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Renal dysfunction and podocyturia in pre-eclampsia may be explained by increased urinary VEGF

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ABSTRACT

Background. Pre-eclampsia has a major impact on renal function as shown by the development of proteinuria and podocyturia. How the systemic, soluble Fms-like tyrosine kinase-1 (sFlt-1)-driven inhibition of vascular endothelial growth factor (VEGF) activity detected in pre-eclampsia directly affects renal function remains unknown. The aim of the study was to clarify whether a non-canonical, renal-centred escape from VEGF inhibition in the case of pre-eclamptic pregnancy might have a direct impact on renal function.

Methods. We evaluated plasma and urinary VEGF and placental growth factor (PlGF), plasma sFlt-1 and carbonic anhydrase IX (CAIX), albuminuria and podocyturia in 18 women with uncomplicated pregnancy, 21 with pre-eclampsia and 18 non-pregnant. The three groups were matched for age and the pregnant groups also for gestational age at enrolment.

Results. Plasma VEGF was reduced in uncomplicated ($P = 0.001$) and pre-eclamptic ($P = 0.0003$) pregnancies when compared with controls. In uncomplicated pregnancy, the dysfunction was balanced by an increase ($P = 0.009$) of plasma PlGF. Increased ($P = 0.0001$) plasma CAIX in pre-eclampsia was in line with hypoxia. Pre-eclampsia resulted in a paradoxical increase ($P = 0.0004$) of urinary excretion of VEGF. Urinary concentrations of VEGF and podocytes were correlated to each other ($r^2 = 0.48$, $P < 0.0005$) but also to plasma sFlt-1 ($r^2 = 0.56$, $P < 0.0001$ and $r^2 = 0.23$, $P = 0.03$, respectively).

Conclusions. In the case of pre-eclampsia, the systemic VEGF inhibition leads the kidney, possibly the podocyte, to increase the VEGF synthesis. The mechanisms leading to local VEGF overproduction or the overproduced VEGF itself are reasonably involved in the pathogenesis of podocyturia and, as a consequence, renal dysfunction in pre-eclampsia.

Keywords: hypoxia, podocyturia, pre-eclampsia, pregnancy, VEGF inhibition

INTRODUCTION

Approximately 2–8% of worldwide pregnancies are complicated by pre-eclampsia [1], a phenomenon characterized by the onset of hypertension as well as by the development of organ damage, particularly focused on the liver and kidneys [2]. Signs of renal involvement consist, apart from the development of high blood pressure, in the appearance of proteinuria, progressive renal insufficiency [2] and urinary excretion of podocytes, so-called podocyturia [3].

Of interest, the recent demonstration that pre-eclampsia is characterized by an increased plasma concentration of soluble Fms-like tyrosine kinase-1 (sFlt-1) [4], a circulating decoy receptor of vascular endothelial growth factor (VEGF), has suggested that VEGF inhibition could be involved in the development of renal damage. This hypothesis is in line with the evidence that systemic anti-VEGF treatment (as in the case of neoplastic diseases) ends up causing side effects such as hypertension, proteinuria and podocyturia, closely reminiscent of the renal involvement in the case of pre-eclampsia [5].

Even though to a far lower extent, increased sFlt-1 plasma concentration and, consequently, VEGF inhibition also occur in uncomplicated pregnancy [6] but in this case, the dysfunction is counterbalanced by the simultaneous augmented secretion of placental growth factor (PlGF, a pro-angiogenic factor [7] sharing several features with VEGF) by the placenta. This compensatory phenomenon is quite reproducible and the measurement of the sFlt-1/PlGF plasma ratio has been recently

KEY LEARNING POINTS

What is already known about this subject?

- escape from systemic soluble Fms-like tyrosine kinase-1-induced inhibition of vascular endothelial growth factor (VEGF) through a selective increase of placental growth factor synthesis is a well-known feature of uncomplicated pregnancy; and
- this pathway is not shared by pre-eclamptic individuals with consequent development of hypertension, proteinuria and podocyturia.

What this study adds?

- here we show that in the case of pre-eclampsia, the VEGF blockade is selectively bypassed at renal level and that this dysfunction could be directly involved in the pathogenesis of renal dysfunction, in particular of podocyturia.

What impact this may have on practice or policy?

- by increasing our knowledge of the mechanisms involved in the pathogenesis of podocyturia associated to pre-eclampsia, this study may help finding novel therapeutic approaches aimed at preventing and/or treating this renal dysfunction.

validated as a suitable tool in the short-term prediction of pre-eclampsia during pregnancy [8].

Quite surprisingly, an increased urinary concentration of VEGF was found in pre-eclamptic patients [6], something that seems to be in striking contrast to the reduced plasma concentration of VEGF that, as described above [4, 6], characterizes these patients, but that could suggest the presence of an alternative, renal-centred, escape from VEGF inhibition.

The mechanisms leading to renal damage in the case of pre-eclampsia remain largely unknown; this study aimed to clarify whether a non-canonical way to escape from VEGF inhibition in case of pre-eclamptic pregnancy might have a direct impact on the renal function.

