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Failure to upregulate VEGF_{165b} in maternal plasma is a first trimester predictive marker for pre-eclampsia

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Short text: VEGF_{165b} and pre-eclampsia

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Abstract

Pre-eclampsia is a pregnancy related condition characterised by hypertension, proteinuria and endothelial dysfunction. VEGF_{165b}, formed by alternative splicing of vascular endothelial growth factor (VEGF) pre-mRNA inhibits VEGF₁₆₅ mediated vasodilatation and angiogenesis, but has not been quantified in pregnancy. Enzyme-linked immunoassays (ELISA) were used to measure mean \pm SEM plasma VEGF_{165b}, soluble endoglin (sEng) and soluble Flt1 (sFlt-1). At 12 weeks gestation the plasma VEGF_{165b} concentration was significantly upregulated in plasma from women who maintained normal blood pressure throughout their pregnancy (normotensive group 4.90 \pm 1.6 ng/ml, $p < 0.01$ Mann-Whitney *U* test) compared with non-pregnant women (0.40 \pm 0.22ng/ml). In contrast, in patients who later developed pre-eclampsia VEGF_{165b} levels were lower than in the normotensive group (0.467 \pm 0.21 ng/ml) but no greater than non-pregnant women. At term plasma VEGF_{165b} concentrations was greater than normal in both pre-eclamptic (3.75 \pm 2.24 ng/ml) and normotensive pregnancies (10.6ng/ml \pm 3.84 ng/ml $p > 0.1$ compared with pre-eclampsia). Patients with a lower than median plasma VEGF_{165b} at 12 weeks, had elevated soluble fms-like tyrosine kinase receptor 1 (sFlt-1) and soluble endoglin (sEng) pre-delivery. Concentrations of sFlt-1 (1.20 \pm 0.07 ng/ml and 1.27 \pm 0.18ng/ml) and sEng (4.4 \pm 0.18 vs 4.1 \pm 0.5) were similar at 12 weeks gestation in the normotensive and pre-eclamptic groups, respectively. Plasma VEGF_{165b} levels were elevated in pregnancy, but this elevation is delayed in women that subsequently develop pre-eclampsia. Low VEGF_{165b} may therefore be a clinically useful first trimester plasma marker for increased pre-eclampsia risk.

Introduction

Pre-eclampsia, the pregnancy related disease of hypertension, proteinuria and oedema is responsible for around 12% of the world's annual 514,000 maternal deaths [1]. Aside from maternal and fetal death, the condition may also result in intra-uterine growth restriction, seizures (eclampsia), renal or liver failure, and placental abruption. Despite much investigation, the pathological processes underlying this disease are still largely undiscovered. Recent investigation had focussed on defective placental implantation as an important aetiological factor, with the resulting release of placentally derived circulating factors, which cause endothelial dysfunction [2, 3, 4]. At the microvascular level, there is a state of vasoconstriction from smooth muscle contraction, increased vascular permeability and anti-angiogenesis [5], which correspond to the clinical findings of high blood pressure, oedema and a characteristically small placenta at delivery of the baby.

The vascular endothelial growth factor (VEGF) family is thought to be one of the important molecular systems involved in the pathogenesis of pre-eclampsia. Conventional VEGF; also known as VEGF-A, is made up of 6 different isoforms formed from alternative exon splicing resulting in proteins of varying amino acid length, termed VEGF_{xxx}. VEGF₁₆₅ is the most common isoform of VEGF_{xxx}, and consists of 165 amino acids. VEGF₁₆₅ acts via its receptor VEGFR-2 to increase vascular permeability, vasodilatation and angiogenesis [6]. Endogenous alternative splicing of the VEGF receptor results in soluble VEGFR-1 (also known as soluble fms-like tyrosine kinase 1 or

sFlt-1), which binds to VEGF and inhibits its function [6]. High levels of sFlt-1 have been documented in pre-eclampsia [7].

VEGF levels in pre-eclampsia have been measured by a number of techniques, with conflicting results according to the technique used. When measured by commercial sandwich Enzyme Linked ImmunoSorbent Assays (ELISAs) which has been proposed to measure only the free, unbound forms of VEGF, levels appear to be reduced in pre-eclampsia [8, 9]. When measured by radioimmunoassay (RIA) or competitive enzyme immuno assay (cEIA), VEGF levels are shown to substantially increase. This discrepancy has been proposed to be due to these latter 2 methods not being affected by circulating binding proteins [10,11].

In 2002, an alternative family of VEGF-A isoforms were identified, termed VEGF_{xxx}b. These are the same size as conventional VEGF-A but are alternatively spliced in exon 8 [12], This alternative splice site selection results in an alternate 6 amino acid C terminus, which affects the property of the isoforms. VEGF₁₆₅b is the most widely studied of these isoforms. VEGF₁₆₅b has been shown to inhibit the effects of VEGF₁₆₅ by binding to its principal receptor VEGFR-2 and preventing it from exerting its physiological effects such as endothelial cell proliferation and migration. VEGF₁₆₅b also binds to and activates Flt-1 (VEGFR-1) resulting in a transient increase in capillary hydraulic conductivity, but no sustained increase in permeability, in contrast with VEGF₁₆₅ [13]. A previous study of VEGF₁₆₅b in term placenta detected a decrease in VEGF_{xxx}b expression in pre-eclamptic placenta compared with control placenta, and an uncoupling of the splicing link between VEGF₁₆₅b and VEGF₁₆₅. To determine whether VEGF₁₆₅b may play a role in the

pathogenesis of pre-eclampsia, we have investigated the expression of VEGF_{165b} in maternal plasma from normotensive and pre-eclamptic pregnancies.

