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PlGF isoform 3 in maternal serum and placental tissue

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ABSTRACT

Objectives: Four isoforms originating from alternative splicing of PGF gene have been reported for placental growth factor (PlGF). Main PlGF isoforms 1 and 2 have been associated with screening and diagnosis of pre-eclampsia (PE). Despite of the vast amount of research around PlGF in PE, protein levels of isoforms PlGF-3 and -4 have not been reported in human serum samples.
Study design: In this study a PlGF-3 specific DELFIA research immunoassay based on a custom recombinant Fab binder was developed and characterized. Serum levels of a third PlGF isoform during pregnancy were determined and screening performance of PlGF-3 for PE and small for gestational age (SGA) was investigated.
Main outcome measures: Levels of serum and placental tissue PlGF 3 and predictive power of PlGF-3 for Pre-eclampsia and SGA.
Results: PlGF-3 was below the detection limit of 1.6 pg/mL in most of the serum samples collected during pregnancy. Detected protein levels of PlGF-3 were not associated to be predictive for PE or SGA. However, measurable, and relatively higher amounts of PlGF-3 was extracted from placental tissue samples.
Conclusion: Data obtained indicates that very low amounts of PlGF-3 is present in blood but significantly higher amounts of protein is present in placental tissue where it is prominently associated with cellular membranes.

1. Introduction

Placental growth factor (PlGF) is a major angiogenic factor which possesses multiple roles in important physiological processes [1]. PlGF has been established as a biomarker for prediction and diagnosis of pre-eclampsia [2–4]. Commercially available immunoassay products are measuring mainly free PlGF-1 isoform. Beside the PlGF-1 isoform a larger splice variant, PlGF-2, originating from the same PGF gene has been described [5]. PlGF-2 has highly basic 21-amino acid insertion (exon VI) that supposedly increases heparin binding capabilities for the isoform [6]. Furthermore, PlGF-2 levels have associated to have predictive properties for pre-eclampsia [7–9]. Same PGF gene transcribes a third isoform, PlGF-3, that was described in 1997 [10]. PlGF-3 contains an in-frame insertion loop of 72-amino acid between exons IV and V, close to C-terminal end of PlGF-1 [10].

Yang et al. [11] reported that all three isoforms are expressed in vitro by trophoblast and HUVEC cells. In addition, they noted that trophoblastic cells express an additional variant of PlGF [11]. Isoform coded as PlGF-4 consists of sequence of PlGF-3, plus the heparin binding domain of PlGF-2 (exon VI). Presence of the heparin binding domain in PlGF-4 suggests that this variant would remain cell membrane-associated and thus could influence trophoblast and endothelial cells in an autocrine manner [11].

PlGF isoforms 1 and 3 primarily bind to VEGFR1 (Flt-1) whereas PlGF-2 and -4 are mainly signalling through NRP1 [1]. PlGF-2 is not binding to KDR/flk-1 but is able to bind to soluble form of Flt-1 (sFlt-1) [12]. PlGF-1 is also able bind to NRP-1 with lower affinity than for sFlt-1. NRP-1 binding is indicated to occur through binding at region of exon VI and VII. Only later is present both in PlGF-1 and -2 [13] leading to differentiated receptor binding towards NRP1. Based on the sequence of PlGF-3 and available data on receptor binding mechanism for primary isoforms 1 and 2, PlGF-3 is thought primarily bind to sFlt-1 but also to lesser extent to NRP1. Glycosylated residues are indicated to play a role in receptor binding kinetics [1].

Based on their product descriptions commercial PlGF kits measure clinically significant free form of PlGF-1. Measurement of free form has been confirmed either by decreased signal of assay when excess amounts of receptor (sFlt-1) is added to test sample or showing that acid-neutralization treatment of sample releases additional analyte from receptor complexes. Measurement of free PlGF-2 with DELFIA research assay has been confirmed using an acid-neutralization treatment of samples [9].

Expression of PlGF-3 transcripts is limited in placental tissue [10] but on protein level only reported data is from in vitro experiment using

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a general anti-PlGF antibody without isoform specificity. Aim of our study was to develop and characterize PlGF-3 specific binders, develop a sensitive immunoassay based on such binder, investigate serum levels during pregnancy and evaluate performance of PlGF-3 for prediction of pre-eclampsia.