MATERIALS AND METHODS

The study was approved by the Ethics Committee of the IRCCS Ospedale San Raffaele, Milan, Italy and written informed consent was obtained from all the study participants. Twenty-one pregnant women affected by pre-eclampsia, 18 women with uncomplicated pregnancy and 18 non-pregnant healthy women were enrolled in the study. Pregnant individuals were recruited at the Obstetrics and Gynaecology Department of IRCCS Ospedale San Raffaele. In accordance with international guidelines [9], pre-eclampsia was diagnosed in the presence of both: new onset of hypertension (systolic blood pressure $\geq$140 mmHg and/or diastolic blood pressure $\geq$90 mmHg, measured on two occasions at least 4 h apart) and proteinuria ($\geq$300 mg of protein per 24-h urine collection or a protein/creatinine ratio $\geq$0.3 mg/dL) after 20 weeks of gestation.

Exclusion criteria for pre-eclamptic pregnant women were: the presence of a pre-existing renal disease, or Type 1, Type 2 or gestational diabetes.

Exclusion criteria for women with uncomplicated pregnancy were the same as above plus the presence of hypertension and/or proteinuria, and the presence of intrauterine growth restriction.

Exclusion criteria for healthy (control) women were: the presence of a pre-existing renal disease, Type 1 or Type 2 diabetes, hypertension and/or proteinuria.

The three groups were matched for age. Pregnant women with and without pre-eclampsia also shared a similar gestational age at the time of blood and urine collections. Paired collections of serum and urine samples were obtained from all the individuals considered for the study. Clinical characteristics (at the moment of blood sample) of the individuals included in the study are described in Table 1.

Enzyme-linked immunosorbent assays (ELISAs) for human unbound VEGF, sFlt-1, PlGF and carbonic anhydrase IX (CAIX) were performed according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Briefly, serum and urine samples were assayed in duplicate in a 96-well plate pre-coated with a capture antibody directed against VEGF, sFlt-1, PlGF and CAIX.

Characteristics of the ELISAs used are the following:

VEGF. For this study, we used the Quantikine® ELISA Human VEGF Immunoassay DVE00 (R&D Systems, Minneapolis, MN, USA). The assay measures the concentration of free VEGF165A in biological fluids. The calibration curve ranges between 15.6 and 1000 pg/mL. At low values, the coefficient of variability is 6.5% (intra-assay) and 8.5% (inter-assay).

PlGF. For this study, we used the Quantikine® ELISA Human PlGF Immunoassay DPG00 (R&D Systems, Minneapolis, MN, USA). The assay measures the concentration of free PlGF in biologic fluids. The calibration curve ranges between 15.6 and 500 pg/mL. At low values, the coefficient of variability is 5.9% (intra-assay) and 13.6% (inter-assay).

Table 1. Clinical characteristics of the individuals involved in the study

Clinical features	Non-pregnant controls	Uncomplicated pregnancy	Pre-eclampsia	P-value
Number of individuals	18	18	21	NS
Age, years	31.9 ± 6.2	36.6 ± 5.4	34.2 ± 6.5	NS
Body mass index, kg/m^2	20.6 ± 2.2	25.2 ± 4.2	25.0 ± 3.7	NS
Gestational age, weeks	–	35.7 ± 4.3	33.4 ± 5.3	NS
Mean blood pressure, mmHg	85.7 ± 5.6	88.9 ± 5.8	109.7 ± 7.7	0.0001
Anti-hypertensive treatment	0/18	0/18	11/21	–

Data are expressed as mean ± SD. NS, not significant.

Flt-1. For this study, we used the Quantikine® ELISA Human VEGFR1/Flt-1 Immunoassay DVR100C (R&D Systems, Minneapolis, MN, USA). The assay measures the concentration of VEGFR in biological fluids. The calibration curve ranges between 31.3 and 2000 pg/mL. At low values, the coefficient of variability is 2.5% (intra-assay) and 6.7% (inter-assay).

CAIX. For this study, we used the Quantikine® ELISA Human CAIX Immunoassay DCA900 (R&D Systems, Minneapolis, MN, USA). The assay measures the concentration of CAIX in biological fluids. The calibration curve ranges between 15.6 and 1000 pg/mL. At low values, the coefficient of variability is 3.8% (intra-assay) and 6.3% (inter-assay).

In case of low values (below the limit of detection), the data were censored and substituted with a constant value, equal to half the limit of detection [10].

Urinary concentration of albumin was measured by immunoturbidimetric technique [at low values, the coefficient of variability is 3.5% (inter-assay)], and urinary concentration of creatinine was measured using the Jaffe picrate alkaline reaction [at low values the coefficient of variability is 2.6% (inter-assay)]; both techniques were performed on a Cobas Mira autoanalyser (Roche, Basel, Switzerland).

Podocyturia was quantified as previously described [11]. Briefly, freshly voided urines were quantified, centrifuged and the sediment was finally resuspended in 1 mL of phosphate-buffered saline. Propidium iodide was added at this stage (1 μM) as viability marker. Aliquots of 100 μL of the resuspended sediment were centrifuged (Shandon cytocentrifuge, Thermo Scientific, Waltham, MA, USA) onto positively charged slides (Superfrost Plus, Menzel Glaser, Braunschweig, Germany). Once fixed and permeabilized, the cells were then stained using a mouse monoclonal anti-podocalyxin primary antibody (Santa Cruz, Dallas, TX, USA) and a fluorescein isothiocyanate (FITC)-conjugated isotype-matched secondary antibody (Jackson, Philadelphia, PA, USA) and finally counterstained with 4,6-diamidino-2-phenylindole (DAPI) for nuclear identification (Sigma, St Louis, MO, USA). Immunostainings were examined under fluorescence microscope (Zeiss AxioImager A1, Zeiss, Jena, Germany). Nucleated, propidium iodide-negative, podocalyxin-positive cells were defined as viable podocytes [11].