Methods

Pregnant subjects were recruited from St Michael's Maternity Hospital, Bristol between June 2006 and December 2007. 18 non-pregnant females aged between 20 and 39 years were recruited from the University of Bristol. The protocol for this study was granted ethical approval by Central and South Bristol Research Ethics Committee. 50 subjects were recruited from routine antenatal clinics in the first trimester of pregnancy. Subjects were aged between 17 and 42 years. Blood was taken from subjects for VEGF_{165b} quantification at recruitment, and at a further 3 times at 28, 34 and 37 weeks gestation. Pre-eclampsia was defined as BP $\geq 140/90$ mmHg on ≥ 2 occasions measured 6 hours apart and ≥ 300 mg of proteinuria/24 hours, in the absence of a urinary tract infection, occurring after 20 weeks gestation. 5 patients who later developed pre-eclampsia had further blood taken at disease diagnosis. Following venepuncture, blood was immediately centrifuged at 4000rpm for 10 minutes, the supernatant removed and stored at -80 °C until protein quantification. Subjects also received fetal growth ultrasound scans at 28, 34 and 37 weeks gestation to screen for intra-uterine growth restriction secondary to pre-eclampsia. During the study period, a further 20 patients who developed pre-eclampsia in the third trimester were recruited into the study at disease diagnosis, and received fetal growth scans at the point of recruitment into the study. In these cases plasma from their first trimesters was obtained from aliquots of frozen plasma stored under the same standard blood storage conditions by the hospital's virology department. Sample size was calculated to see an 80% change in mean VEGF_{165b} levels

at $p < 0.05$ with a power of $> 90\%$ given a standard deviation equivalent to the mean (calculated using G Power).

VEGF₁₆₅b ELISA

Anti-VEGF_{xxx}b antibody (MAB3045, clone 56/1, R&D systems) was coated onto the surface of a sterile Immulon-2HB 96 well plate at a concentration of 200 µg/ml overnight. This antibody recognises an epitope within a 9 amino acid sequence at the C terminus of human VEGF₁₆₅b. The plate was washed 3 times with PBS-Tween 0.05%, 100 µl/well. The plate was blocked for 12 hours with Superblock (250 µl/well, Pierce 37515). Serial dilutions of recombinant VEGF₁₆₅b standards (R&D systems) diluted in PBS/BSA up to a concentration of 16 ng/ml were then added to the wells in triplicate (200 µl/well). Plasma samples were also added in triplicate (200 µl/well). Plates were then incubated at room temperature with shaking for 2 hours and then washed as above. 50 ng/ml of anti-human VEGF biotinylated affinity purified polyclonal antibody (BAF293, R&D systems) was added (200 µl/well) and incubated at room temperature with shaking in foil for 2 hours as a detection reagent. Following a further wash, 100 µl HRP-streptavidin diluted 1 in 200 in PBS was added for 20 minutes protected from light, and then substrates A and B (100 µl/well) were added following washing. After 25 minutes the colour change was stopped on addition of 1M H₂SO₄ (50 µl/well) and plates were read immediately at a wavelength of 450 nm using a plate photospectrometer (Dynex Technologies). Revelation Quicklink 4.25 software was used to construct a standard curve from mean absorbance values of VEGF₁₆₅b standards, which enabled estimation of VEGF₁₆₅b concentration in plasma samples. VEGF₁₆₅b sample concentrations were quantified at multiple different

concentrations to ensure values were in the range of the ELISA, in triplicate. This sandwich ELISA measures total circulating VEGF₁₆₅b. It has been shown not to detect VEGF₁₆₅ and sFlt-1 is known not to interfere due to the use of antibodies against the VEGF₁₆₅b molecule with epitopes at different parts of the molecule²⁵. The coefficients of variation (CV) of this assay in quantifying VEGF_{xxx}b was 17% for within subject variation (samples taken at least a week apart), and 7% for within sample variation, whereas between sample CV was >200%, indicating consistency of assay, and significant variation amongst the population. VEGF₁₆₅b concentration in maternal plasma was quantified at 8-12 weeks, 28, 34 and 37 weeks gestation in 45 normotensive subjects and 4 subjects recruited in the first trimester who later developed pre-eclampsia in the third trimester. VEGF₁₆₅b concentration was also quantified in 21 pre-eclamptic patients at 12 weeks gestation, and again in the third trimester at disease diagnosis. This ELISA is now available as a DuoSet Kit from R&D Systems.

Endoglin and sFlt-1 ELISAs

ELISAs for soluble endoglin and sFlt-1 were carried out on maternal plasma samples using commercial ELISA kits from R&D Systems (DNDG00 and DVR100B respectively) according to the manufacturer's instructions.

Total VEGF ELISA and EIA

Total circulating VEGF was quantified by commercial ELISA (Alpco Diagnostics, 45-VEGFH-0111) and by competitive enzyme immunoassay (Calbiochem, QIA69). The EIA measures both bound and free forms of VEGF. Competitive EIAs for total VEGF quantification have not been commercially available since 2006 and we had access to only a single 96 well EIA. For this reason total VEGF quantification was possible in only

10 patients. For each plasma sample, VEGF concentration was determined both by ELISA and EIA.

