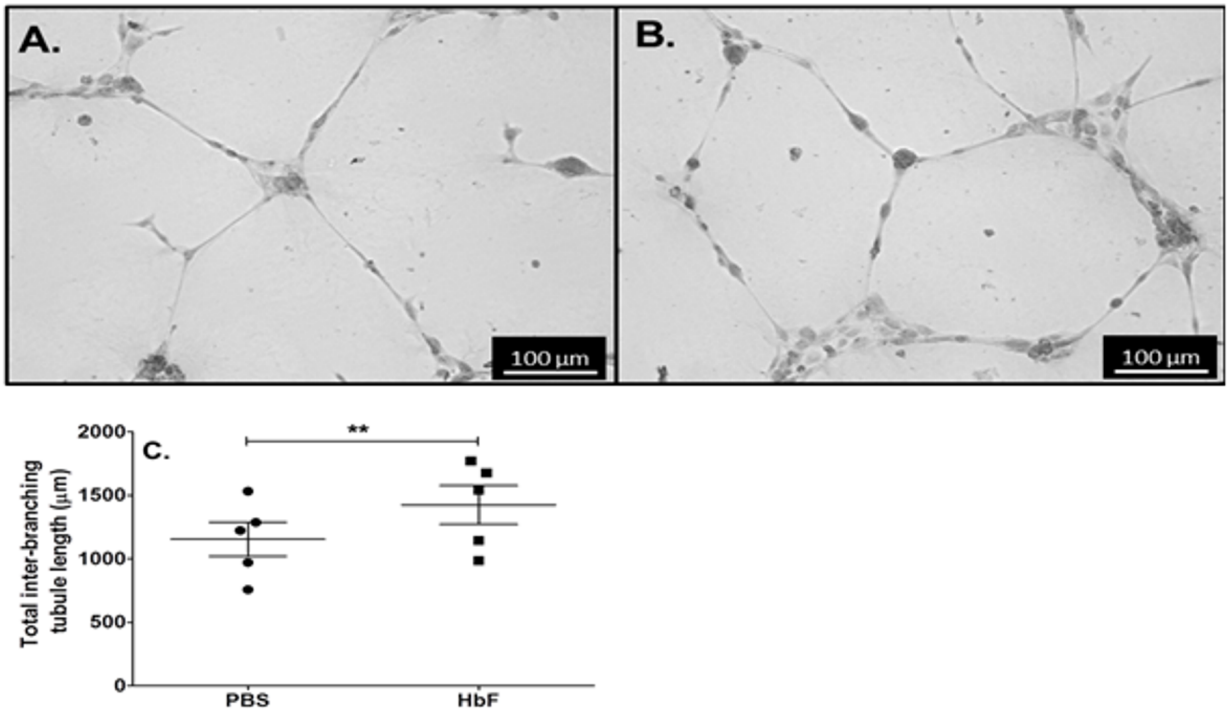
Abbreviations: DKK-4 = Dickkoph WNT signalling pathway 4; FABP1 = fatty acid binding protein 1; Hif-1alpha = hypoxia-inducible factor 1 alpha; NFKappaB1 = nuclear factor kappa-light-chain-enhancer of activated B cells.



Supplemental Figure 1: EPO and heme handling assays. Erythropoietin (EPO), hemopexin, haptoglobin and α1-microglobulin in the fetal plasma of normal (closed circles) and fetal growth restricted (FGR; closed squares) pregnancy groups. a. EPO values for normal: 5.58 (3.59, 13.96; N=11) and FGR (20.13 (8.04, 35.72; N=10) groups (P=0.05); b. hemopexin values for normal: 127.3 (74.7, 155.8; N=13) and FGR (31.6 (21.7, 70.8; N=13) groups (Mann-Whitney test: P=0.0069); c. haptoglobin values for normal: 22.4 (14.8, 57.4; N=13) and FGR (35.0 (3.0, 95.4; N=13) groups (P=0.97); d. α1-microglobulin values for normal: 4.8 (3.2, 61.0; N=13) and FGR (63.6 (38.9, 199.3; N=13) groups (P=0.01). All data is presented as median (25th, 75th centiles; N) and data was tested non-parametrically with Mann-Whitney tests.



Supplemental Figure 2: Spectral survey scan of fetal haemoglobin. Demonstrating shift in waveform of 4.7µmol/L fHbF on the reduction of fetal oxyhemoglobin (solid line) to methemoglobin (dashed line), occurring rapidly after the addition of the NO donor, DEA NONOate (120 µmol/L), measured at T=5 minutes (when samples were in a steady-state).



Supplemental Figure 3: Angiogenesis assay. Effect of fHbF on angiogenesis in Matrigel-grown human chorionic plate artery endothelial cells (HPAECs). Representative light microscopy images showing the effects of PBS (control; A.) and fHbF (B.). 4.7 µmol/L fHbF promoted angiogenesis (branching and non-branching) compared to PBS vehicle control. Tubule formation assessed as the net number of branches multiplied by the mean tubule length (µm) across four fields of view (C.; paired t-test: **P≤0.01; n=5 paired cell lines; Image Pro 7 software, MediaCybernetics Inc.,MD, USA). Data shown as mean +/- SEM.