Immunofluorescence for podocin (goat polyclonal anti-podocin antibody, Santa Cruz) and VEGF (Abcam, Cambridge, UK) was performed on sections of paraffin-embedded renal autoptic tissue obtained through the Dutch Pathology Registry as previously described [12]. The sections of autoptic kidneys used for immunofluorescence came from patients independent from the population tested for the plasma and urine biomarkers [12]. Isotype matched secondary antibodies, TRITC- or FITC-conjugated (all from Jackson) were used along with DAPI counterstaining (Sigma). Renal sections were examined by confocal microscope (TCS SP2, Leica).

Statistical analysis

Data are shown as arithmetical means [standard deviation (SD)]. Comparisons between groups were addressed by analysis of variance, and multiple comparisons were performed with the Tukey–Kramer test (JMP software for the Apple Macintosh; SAS Institute, Cary, NC, USA). Correlations were sought by simple linear regression. The null hypothesis was rejected for two-tailed $P < 5\%$.

RESULTS

sFlt-1, VEGF and PlGF plasma concentrations in the study population

Plasma sFlt-1 concentration in pre-eclampsia (9870.9 ± 5366.0 pg/mL, mean ± SD) was higher when compared with both uncomplicated pregnancy (3357.2 ± 2181.4, $P < 0.0001$) and non-pregnant controls (62.2 ± 7.3, $P < 0.0001$). A significant increase of sFlt-1 in uncomplicated pregnancy, when compared with non-pregnant controls ($P = 0.04$), could also be demonstrated (Figure 1A).

As described in the literature [13], the total serum concentration of VEGF is increased in pre-eclampsia. In our case, we used an ELISA that specifically reads free VEGF (the amount not bound to sFlt-1). Plasma concentration of free VEGF was higher in non-pregnant controls (28.0 ± 35.8 pg/mL, mean ± SD) when compared with both uncomplicated pregnancy (2.5 ± 3.7, $P = 0.001$) and pre-eclampsia (0.5 ± 0.8, $P = 0.0003$), suggesting that the slight, but significant increase of sFlt-1 found in uncomplicated pregnancy was sufficient to substantially blunt the amount of circulating VEGF. No difference in plasma concentration of VEGF could instead be shown between uncomplicated pregnancy and pre-eclampsia ($P = 0.9$; Figure 1B).

As described in the literature [14], the total serum concentration of PlGF is not increased in pre-eclampsia. In our case, we used an ELISA that specifically reads free PlGF (the amount not bound to sFlt-1). Plasma concentration of free PlGF was higher in uncomplicated pregnancy (342.1 ± 473.5 pg/mL, mean ± SD) when compared with both non-pregnant controls