Results

During the study period, 100 patients were recruited. 25 patients had pre-eclampsia, and 45 remained normotensive. Of the 30 recruits who were excluded from the study, 5 developed pregnancy induced hypertension, 1 developed idiopathic fetal growth restriction, 9 patients chose not to attend follow up appointments due to social reasons, 2 patients experienced intrauterine deaths at 21 and 28 weeks gestation, 3 patients experienced preterm labour in the absence of pre-eclampsia, and in 10 patients with pre-eclampsia no first trimester blood sample was available.

Table 1 shows the clinical characteristics of the study participants.

Characteristic	Normotensive (n=45)	Pre-eclampsia (n=25)
Maternal age (yrs)	30±0.8	30±1.3
Gestational age at diagnosis (wks)	NA	34+5±0.6
Gestational age at birth (wks)	39+3 ±0.17	36+3 ±0.47
Systolic BP (mmHg)	<140	151±3.1
Diastolic BP (mmHg)	<90	98±1.7
Proteinuria (g/24 hours)	<0.3	1.3±0.17
Primiparous (%)	58	52
Birth weight (g)	3495±481	2513±166
Platelet count (10 ⁹ /l)	259±10	206±17
Creatinine (mmol/l)	60±1.3	79±2.5

Table 1 Clinical characteristics of the study participants. Values shown are mean \pm SEM.

NA, not applicable.

The mean maternal age within the normotensive (n=45) and pre-eclamptic (n=25) groups were 30 \pm 0.8 and 30 \pm 1.3 years respectively. There were no differences in smoking status or ethnicity between the groups. Within the pre-eclampsia group, the mean gestational age at diagnosis was 34+5 \pm 0.6 weeks, the mean proteinuria was 1.3 \pm 0.17g/24 hours, and the mean blood pressure was 151/98 \pm 3.1/1.7mmHg. Mean birth weight within the pre-eclamptic and normotensive groups was 2513 \pm 166g and 3495 \pm 481g respectively. Of the 25 pre-eclamptic patients, 6 developed early-onset pre-eclampsia (<34 weeks gestation) and 12 developed pre-eclampsia between 34 and 37 weeks gestation. The remaining 7 patients developed pre-eclampsia at full term. 5 of the 25 pre-eclamptic patients developed severe pre-eclampsia (according to the Royal College of Obstetricians and Gynaecologists criteria: systolic BP >169mmHg or diastolic BP >109mmHg with > 1 gram proteinuria in 24 hours; or the occurrence of HELLP syndrome). 5 of the 25 fetuses born to pre-eclamptic mothers had growth restriction (ultrasonically defined as estimated fetal weight < 10th percentile for gestational age with further evidence of placental insufficiency such as oligohydramnios or abnormal umbilical artery Dopplers³³).

Increased VEGF_{165b} in pregnancy

Plasma VEGF_{165b} concentration from non-pregnant women was 0.4 \pm 0.22ng/ml. In the normotensive group, circulating plasma VEGF_{165b} at 12 weeks gestation was significantly raised (4.90 \pm 1.66 ng/ml, p<0.001), and remained so throughout pregnancy.

Reduced first trimester VEGF_{165b} in patients who later develop pre-eclampsia

At 12 weeks gestation the plasma VEGF_{165b} concentration was significantly lower in patients who later developed pre-eclampsia (0.467 ± 0.209 ng/ml) compared with plasma from normotensive pregnancies [$p < 0.0047$, as determined by Mann-Whitney *U* test, Figure 1a and 5a]. When the severe, early onset pre-eclampsia sub group was analysed, low first trimester VEGF_{165b} concentration was also predictive at 12 weeks (Mann Whitney *U*, $p = 0.008$). In contrast, at term there was no significant difference in plasma VEGF_{165b} concentrations between pre-eclamptic (3.75 ± 2.24 ng/ml) and normal pregnancies (10.58 ± 3.74 ng/ml). Thus, pre-eclampsia was associated with an 8 ± 1.8 fold increase in plasma VEGF_{165b} from first trimester to pre-delivery, compared with a 2 ± 0.3 fold increase in normotensive plasma [$p < 0.0012$, Mann-Whitney *U* test, Figure 1B].

Patients with a lower than median plasma VEGF_{165b} at 12 weeks, had elevated sFlt-1 and sEng just before delivery. Concentrations of sFlt-1 (1.20 ± 0.07 ng/ml and 1.27 ± 0.18 ng/ml) and sEng (4.4 ± 0.18 vs. 4.1 ± 0.5 ng/ml) were similar at 12 weeks gestation in the normotensive and pre-eclamptic groups, respectively [Figure 2a]. Therefore, at 12 weeks gestation, neither sFlt-1 nor sEng were able to predict the onset of pre-eclampsia later in the pregnancy [Figure 5b and 5c]. At disease diagnosis however, both sEng and sFlt-1 were significantly up-regulated in comparison with healthy patients [$p < 0.001$, Mann Whitney *U* test, Figures 2b and 2c].

VEGF_{165b} predicts sFLT-1 and sEndoglin

The reduced first trimester levels of VEGF_{165b} are able to predict the elevated sFLT-1, which occurs with the onset of pre-eclampsia [$p = 0.028$, Mann-Whitney *U* Test].

However, VEGF_{165b} concentration in the first trimester does not correlate with the elevated sEndoglin of pre-eclampsia.

Commercial total VEGF ELISAs underestimate total VEGF levels.