2. Methods

A custom PlGF-3 recombinant antibody was developed in collaboration with AbD Serotec (a Bio-Rad company, München, Germany). Development was initiated with a peptide designed based on the additional 72-amino acid insertion between exons IV and V. Multiple recombinant Fab binders were generated upon first library screening round of recombinant antibody fragments. Screening procedure is based on methods described by Knappik et al. in 2000 [14]. However, as affinities were not in the targeted level an additional maturation process was performed based on the identified recombinant Fabs having desired isoform-specificity. Three Fab clones (ID's 19, 24 and 27) were selected for further testing.

Affinities were determined using Octet red 96 biosensor (Fortebio, USA). Streptavidin sensor tips were used to measure binding of biotinylated Fabs. Recombinant PlGF-3 concentrations ranging from 0.3 nM to 30.0 nM were used to confirm binding constant and kinetic profile (ON- and OFF-rates) of target binding. Unglycosylated E-coli produced PlGF-3 protein (Peprotech, UK) was used for affinity studies. Glycosylated recombinant PlGF-3 protein (Reliatech, Germany) was used as a calibrator in the subsequent immunoassay studies. Although glycosylation can be important for receptor binding closer investigation shows that there is one asparagine in the insertion loop of PlGF-3, but according to antigenicity plotting based on amino acid sequence this putative glycosylation site is in least immunogenic part of PlGF-3. We confirmed that in this case the developed Fab binders, did not showed any functional (affinity, kinetics) difference between glycosylated and un-glycosylated protein.

Dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA) [15] research assay was set up using Fab clone 19 as a capture binder. Tracer antibody for this research assay was anti-PlGF Fab-IgG2a chimera having specificity against all PlGF isoforms by binding into epitope in the common region. Tracer antibody was labelled with DELFIA Eu chelate (PerkinElmer). Glycosylated recombinant PlGF-3 (Reliatech, Germany) antigen was used as a calibrator material. Biotinylated monomeric-Fab binder was first attached to streptavidin-coated plate (PerkinElmer, Turku, Finland). In second incubation step sample or calibrators were incubated on plate for two hours together with Eu-labelled tracer in assay buffer containing background-reducing agents and various blockers for unspecific binding. After second incubation plate was washed four times followed by five-minute incubation with enhancement solution before europium measurement in Victor 2D multilabel reader (PerkinElmer, Turku, Finland).

PLGF-1 was assayed using commercialized AutoDELFIA™ PlGF kit (PerkinElmer, Turku, Finland) and PlGF-2 was assayed using a DELFIA research kit [9] (Hurskainen and Sairanen, 2015) that was also generated using a custom capture antibody from AbD Serotec (Munich, Germany). In short, DELFIA PlGF-2 assay measures free form of PlGF-2 having a cross-reactivity against glycosylated human recombinant PlGF-1 and PlGF-3 4.4% and 0.8%, respectively and limit of detection at 7.1 pg/mL.

Normal pregnancy serum samples for assessing presence of PlGF-3 and samples to assess PlGF-3 levels in pregnancies complicated by pre-eclampsia (PE) and small for gestational age (SGA) were received from a biobank in Finland and United Kingdom, respectively. Sixteen normal pregnancy samples were used to roughly preliminarily evaluate the serum levels and possible changes throughout gestational age. For evaluating clinical prediction power of PlGF-3 250 samples from normal, 50 samples from pregnancies complicated by SGA and 50 samples from pregnancies complicated by term PE were measured.

Table 1
Maternal characteristics in the cases and controls. Values are given as medians (IQR) or n(%). Statistical significance at p-value of < 0.05 is marked with *.

Characteristics	Control (n = 250)	PE (n = 50)	SGA (n = 50)
Maternal Age, years	31.2 (27.6–34.9)	29.8 (24.2–33.8)	30.7 (27.4–36.3)
Maternal Weight, kg	76.9 (69.6–85.6)	77.2 (67.9–90.1)	70.6 (63.9–80.3)*
Maternal Height, cm	165 (161–169)	163 (158–166)	164 (158–167)
Ethnicity			
Caucasian	123 (49.2)	24 (48.0)	27 (54.0)
Afro-Caribbean	100 (40.0)	20 (40.0)	16 (32.0)
South	9 (3.6)	1 (2.0)	5 (10.0)
East	10 (4.0)	2 (4.0)	1 (2.0)
Mixed	8 (3.2)	3 (6.0)	1 (2.0)
Parity			
Nulliparous	128 (51.2)	30 (60.0)	28 (56.0)
Parous with no previous PE	119 (47.6)	16 (32.0)*	17 (34.0)*
Parous with previous PE	3 (1.2)	4 (8.0)*	5 (10.0)*
Cigarette Smoker	16 (6.4)	4 (8.0)	11 (22.0)*
Family history of PE	10 (4.0)	5 (10.0)	4 (8.0)
Conception			
Spontaneous	246 (98.4)	48 (96.0)	48 (96.0)
Assisted	4 (1.6)	2 (4.0)	2 (4.0)
History of Chronic Hypertension	0	5 (10.0)*	0