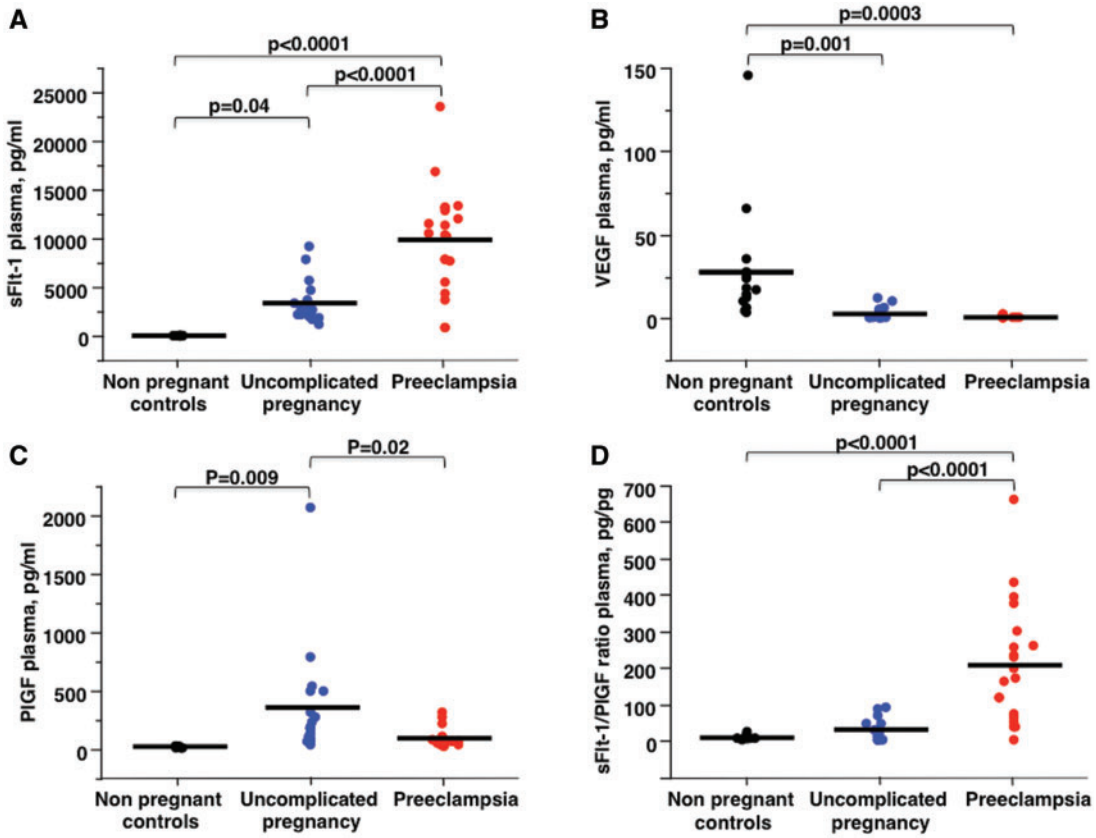

FIGURE 1: Plasma concentrations of sFlt-1, VEGF and PlGF in the study population. (**A**) Concentration of plasma sFlt-1 in non-pregnant controls, uncomplicated pregnancy and pre-eclampsia. (**B**) Concentration of plasma VEGF in non-pregnant controls, uncomplicated pregnancy and pre-eclampsia. (**C**) Concentration of plasma PlGF in non-pregnant controls, uncomplicated pregnancy and pre-eclampsia. (**D**) sFlt-1/PlGF ratio in the plasma of non-pregnant controls, uncomplicated pregnancy and pre-eclampsia.

(9.7 ± 2.9, $P = 0.009$) and pre-eclampsia (81.4 ± 80.7, $P = 0.02$). No difference in plasma concentration of PlGF could instead be shown between non-pregnant controls and pre-eclampsia ($P = 0.8$) (Figure 1C).

As a consequence of these findings, the plasma ratio sFlt-1/PlGF was increased in pre-eclampsia (208.6 ± 164.3 pg/pg, mean $\pm$ SD) when compared with both non-pregnant controls (7.8 ± 5.7, $P < 0.0001$) and uncomplicated pregnancy (28.2 ± 28.9, $P < 0.0001$). No difference could instead be shown between uncomplicated pregnancy and non-pregnant controls ($P = 0.9$) (Figure 1D).

Urinary excretion rates of albumin and podocytes

The well-established impact of pre-eclampsia on renal function was confirmed by the increased urinary albumin excretion rate (evaluated as albumin/creatinine ratio) found in pre-eclampsia (232.3 ± 205.8 µg/mg, mean $\pm$ SD) when compared with both uncomplicated pregnancy (8.4 ± 8.6, $P < 0.0001$) and non-pregnant controls (5.3 ± 4.5, $P < 0.0001$). No difference could instead be shown between uncomplicated pregnancy and non-pregnant controls ($P = 0.9$; Figure 2A).

In parallel with another well-known marker of pre-eclampsia, the urinary excretion of viable podocytes, so-called podocyturia (evaluated as number of podocytes/creatinine ratio) was increased in pre-eclampsia (72.7 ± 44.9 *n*/mg, mean $\pm$ SD) when compared with both uncomplicated pregnancy (1.6 ± 2.3, $P < 0.0001$) and non-pregnant controls (0.2 ± 0.3, $P < 0.0001$). No difference could instead be shown between uncomplicated pregnancy and non-pregnant controls ($P = 0.9$; Figure 2B).

Plasma concentration of the hypoxia marker CAIX

To clarify whether the resistance to escape from VEGF inhibition has a systemic impact, we investigated the plasma concentration of CAIX, a well-established marker of hypoxia [15]. As a result, plasma concentration of CAIX was increased in pre-eclampsia (109.1 ± 73.1 pg/mL, mean $\pm$ SD) when compared with both non-pregnant controls (27.9 ± 13.9, $P < 0.0001$) and uncomplicated pregnancy (19.6 ± 14.4, $P < 0.0001$). No difference could instead be shown between non-pregnant controls and uncomplicated pregnancy ($P = 0.9$; Figure 2C).

Urinary excretion rates of VEGF and PlGF and correlation with the respective plasma concentrations

These results suggest that pre-eclampsia is characterized by a systemic (and not by-passed) inhibition of VEGF coupled to a significant hypoxia. To verify the impact of these dysfunctions on the kidney, we evaluated the urinary excretion of VEGF (measured as VEGF/creatinine ratio). The ratio was increased in pre-eclampsia (193.4 ± 145.2 pg/mg, mean $\pm$ SD) when