Total circulating VEGF was quantified in the same plasma samples both by commercial ELISA and EIA. When quantified by ELISA, VEGF concentrations were on average 2500 fold lower than when quantified by EIA [$p < 0.0001$, Figure 3a and 3b].

VEGF_{165b} accounts for the majority of total circulating VEGF in the third trimester in pre-eclamptic pregnancy.

In 5 patients from each group we were able to quantitate VEGF_{165b} and total VEGF in the same samples. VEGF_{165b} expression increased in both pre-eclampsia and normotensive pregnancy with increasing gestational age. At 12 weeks gestation, VEGF_{165b} accounts for $10.5 \pm 20\%$ of total plasma VEGF in patients that went on to develop pre-eclampsia, compared with $18.1 \pm 10\%$ in control patients (figure 5). With the onset of pre-eclampsia, VEGF_{165b} accounted for the majority of total circulating VEGF, comprising $69.3 \pm 21\%$ of total plasma VEGF in the patient group and $49 \pm 12\%$ in the control group.

VEGF_{165b} levels at 12 weeks predict pre-eclampsia.

To determine which of VEGF_{165b}, sFlt1 and endoglin are more accurate prognostic factors, receiver operating characteristic (ROC curves) were generated by calculating sensitivity (proportion of times that the test predicts pre-eclampsia) and specificity (proportion of times that the test excluded pre-eclampsia). Thus a high sensitivity value

would include all patients, but if not discriminatory, would provide a low specificity value (would include false positives). Thus non discriminatory test would give a straight line with a slope of 1 and area under the curve of 0.5 (dotted line). A perfect discriminatory test would have an area under the curve of 1.0. It can be seen from figure 5A that VEGF_{165b} levels have an area under the curve significantly greater than 0.5, in contrast to sflt and sEng.

Discussion

There have been a number of studies investigating the VEGF family of proteins in pre-eclampsia [4,14,20], which have suggested that they may play a role in its pathophysiology [21,22]. The total VEGF levels measured by EIA are consistent with those previously measured using this assay methodology [11], and by those using an independent method, the radioimmunoassay [10]. In contrast the ELISA results from the same samples gave much lower readings, consistent with previous ELISA reports of plasma VEGF [23]. These experiments therefore highlight the previously reported discrepancy between measurements of total circulating VEGF in plasma by commercial ELISAs compared with cEIA, or RIA [24]. The antibodies used in the ELISA are two monoclonals raised against the VEGF peptide sequence and thus may be raised against a similar or identical epitope. The ELISA appears to yield artificially low results, presumably as VEGF is bound by agents in plasma which prevent its detection by both antibodies simultaneously. sFlt-1 does not affect this ELISA when given as recombinant protein [25], but the effect of endoglin or other plasma constituents have not been tested. The discrepancy was particularly striking after measurement of VEGF_{165b} levels, using an ELISA that detects plasma VEGF_{165b} using two antibodies that have epitopes on

completely separate parts of the antigen (VEGF) molecule. It is therefore rather disturbing that the cEIA is no longer commercially available and was withdrawn from sale by all known suppliers between 2006 and 2007.

Of the VEGF family, VEGF₁₆₅ the most widely studied form [6] is known to increase vascular leakage, induce vasodilatation and promote angiogenesis. Although this isoform is up-regulated in pre-eclampsia, its metabolic activities may be blocked by other proteins, which bind to VEGF and inhibit its function. sFlt-1 and sEng both bind to VEGF and prevent it from exerting its physiological effects [15]. sFlt-1 is an anti-angiogenic molecule that is able to induce a pre-eclampsia-like syndrome of hypertension and proteinuria when administered to pregnant rats [7]. sEng is an anti-angiogenic protein that inhibits transforming growth factor β_1 and β_3 signalling and increases the severity of pre-eclampsia occurring in pregnant rats treated with sFlt-1 [16]. However, neither molecule can be used clinically as a first trimester marker of pre-eclampsia as sFlt-1 levels are seen to rise only 5 weeks before the onset of the clinical disease [17], and sEng concentrations become elevated at 17 weeks gestation [15].

In 2002 VEGF_{165b} was identified in normal renal cortex, and subsequently shown to be present in many different tissues, and forms the majority of VEGF in tissues such as human colon [25], and vitreous [26]. VEGF_{165b} is relatively downregulated in many conditions, including prostate, renal, bowel, and skin cancers [12,18,25,27], diabetic retinopathy [26], Denys Drash Syndrome [28] and in the placenta of patients with pre-eclampsia [29]. The mechanisms underlying these changes in expression are still under investigation, but the reduction is associated with excess angiogenesis. VEGF_{165b} has been shown to be anti-angiogenic in animal models of VEGF₁₆₅ induced blood vessel

growth in the cornea [30], mouse subcutaneous tissue [31] and rat mesentery [18] and inhibits physiological [32], and pathological [25, 30,32] angiogenesis. Studies have also shown that VEGF_{165b} transiently, but not chronically increases hydraulic conductivity [18].

The results shown here indicate that VEGF_{165b} fails to be upregulated in the first trimester in those pregnancies that will later be complicated by pre-eclampsia. It can be concluded that VEGF_{165b} may be a clinically useful first trimester marker for increased pre-eclampsia risk, providing for instance a guide to commencement of first trimester oral aspirin therapy, as this decreases the incidence of pre-eclampsia by 15% [19].