PlGF, PAPP-A and free hCG-β values of control and PE samples have been previously published [16]. Full maternal characteristics of these samples and those from pregnancies complicated by SGA are presented in Table 1. All samples were collected during 2011–2012 under ethical approval by the hospital and written informed consent from donor/patients was obtained. In short, definition of PE was that of the International Society for the Study of Hypertension in Pregnancy [17] and for SGA diagnosis was done as described in Poon et al. [18].

Placental tissue samples were obtained from Innovita Research (Vilnius, Lithuania). Tissue samples were frozen with liquid nitrogen and homogenized using Dismembrator, a vibrating cell disrupter instrument (B. Braun Biotech International, Germany). Pulverized (300 mg) placental tissue was centrifugated with ice-cold (2 mL) 10 mM HEPES, 24 mM KCl – solution including protease-inhibitors. After centrifugation supernatant was collected and put in to freezer. Pellet was re-homogenized with using Ultra Turrax T8 (IKA Labortechnik, Germany) in 2 mL 1 mM Triton X-100 solution. This was done to separate membrane bound proteins. After centrifugation supernatant was collected and frozen with previously collected supernatant for PlGF-3 determination.

Statistical analysis was performed using TIBCO Spotfire version 7.5 (TIBCO Software Inc., Palo Alto, CA, USA). Analysis of variance (ANOVA) was used with continuous variables and χ^2 test for categorial variables to determine statistical. Pearson correlation coefficient (R) was determined to analyse relationship between variables when required. Statistical significance was considered when *p*-value was below 0.05.

3. Results

Recombinant Fab characterization showed that affinity constant (Kd) for Fab clone 19 was 0.160 nM, for Fab clone 24 Kd = 0.032 nM and for Fab clone 27 Kd = 0.037 nM. Cross-reactivity of Fab clone 19 to PlGF-1 was 0.1% and PlGF-2 was 1.0%. Cross-reactivity of Fab clone 24 to PlGF-1 was 0.1% and PlGF-2 was 7.0%. Cross-reactivity of Fab clone 27 to PlGF-1 was 1.0% and PlGF-2 was 13.0%.

DELFIA research assay was setup using Fab clone 19 although affinity of all clones were high but a high specificity over other isoforms was needed. Limit of detection was determined to be 1.6 pg/mL and CV

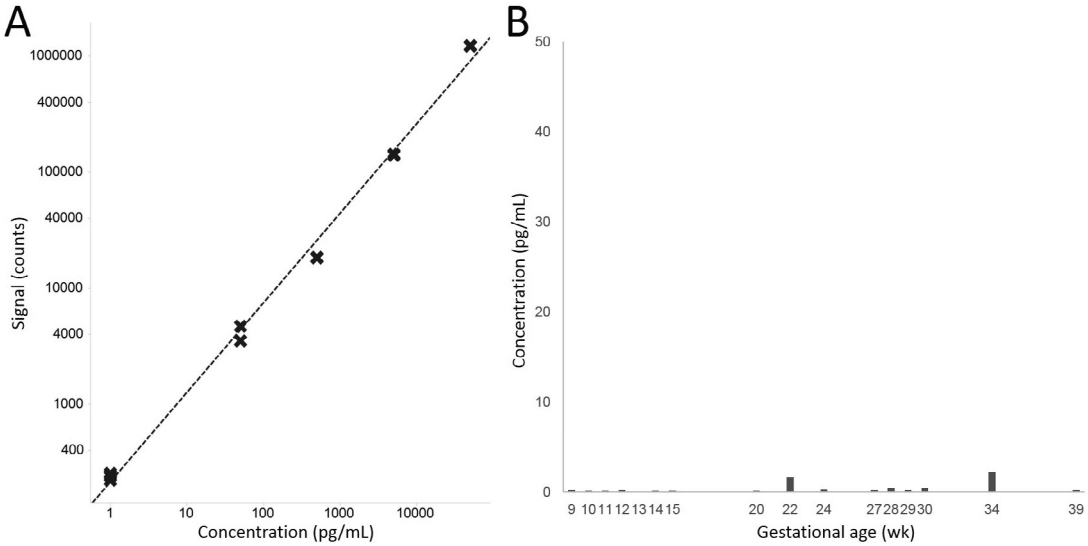

Fig. 1. A) Calibration curve from DELFIA research assay based on Fab clone 19. B) Serum PlGF-3 measured from normal pregnancy samples across gestational age.