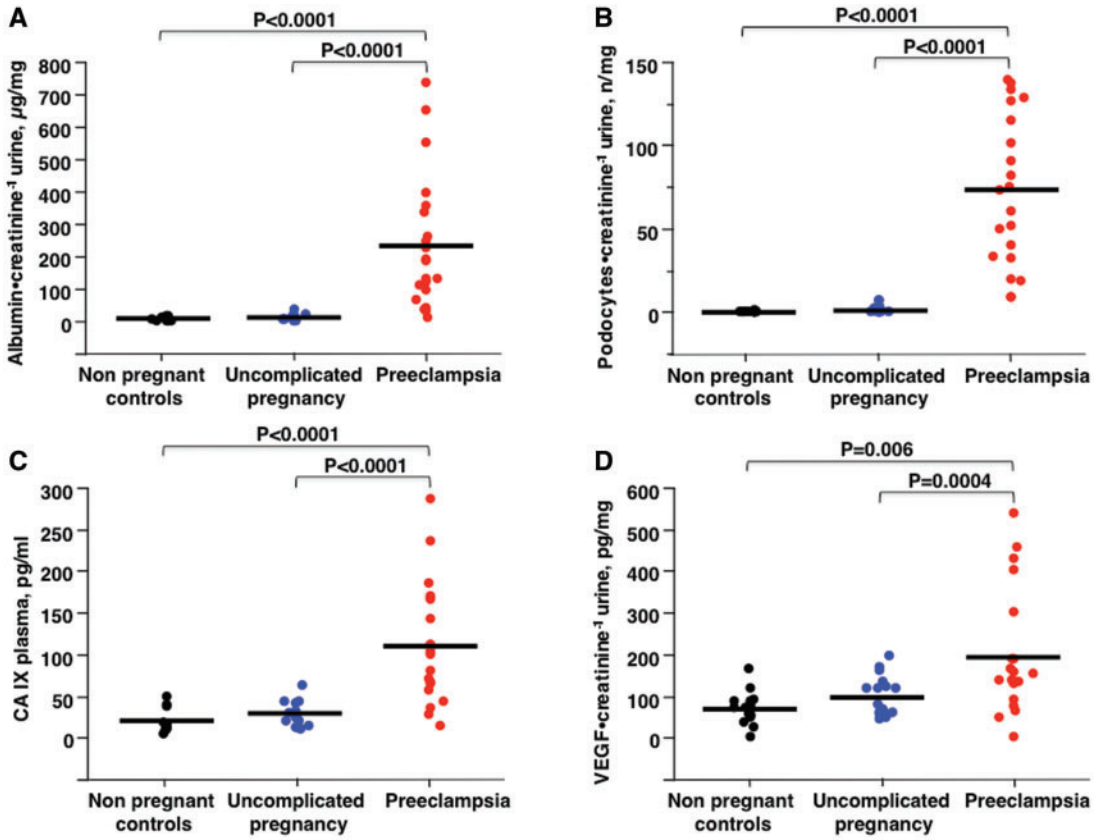

FIGURE 2: Urinary excretion of albumin, podocytes and VEGF along with plasma concentrations of CAIX in the study population. (**A**) Albumin/creatinine ratio measured in the urine of non-pregnant controls, uncomplicated pregnancy and pre-eclampsia. (**B**) Podocyte number/creatinine ratio measured in the urine of non-pregnant controls, uncomplicated pregnancy and pre-eclampsia. (**C**) Concentration of plasma CAIX in non-pregnant controls, uncomplicated pregnancy and pre-eclampsia. (D) VEGF/creatinine ratio measured in the urine of non-pregnant controls, uncomplicated pregnancy and pre-eclampsia.

compared with both non-pregnant controls (68.6 ± 36.3, $P = 0.006$) and uncomplicated pregnancy (95.5 ± 46.6, $P = 0.0004$). No difference could instead be shown between non-pregnant controls and uncomplicated pregnancy ($P = 0.7$; Figure 2D).

These results suggest that, VEGF being substantially absent in the pre-eclamptic plasma, the increased amount of VEGF found in these patients is synthesized locally in the kidney reasonably by the podocytes, the only VEGF-secreting cells detectable in the renal glomerulus [16].

Plasma and urinary concentrations of VEGF show a specular behaviour in pre-eclampsia (visible by comparing Figures 1B to 2D). PlGF instead, because of its small dimension(30 kDa) [6], is freely excreted with urine and therefore its urinary concentrations directly reflect the ones measured in the plasma: urinary concentration of PlGF (measured as PlGF/creatinine urinary ratio) was increased in uncomplicated pregnancy (152.3 ± 217.8 pg/mg, mean $\pm$ SD) when compared with both non-pregnant controls (11.7 ± 7.9, $P = 0.004$) and pre-eclampsia (28.9 ± 22.1, $P = 0.009$). No difference could instead be shown between non-pregnant controls and pre-eclampsia ($P = 0.9$; Figures 1C and 3A).

Accordingly, plasma and urinary concentrations of PlGF were strictly correlated ($r^2 = 0.81$, $P = 0.0001$; Figure 3B).

The correlation between urinary concentrations of VEGF and PlGF unveils the different ways to escape VEGF inhibition in uncomplicated pregnancy and pre-eclampsia

By comparing the urinary concentrations of VEGF and PlGF, it is possible to immediately distinguish between the 'PlGF-mediated' escape from VEGF inhibition in uncomplicated pregnancy (open dots, Figure 3C) and the 'VEGF-mediated' escape from VEGF inhibition in pre-eclampsia (triangles, Figure 3C).

Correlation between urinary concentrations of VEGF and podocytes

Of interest, the urinary concentrations of VEGF and podocytes were significantly correlated ($r^2 = 0.48$, $P < 0.0005$) in pre-eclamptic patients (Figure 3D), in line with the hypothesis that the two phenomena might share a common pathogenic background.