It is not clear what mediates the upregulation of VEGF_{165b} in early pregnancy, nor what prevents it in women who will develop pre-eclampsia, and further work must be done to investigate this finding. The failure of upregulation may be reflective of the aetiology, or could be contributory to the subsequent pre-eclampsia. For instance, in the first trimester the reduced anti-angiogenic VEGF_{165b} compared with normal pregnancy may reflect a maternal vasculature response to try and correct the defective implantation processes underlying the disease, or the failure to upregulate VEGF_{165b} may contribute to defective implantation.

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Figure 1. Measurement of VEGF_{165b} levels in human plasma. A. At 12 weeks gestation VEGF_{165b} was increased in plasma from pregnant women who went on to have normotensive pregnancies (n=45). This was not the case in patients who

subsequently developed severe, early onset and non severe pre-eclampsia ($n=25$). One way anova, Kruskal Wallis test $p=0.0003$. Subgroup analysis of severe/early onset pre-eclampsia ($n=9$) vs normotensive patients also showed that VEGF_{165b} is significantly lowered, Mann-Whitney U test, $p=0.008$. B. VEGF_{165b} levels in both pre-eclamptic and normal patients was increased in the third trimester ($n=25$). Mann-Whitney U Test, $p=0.0012$.

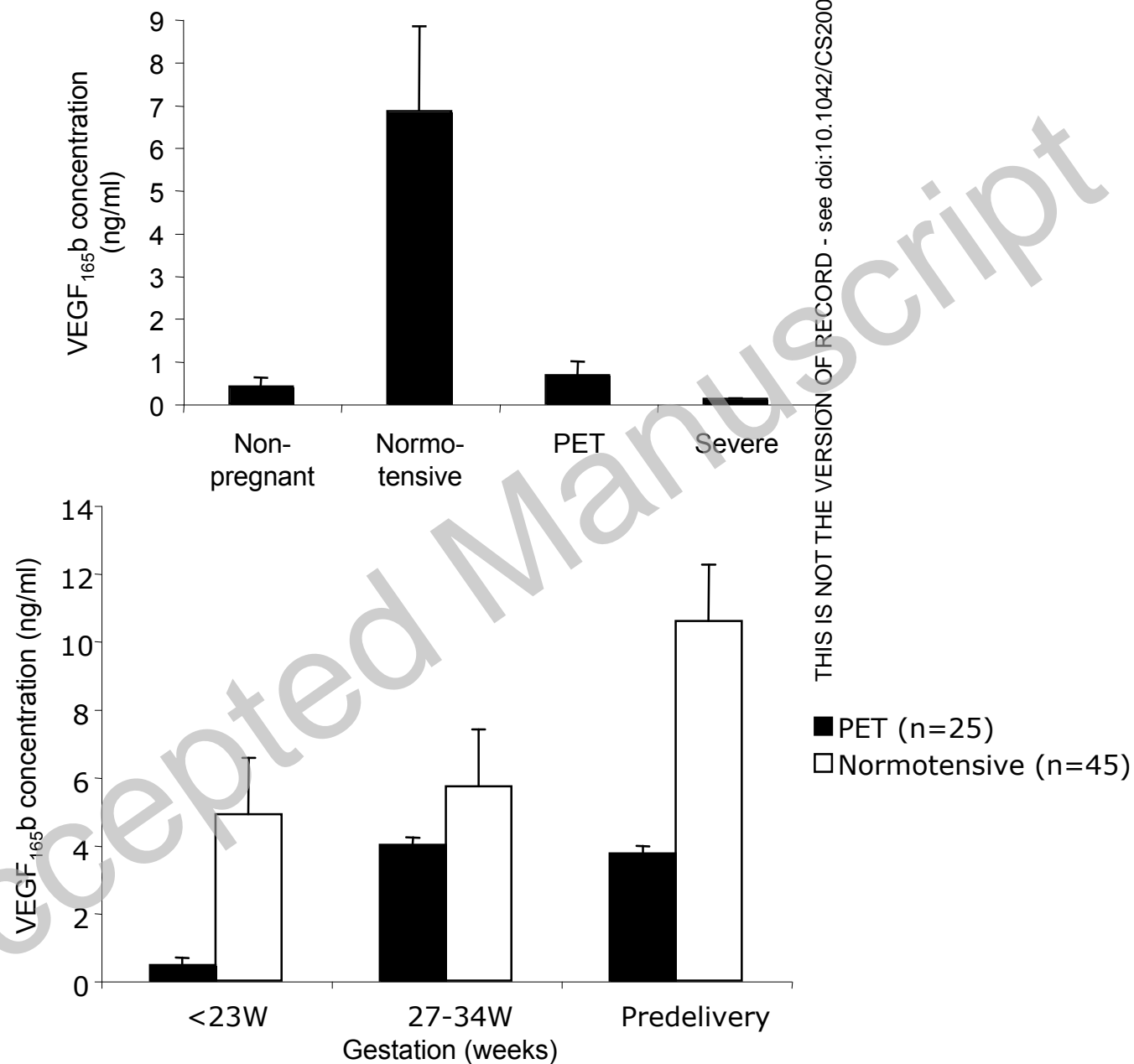
Figure 2. First trimester sFlt-1 and sEng do not predict increased risk of pre-eclampsia. At 12 weeks gestation, healthy subjects and subjects who later developed pre-eclampsia had similar levels of both sFlt-1 and sEng. Neither plasma marker was able to predict pre-eclampsia at 12 weeks gestation. Pre-eclampsia is associated with up-regulation of maternal plasma levels of B. sFlt-1 and C, sEng relative to first trimester levels. In normotensive pregnancy plasma levels of both molecules increase with advancing gestational age by 2.8 fold (sEng) and 5.3 fold (sFlt-1). Mann-Whitney U Test, $p<0.001$.