% of 9.1%. (Fig. 1A). Assay is linear up to 50,000 pg/mL.

Concentration of PlGF-3 from 16 pregnancy samples across various gestational ages was measured. Results indicated that very low amounts, if any, of PlGF-3 was present in pregnancy serum samples. All first trimester samples (n = 7) were below limit of detection (1.6 pg/ml). Only one second trimester (n = 4) and one third trimester (n = 5) sample were above limit of detection, 1.7 pg/mL and 2.3 pg/mL, respectively.

We wanted to investigate the role of PlGF-3 in prediction of pre-eclampsia and small for gestational age. Third trimester serum samples from 50 pregnancies with subsequent pre-eclampsia and 50 SGA pregnancies were analyzed. Results were compared with PlGF-3 results from 250 serum samples from uncomplicated pregnancies. There was no statistically significant difference between groups (Fig. 2A). Most (62%) of the measurements were below LoD. Only 131 samples overall gave signal above 1.6 pg/mL, including 13 PE and 14 SGA samples. Statistical analysis (ANOVA) with samples within measuring range gave p-value of 0.67 for PE comparing to control pregnancies and for SGA pregnancies p-value was 0.58.

For comparison AutoDELFIA PlGF kit and DELFIA PlGF-2 research assay were also assayed from same samples. Correlation (R) between the two assays was 0.74 (Fig. 3A). In third trimester serum samples median free PlGF-2 concentrations were 35% higher than AutoDELFIA PlGF measuring free PlGF-1 (Fig. 3B). Significantly ($p < 0.05$) decreased concentration with both assays were measured from pregnancy serum samples compromised with PE or SGA (Fig. 3B).

Presence of PlGF-3 protein in placental tissue was investigated. Placental levels of PlGF-3 protein showed that low levels of PlGF-3 were present in protein extracts from placental tissue (cotyledon) homogenates. PlGF-3 levels ranged 2–29 pg/mL and were on average about 10-times higher than in third trimester serum samples (Fig. 3). Significantly, up to 2–32-fold (on average 9 fold), higher levels of PlGF-3 compared to the first extract were measured from tissue homogenate extracts obtained with Triton X-100 (Fig. 4) than from untreated samples. When placental homogenate samples were analysed with PlGF-1 and PlGF-2 specific assays the same Triton X-100 treated samples gave about 25% decreased and 100% increased levels, respectively, compared to tissue samples prior triton X-100 treatment (unpublished data).

4. Discussion

Vast literature exists on PlGF as a biomarker for prediction and diagnosis of pre-eclampsia. Reports on first trimester treatment