Plasma sFlt-1 as the driving force of urinary excretion of VEGF and podocytes

This assumption was further confirmed by the evidence that, in the case of pre-eclampsia, the urinary concentrations of both podocytes (Figure 4A, $r^2 = 0.23$, $P = 0.03$) and VEGF (Figure 4B, $r^2 = 0.56$, $P < 0.0001$) were correlated to plasma sFlt-1

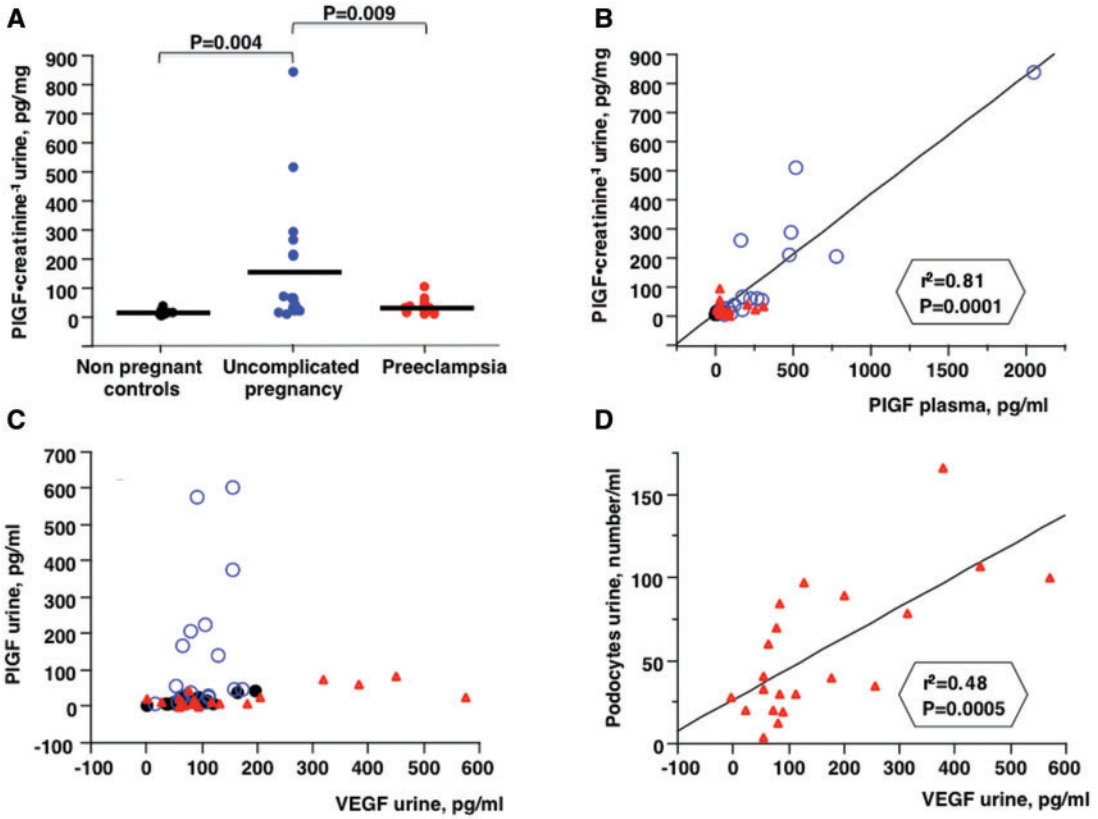

FIGURE 3: Urinary excretion of PlGF in the study population and different correlations between plasma and urinary cytokines and urinary podocytes. (**A**) PlGF/creatinine ratio measured in the urine of non-pregnant controls, uncomplicated pregnancy and pre-eclampsia. (**B**) Correlation between concentration of plasma PlGF and urinary PlGF/creatinine ratio in non-pregnant controls (filled dots), uncomplicated pregnancy (open dots) and pre-eclampsia (triangles). (**C**) Correlation between concentration of urinary VEGF/creatinine ratio and urinary PlGF/creatinine ratio in non-pregnant controls (filled dots), uncomplicated pregnancy (open dots) and pre-eclampsia (triangles). (**D**) Correlation between urinary VEGF concentration and urinary podocyte number in pre-eclamptic patients.

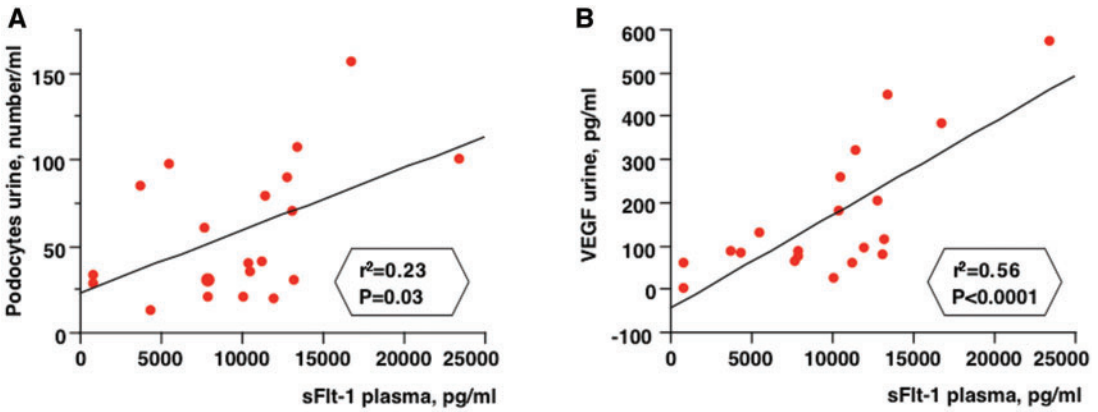

FIGURE 4: Correlations between plasma sFlt-1 and urinary VEGF and podocytes. (**A**) Correlation between plasma sFlt-1 concentration and urinary podocyte number in pre-eclamptic patients. (**B**) Correlation between plasma sFlt-1 concentration and urinary VEGF concentration in pre-eclamptic patients.

concentration, confirming the role of the increased plasma concentration of sFlt-1 as the origin of renal dysfunction in pre-eclampsia.