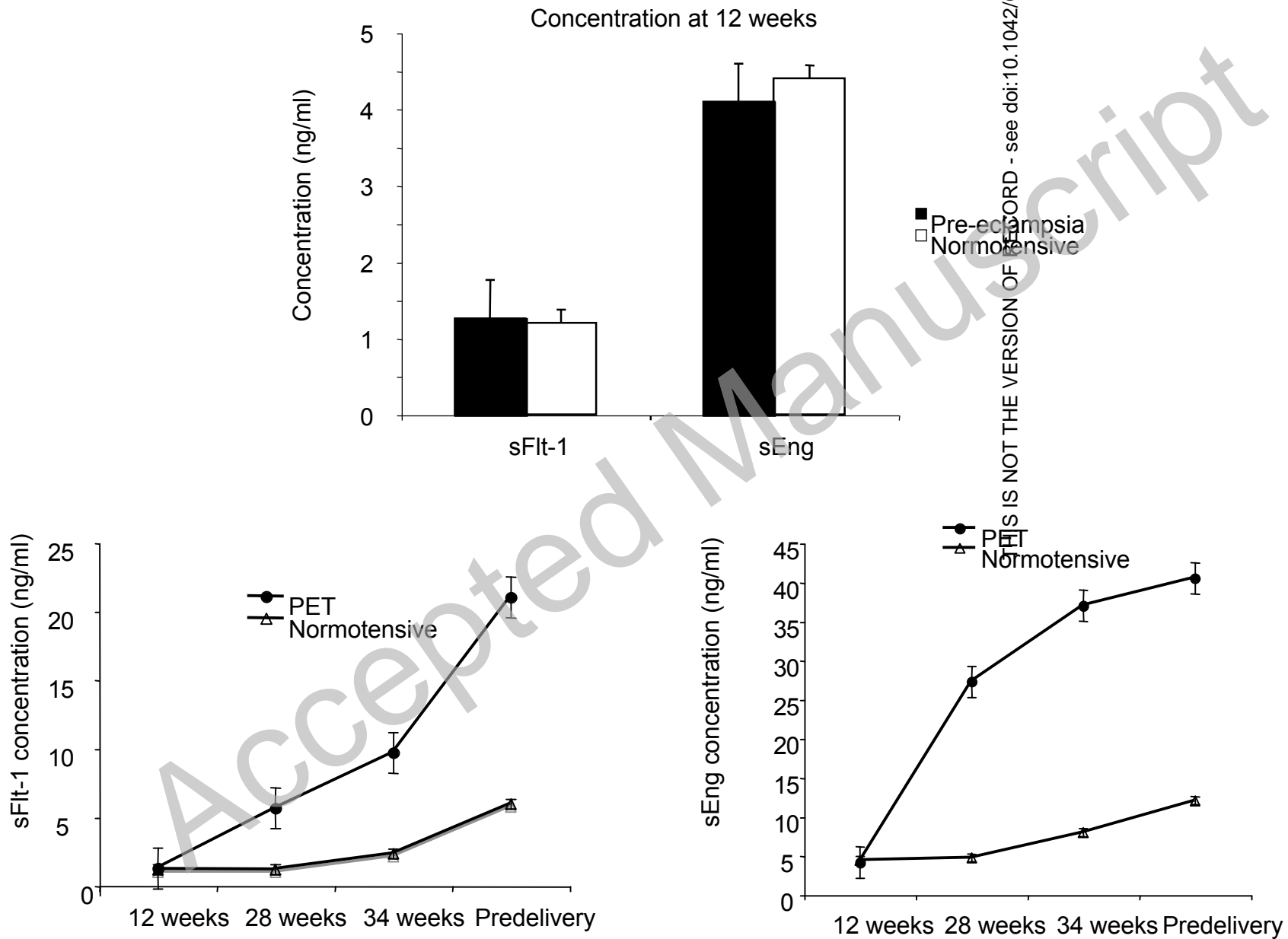
Figure 3. Lack of upregulation of VEGF_{165b} in the first trimester is able to predict the elevated sFlt-1 concentration occurring with the onset of pre-eclampsia. Mann-Whitney U test, 0.028. However, first trimester VEGF_{165b} does not correlate with sEndoglin concentration at pre-eclampsia diagnosis.