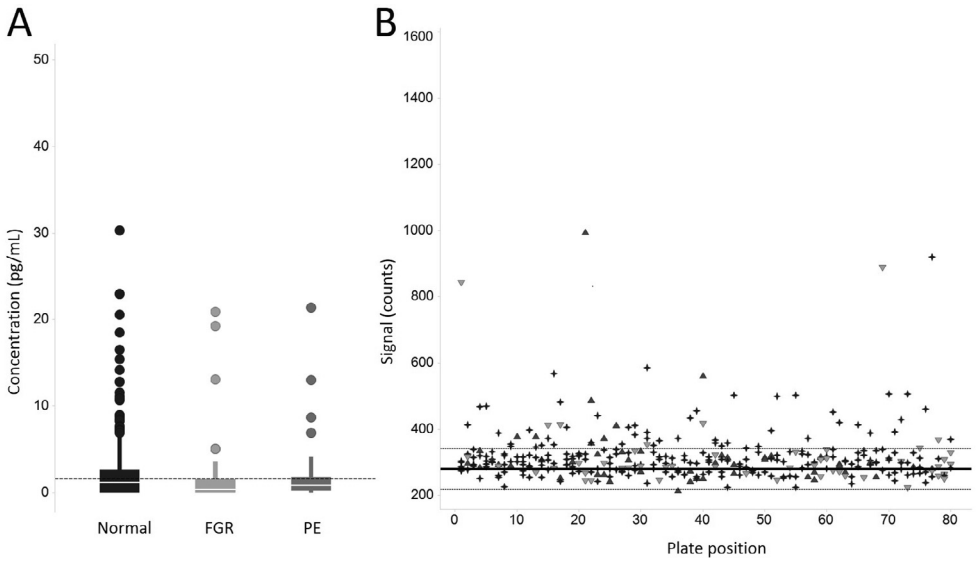

Fig. 2. A) Third trimester serum PlGF-3 levels in samples from normal pregnancies (n = 250), and pregnancies complicated with SGA (n = 50) or PE (n = 50). LoD (1.6 pg/mL) marked as a dotted line. B) Signal level from samples assayed with DELFIA PlGF-3. In x-axis a plate position of the sample is listed to visualise possible drift or pattern. Average background signal and 2*SD limits visualized. Eighty percent of the samples are below LoD. Black stars are normal samples, light grey downward triangle is SGA and dark grey upward triangle PE samples.

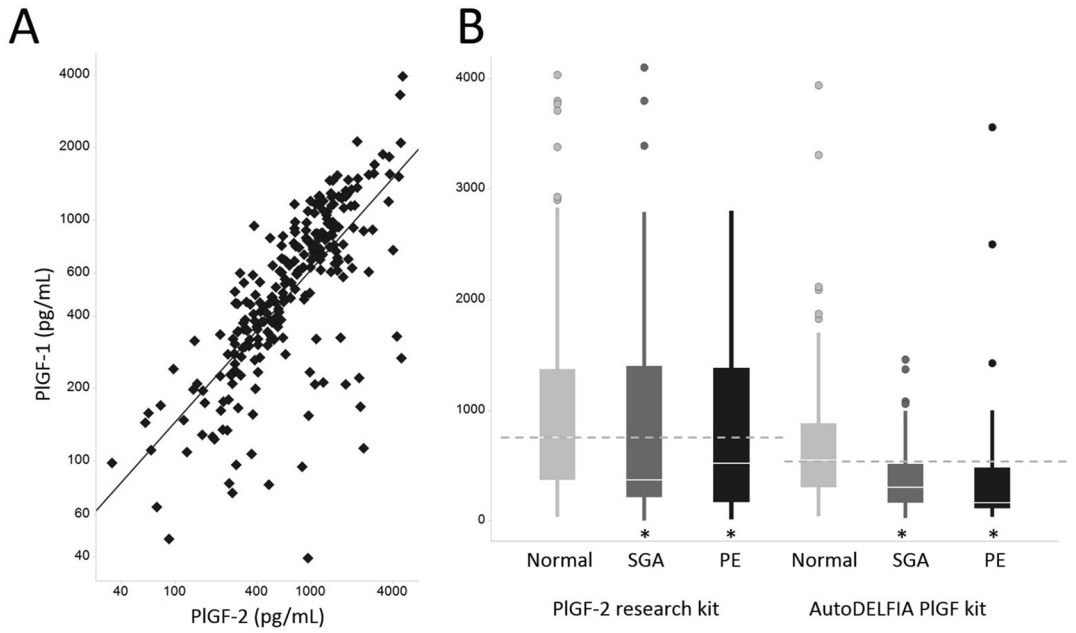

Fig. 3. A) Correlation between PlGF-1 and PlGF-2 on third trimester normal pregnancy samples (R = 0.73). B) PlGF-2 and PlGF-1 concentration in third trimester SGA and term PE samples. *p < 0.05 compared to normal samples.

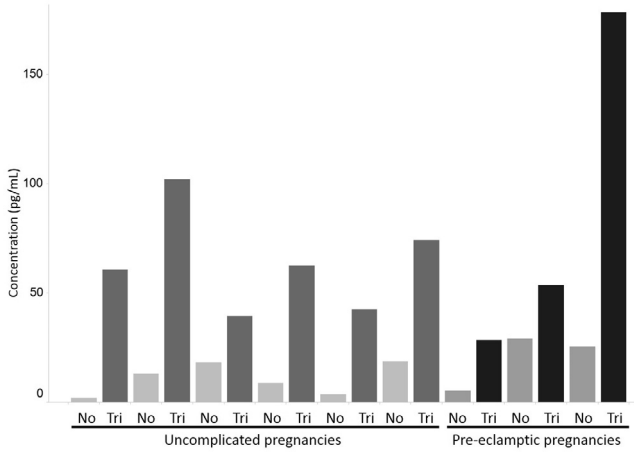

Fig. 4. PlGF-3 levels from placental tissue, cotyledon, homogenates. Six placentas from uncomplicated pregnancies and three from pre-eclamptic pregnancies were assayed for PlGF-3 levels. One sample was after normal HEPES-KCl treatment (No) and second sample was taken after Triton X-100 treatment (Tri) of the remaining pellet from first extraction.

