Title: Circulating soluble fms-like tyrosine kinase-1 is placentally derived in normal pregnancy : First *in vivo* evidence

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Abstract: Circulating sFlt-1 increases significantly in pregnancy compared to the non-pregnant state and even more so in the event of preeclampsia. While *in vitro* studies have shown sFlt-1 production by normal placental explants, *in vivo* studies have

shown a significant gradient between the uterine artery and peripheral vein in preeclampsia, but not in normal pregnancy. We set out to determine if circulating sFlt-1 is placentally derived in normal pregnancy. Paired uterine and peripheral vein samples were collected at time of caesarean section. A follow up peripheral sample was collected in the postpartum period. There was a significant sFlt-1 gradient between uterine and peripheral veins. sFlt-1 levels dropped significantly when the placenta has been removed. i.e. postnatally. This is in keeping with the placenta being the main site of sFlt-1 production in normal pregnancies.

Keywords: placenta, pregnancy, sFlt-1, uterine vein, preeclampsia

Introduction: Soluble fms-like tyrosine kinase-1 (sFlt-1) is an anti-angiogenic factor that increases significantly in the maternal circulation during normal pregnancies and even more so in preeclampsia (PE)[1]. Its physiologic role in normal pregnancies is not known. However, when present in extremely high levels, it causes widespread

endothelial dysfunction leading to the development of PE and it is now used as a circulating biomarker for this disease. The study of placental and sFlt-1 biology, is fundamental for the understanding of preeclampsia. Numerous studies have shown *in vitro* sFlt-1 expression and secretion in normal and preeclampsia placentas (higher in PE placentas)[2]. Other sources of sFlt-1 have also been proposed such as smooth muscle cells, endothelial cells and PBMCs[3, 4]. In vivo assessment of sFlt-1 production has been less well characterized. A limited number of studies have assessed both normal and preeclampsia pregnancy. In preeclampsia, a gradient of sFlt-1 was demonstrated between uterine vein and peripheral vein supporting the hypothesis that in preeclampsia the placenta is the source of this molecule. Conversely, the previous work in normal pregnancy did not reveal a gradient between uterine and peripheral vein. This was proposed to argue against placental production in the absence of PE [5-7]. These normal pregnancy studies did not a) adjust for placental location, b) interrogate postpartum levels or c) use automated assays. We set out to determine whether maternal sFlt-1 is placentally derived in normal pregnancy by accounting for these factors.

Methods: Ultrasound to assess placental location was performed prior to elective cesarean section in women with normal pregnancy course. We aimed to find patients with extreme lateral placentas where we could sample both uterine veins, and all patients who consented for the study had their placenta location recorded. Serum samples from the antecubital (peripheral) and uterine vein (UV) were collected during caesarean section prior to delivery of the fetus. Accessing bilateral samples was technically more difficult but was performed in a small number of patients with extreme lateral placentas (and normal pregnancies) who consented to the procedure, in whom both uterine veins were surgically and safely accessible (n=3).

In the other cases, the obstetrician performing the caesarean section collected samples either from the left or the right uterine vein at their discretion. Samples were classified as contralateral or ipsilateral depending on the relative placental location to the UV. Centrally located placentas were excluded as well as cases where there was any uterine activity (i.e. contractions). Post-natal serum samples from these patients were collected at day 1-2 from the peripheral vein. Postnatal samples were collected in 8 patients and sFlt-1 levels were compared to their own periphery levels obtained at the time of Caesarean section. Sample collection is schematically represented in Supplemental Figure 1. The study was conducted at the John Radcliffe Hospital, Oxford, United Kingdom and all patients gave written informed consent (Central Oxfordshire Research Ethics Committee C). sFlt-1 was measured on a Roche e411 analyzer (Roche Diagnostics Limited, Burgess Hill, UK). Inter-assay percentage coefficient of variation was 5.1% at 102 pg/mL and 2.8% at 1043 pg/mL.