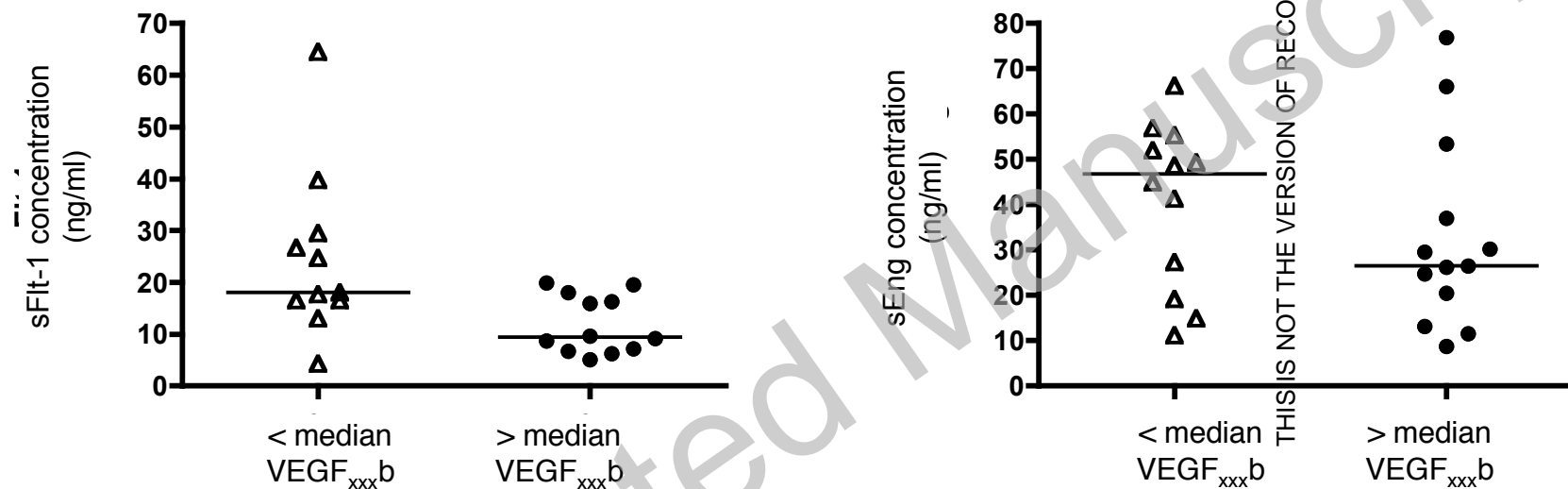
Figure 4. Total VEGF was quantified both by EIA (shown in grey) and ELISA (shown in black) in maternal plasma from normotensive and pre-eclamptic pregnancies ($n=10$). Detectable levels are 2500 fold lower when measured by ELISA compared to EIA, $p<0.0001$.

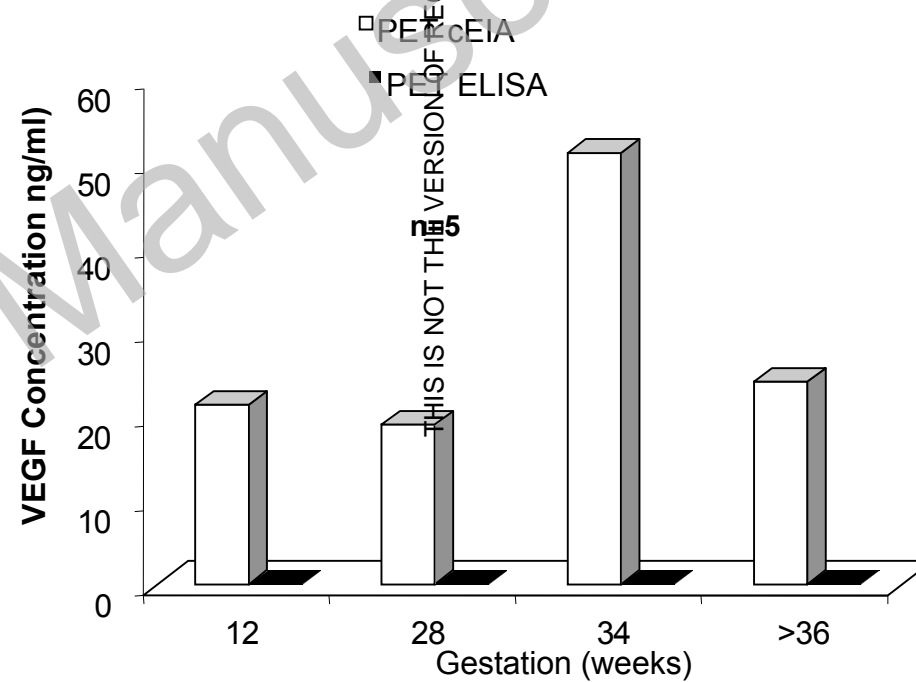
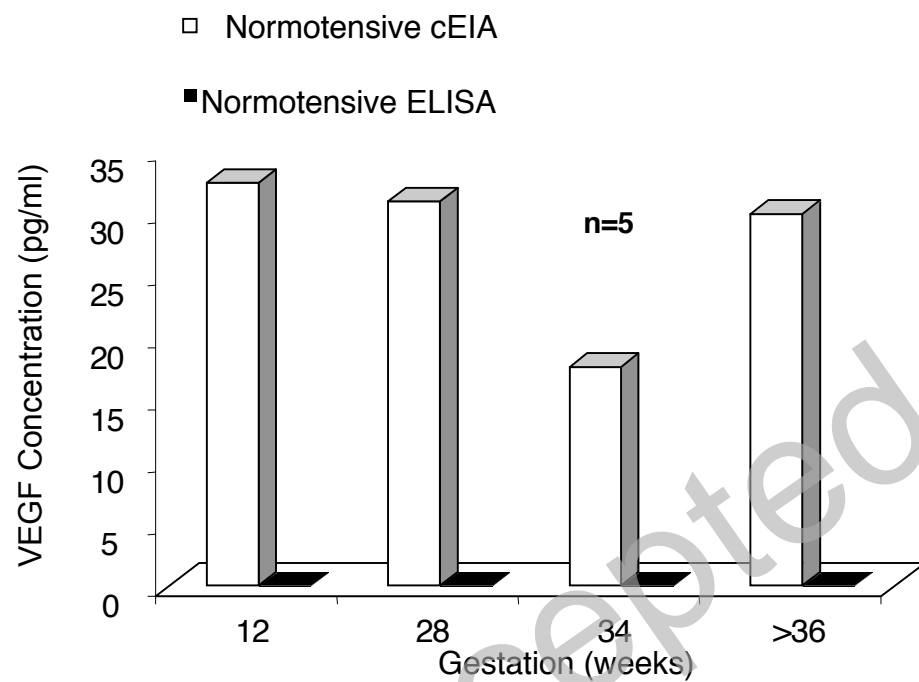
Figure 5. The increase in VEGF levels seen during pregnancy are primarily due to increased VEGF_{165b}. (A). At 12 weeks, only a small proportion of total VEGF (10-18%) was VEGF_{165b} ($n=10$). (B). In contrast at term, approximately half the VEGF was VEGF_{165b} in normals, and In pre-eclampsia, 70% of total VEGF was VEGF_{165b}.