availability and existing in vitro diagnostic kits are paving way for routine prediction and prevention of this devastating disease. Few publications have investigated the role of PlGF-2 isoform in pregnancy [7,9] but studies on human translation products of PGF splice variants 3 and 4 have not been reported. Here we describe first high-sensitivity PlGF-3-specific research immunoassay based on DELFIA technology. Furthermore, PlGF-3 protein levels in human pregnancy serum and placental tissue.

Using recombinant HuCal library technology a selective and high affinity binder against PlGF-3 was generated. Multiple high-affinity Fab clones were developed and one with lowest cross-reactivity against PlGF-1 and -2 was selected for DELFIA research assay. Research assay had very low LoD and a wide measuring range.

Measuring a small serum sample set across gestational age and larger set from third trimester indicate that very low limits of PlGF-3, if any, is present in pregnancy serum samples. Most of the serum samples fell under the measuring range and although high specificity of the assay detected serum signals might be due to low but existing cross-reactivities to PlGF-1 and PlGF-2, which range from 100's to 1000's pg/mL in third trimester samples (Fig. 3B in Nucci et al. [8]). It seems that with currently used technology role and existence of sub-pg/mL levels of PlGF-3 in human serum remains open. Alternative methods might confirm in future the presence and role of PlGF-3 isoform in human circulation. At this point these results indicate that possible cross-reactivity of commercial PlGF assays towards PlGF-3 is clinically insignificant.

Previously we have reported that first trimester serum levels of free PlGF-2 were approximately 40% lower than PlGF-1 levels measured from the same samples [9]. Correlation at first trimester between free PlGF-1 and PlGF-2 assays was about 60% and free PlGF-2 concentrations were significantly lower in pregnancies complicated subsequently by pre-eclampsia [9]. Nucci et al. reported approximately 4 times higher PlGF-2 levels compared to commercial PlGF assay which measure mainly PlGF-1 isoform [7]. However, they did not claim cross-reactivity of their PlGF-2 assay or whether it measured free or total PlGF-2. Recent study [19] indicates that PlGF-2 assay used in Nucci et al. [7] has significant cross-reactivity with PlGF-1 and PlGF-3. Using spiking experiments using sFlt-1 and NRP-1 we have confirmed that our isoform specific assays measure free form of target protein in circulation.

Results from the placental tissues were highly interesting as firstly, they prove that expressed PlGF-3 is translated in placental tissue. Secondly, PlGF-3 proved to be associated with cellular membranes. However, these results include a possibility that the protein identified from the placental tissue samples after Triton X-100 treatment is actually PlGF-4 as it includes the same 72-amino acid insertion as PlGF-3. In PlGF-4 there is the heparin binding loop that would generate the preference to associate in membranes. In addition, as PlGF-2 assay gave higher concentrations from tissue samples some of the detected protein can be PlGF-4 due to a common epitope at exon VI. Further studies are required to confirm this.

Strengths of this study were the high affinity and specific binders for PlGF-3 that were identified, the characterized DELFIA immunoassay that was used to test validated samples to evaluate role of PlGF-3 in prediction of PE and SGA. Weaknesses of this study were the low number of placental tissue samples and lack of tools to confirm more

detailed localization of protein in tissue samples and to what extent the detected protein is PlGF-3 or PlGF-4.

As a conclusion, novel sensitive and PlGF-3-specific DELFIA assay was developed. PlGF-3 levels were extremely low in pregnancy serum samples. Significant association of PlGF-3 in prediction of pre-eclampsia or small for gestational age was not seen. However, protein bound by PlGF-3 specific assay was found from placental tissue samples where it was mainly bound to cellular membranes.

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No external funding was obtained for this research.

Declaration of Competing Interest

HF, PH and MS are employees of PerkinElmer developing DELFIA kits for clinical use. KN has no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.preghy.2019.08.001.

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