VEGF secretion in pre-eclamptic glomeruli

Immunofluorescence performed in the autoptic renal tissue of one woman with uncomplicated pregnancy shows that podocytes are clearly detectable as demonstrated by the staining for the podocyte marker podocin, while VEGF secretion by podocytes can be barely appreciated (Figure 5, upper panel).

In the case of pre-eclampsia, the colocalization of podocin and VEGF stainings is clearly detectable in line with the hypothesis that the cytokine is actively secreted by podocytes (Figure 5, lower panel).

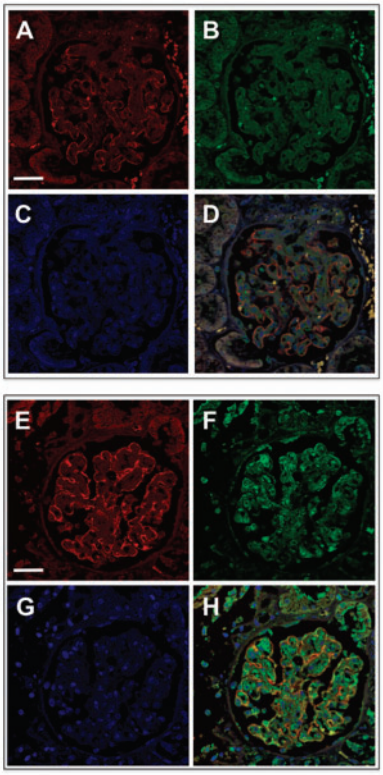

FIGURE 5: Podocin and VEGF expression (immunofluorescence) in sections of autoptic kidneys of a woman with uncomplicated pregnancy (upper panel) and a woman with pre-eclampsia (lower panel). Upper panel (uncomplicated pregnancy). (**A**) Podocytes are detectable as podocin-expressing cells inside a renal glomerulus. (**B**) Weak, if any, VEGF expression inside the same glomerulus. (**C**) Cell nuclei are counterstained with DAPI. (**D**) The merged figure shows substantially no VEGF expression by podocytes. Lower panel (pre-eclampsia). (**A**) Podocytes are detectable as podocin-expressing cells inside a renal glomerulus. (**B**) VEGF expression is detectable inside the same glomerulus. (**C**) Cell nuclei are counterstained with DAPI. (**D**) The merged figure shows a clear VEGF expression by podocytes. Scale bar, 20 μm.

DISCUSSION

The results of this study show that, despite the evidence that the amount of circulating sFlt-1 in pre-eclampsia exceeds the one detectable in uncomplicated pregnancy, plasma of VEGF is similarly (and significantly) reduced in both cases when compared with non-pregnant controls. The substantial absence of plasma VEGF is balanced, in case of uncomplicated pregnancy, by the parallel increase of PlGF. The success of the compensation is confirmed not only by the evidence, in line with the literature [8], that the sFlt-1/PlGF ratio does not differ between non-pregnant controls and uncomplicated pregnancy (Figure 1D), but also by the novel finding that, in these same two groups of individuals, the plasma concentrations of the hypoxia marker CAIX [15] are similar (Figure 2C).

The problem, however, is far from being solved in the case of pre-eclampsia where the lack of escape from VEGF inhibition has a major systemic effect as shown by the sFlt-1/PlGF ratio (Figure 1D) and plasma concentrations of CAIX (Figure 2C), which are both significantly increased when compared with uncomplicated pregnancy and non-pregnant controls.

Of interest, both VEGF [17] and CAIX [18] are downstream targets of hypoxia-inducible factor-1, a key regulator of cellular metabolism and response to hypoxic conditioning [19] that has been associated to the pathogenesis of pre-eclampsia [20]. The apparent discrepancy represented by the simultaneous reduction of VEGF and increase of CAIX in the plasma of pre-eclamptic patients is actually explained by the evidence that VEGF, just like CAIX, is reasonably overproduced in the case of pre-eclampsia but its final plasma concentration is blunted due to the sequestration implemented by the excess of sFlt-1.

At renal level, the major impact of the systemic inhibition of VEGF and consequent increased hypoxia is reasonably localized in the glomerulus [21–23].

A reasonable question at this point would be: what concentration of VEGF would be expected in the urine of a typical pre-eclamptic woman, taking into account the molecular weight of VEGF, the fact that the syndrome leads to a glomerular proteinuria, and the filtered load of VEGF?