Figure 6. Receiver–operating characteristic (ROC) curves for first trimester VEGF_{165b}, sFlt-1 and sEng in the prediction of pre-eclampsia. Area under the curve (AUC) was highest for VEGF_{165b} (0.705, $p=0.0047$ compared with random (0.5)). AUC for sEng and sFlt-1 were 0.59 ($p=0.34$) and 0.56 ($p=0.43$) respectively, not different from random (0.5).









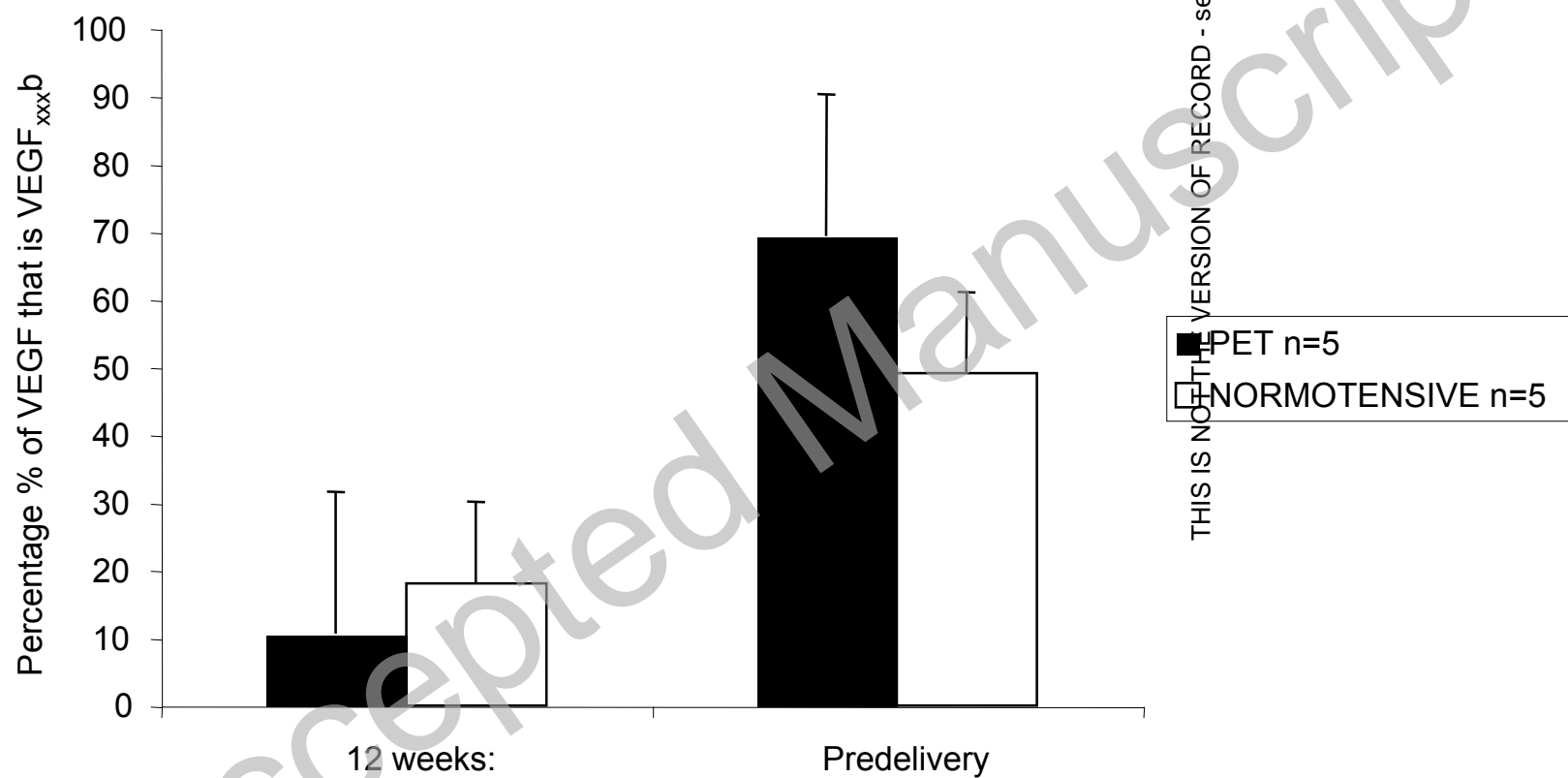


Figure 3