Results: Seventeen (uterine and peripheral) paired samples were collected in total. Median gestational age was 39.4 (IQR 39.1-40) weeks. We first analysed bilateral samples from 3 patients with extreme lateral placentas. In these patients, sFlt-1 levels from ipsilateral samples were consistently higher than from contralateral ones (Figure 1A). In order to further validate the hypothesis that placentally derived factors show a concentration difference between the ipsilateral and contralateral side, we measured hCG in these 3 samples. hCG was consistently higher in the ipsilateral samples than in the contralateral ones (20327 vs 19668 UI/L; 14374 vs 13309 UI/L and 21329 vs 20339 UI/L respectively). We then proceeded to analyse the entire study group. Overall median UV sFlt-1 was 5276 pg/ml (IQR 4384-8488) and overall median peripheral sFlt-1 was 4573 pg/ml (IQR 3150-6940); (n=17 paired samples, paired *Wilcoxon* test $p=0.0004$) (Figure 1B).

The sFlt-1 level from ipsilateral UVs was 1.30 (1.15-1.6) times higher than their peripheral counterparts whilst from contralateral UVs it was 1.12 (1.04-1.34) times higher. The median difference on sFlt-1 level between UVs and periphery was 1580 pg/ml (703-2833) on ipsilateral (n=11) samples and 429 pg/ml (157-2413) on contralateral ones (n=6). Postnatal samples (Figure 1C) showed an 80% drop of sFlt-1 postpartum [(median sFlt-1: 688.5 pg/ml (IQR 644.8-1147.5); n=8; paired *Wilcoxon* test $p=0.011$)].

Discussion: We first demonstrated that, in cases of extreme lateral placentas, samples acquired from the uterine vein nearest to the placenta (ipsilateral) have a higher concentration than the contralateral vessel. The likely placental origin was further confirmed by the concomitant measurement of beta-HCG. These samples also showed that there is an overall sFlt-1 gradient between the uterine vein and the periphery. Accordingly, there was a significant difference in the sFlt-1 concentration between the uterine and peripheral veins in the entire population (n=17 paired samples). Importantly, after the placenta is removed (i.e. postnatal period), sFlt-1 levels drop significantly. *In vitro* studies have also shown significant expression and secretion of sFlt-1 in normal placentas [2, 8]. Taken together, this data is in keeping with the placenta being the main site of sFlt-1 production in normal pregnancy..

Peripheral sources may also contribute, although their contribution is estimated to be minimal as there is no difference between sFlt-1 in the radial artery vs radial vein[6]. Previous studies have not shown a gradient in uterine vs. peripheral samples. We believe the discrepancy is due to methodology. We accounted for placental location and used an assay with a low CV. These studies are also scarce and relatively small sized (mostly due to difficulty in obtaining these samples) and larger studies would be ideal. To our knowledge, this is the first study to show, *in vivo*, that the placenta is

a significant source of circulating maternal sFlt-1. This data will allow the generation of mathematical models to better understand sFlt-1 biology (fundamental for understanding preeclampsia), namely the contribution of placental and other potential sources, distribution and rate of sFlt-1 clearance in maternal circulation.

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Figure Captions:

Figure 1: sFlt-1 levels in uterine and peripheral veins.

Figure 1A: shows three patients who had simultaneous bilateral uterine vein samples and a peripheral (PERI) vein sample taken at caesarean section. The placenta was located by ultrasound prior to the procedure. The uterine vein sample taken from the ipsilateral side as the placenta was identified as IPSI and the contralateral side to the placenta was identified as CONTRA. Figure 1B shows sFlt-1 (pg/ml) measured in paired uterine vein and peripheral vein (PERI) (n=17 paired samples). Figure 1C shows sFlt-1 (pg/ml) taken from the peripheral vein just prior to delivery (DELIVERY) and day 1-2 post-natally (POSTNATAL), n=8 paired samples these are 8 patients where all samples were available: uterine vein, peripheral samples (named as delivery in Figure 1C) and postnatal). Significance was determined to be $p<0.05$.

Supplemental Figure 1: Schematic representation of sample collection. . Blue lines represent veins (2 uterines and 1 periphery). CONTRA: contralateral; IPSI: ipsilateral; PERI: periphery.