To answer the question we have first to take into account that the molecular weight threshold for glomerular filtration is estimated to be ∼50 kDa [24, 25]. A protein such as albumin (∼50 kDa) is typically not filtered in normal conditions [26]. VEGF, with a calculated molecular weight (as a dimeric protein) of 45 kDa [27], should be able to cross the normal barrier. In our non-pregnant controls, a plasmatic concentration of VEGF equal to 28.0 ± 35.8 pg/mL(Figure 1B) corresponds to a urinary concentration of 82.0 ± 23.3 pg/mL(i.e. after the correction for urinary creatinine gives rise to a VEGF/creatinine ratio of 68.6 ± 36.3 pg/mgas shown in Figure 2D). Pre-eclampsia is characterized by unselective proteinuria, for this reason, there should be no obstacle for plasma VEGF to be excreted with urine. As shown in Figure 1B, in case of pre-eclampsia, the plasma concentration of free VEGF (as measured by ELISA) is, however, close to zero (0.5 ± 0.8 pg/mL). As a consequence also the filtered load of free VEGF (equal to the glomerular filtration rate times the VEGF's plasma concentration: 125 mL/min × 0) should be substantially negligible.

The well-being and fenestration of the glomerular endothelium depend on an 'excess' of VEGF coming from a double source: the plasma on one side of the glomerular basement membrane, and the podocytes on the other [28]. Podocytes in particular were shown to be able to synthesize and 'send back' VEGF to the glomerular endothelium in a countercurrent way with respect to the flux of the glomerular filtrate [29].

In the absence of VEGF coming from the plasma, as in case of pre-eclampsia, the podocyte is realistically asked to overproduce the cytokine but, the slit diaphragm being damaged, as demonstrated by the presence of albuminuria (Figure 2A), locally synthesized VEGF is lost in large amounts with urine and this may well explain the increased amount of urinary excretion of VEGF found in pre-eclamptic patients (Figure 2D).

The evidence that pre-eclampsia is characterized by a significant reduction of plasma VEGF paralleled by a paradoxical increase of urinary excretion of the same cytokine strongly

supports the hypothesis that, the PlGF-mediated bypass of VEGF inhibition being blocked in this disease, a 'second-intention' VEGF-mediated escape from VEGF inhibition is implemented at the glomerular (podocyte) level.

Taking into account that PlGF, because of its small dimension, is freely filtered with urine even in case of normal renal function (and in effect plasma and urinary concentrations of PlGF are strongly correlated as shown in Figure 3B), we were able to confirm the above-described hypothesis by comparing VEGF and PlGF urinary excretion in the entire population studied (Figure 3C). In this way, it is possible to separate women with uncomplicated pregnancies that escaped the VEGF inhibition through an increased systemic synthesis of PlGF (open dots) from pre-eclamptic women that escaped the VEGF inhibition through an increased renal synthesis of VEGF (triangles).

Of interest, the urinary excretion of VEGF in pre-eclampsia correlates with the excretion of podocytes (podocyturia), raising the hypothesis that the mechanisms leading to the glomerular overproduction of VEGF (hypoxia) or the overproduced VEGF itself could be involved in podocyte detachment.

The finding that VEGF expression by the podocytes is barely detectable in uncomplicated pregnancy (Figure 5, upper panel), while it can be easily appreciated in case of pre-eclampsia (Figure 5, lower panel), supports in some way the above-described hypothesis.

The pathogenesis of podocyturia (urinary excretion of viable podocytes) in pre-eclampsia (and in other diseases) remains unclear. Hypoxia by itself could be the reason for detachment, but in this case, we would expect that few, if any, living podocytes would end up being excreted with urine. This would be in contrast to the finding, which we share with other research groups, that urinary podocytes are alive, show strong signs of dedifferentiation and, when put in culture, actively proliferate *in vitro* [28, 30].

An alternative and intriguing hypothesis is based on the concept that the increased urinary excretion of VEGF found in pre-eclampsia could be directly involved in the detachment of podocytes from the glomerular basement membrane. The excess urinary VEGF (reasonably produced by the podocytes) could actually act in an autocrine way on the podocyte itself through the VEGFR2 receptor, thus causing dedifferentiation and, as a consequence, detachment. This hypothesis, although in line with other studies suggesting the existence of an 'autocrine VEGF system in podocytes' [30, 31] remains, however, to be verified, mostly because at the moment it is still a matter of discussion whether the podocyte actually expresses [32] or not [33] the VEGFR2 receptor.

In conclusion, the results of our study suggest that the impossibility in the case of pre-eclampsia to bypass, at the systemic level, the sFlt-1-induced VEGF inhibition ends up by prompting the development of an alternative, glomerular-centred, escape from the inhibition that could be directly responsible for the development of the renal dysfunction associated to the development of pre-eclampsia. Whether a similar pathogenic mechanism could be shared with other diseases characterized by the presence of podocyturia remains to be clarified.

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AUTHORS' CONTRIBUTIONS

L.V., G.Z. and A.G. contributed to the design of the study; L.V., M.S., A.G. and A.F. conducted the clinical study; D.G., S.M., S.G. and I.V. carried out the experiments; G.Z., L.V., R.B. and G.D.A contributed to the analysis and interpretation of the data. H.J.B. contributed to the analysis of results and provided the renal sections of uncomplicated and pre-eclamptic individuals. G.Z. wrote the draft of the article and had full access to the data of the study.

CONFLICT OF INTEREST STATEMENT

The authors declare to have no conflict of interest. The results presented in this article have not been published previously in whole or part, except in abstract format.

DATA AVAILABILITY STATEMENT

The data underlying this article will be shared on reasonable request to the corresponding author.

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