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Keywords:	Procalcitonin, Placenta, Pre-eclampsia, Adalimumab, CGRP

PRE-ECLAMPSIA AFFECTS PROCALCITONIN PRODUCTION IN PLACENTAL TISSUE

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Running Head: Circulating factors up-regulate PCT in PE

Abbreviations: PCT, procalcitonin; CGRP, calcitonin gene-related peptides; PE, Pre-eclamptic; DEC, decidual endothelial cell; DSC, decidual stromal cell; EVT extravillous trophoblast.

Abstract

Problem

Procalcitonin (PCT) is the prohormone of calcitonin which is usually released from neuroendocrine cells of the thyroid gland (parafollicular) and the lungs (K cells). PCT is synthesized by almost all cell types and tissues, including monocytes and parenchymal tissue, upon LPS stimulation. To date there is no evidence for PCT expression in the placenta both in physiological and pathological conditions.

Method

Circulating and placental PCT levels were analysed in PE and control patients. Placental cells and macrophages (PBDM), stimulated with PE sera, were analysed for PCT expression. The effect of anti-TNF-α antibody was analysed.

Results

Higher PCT levels were detected in PE sera and in PE placentae compared to healthy women. PE trophoblasts showed increased PCT expression compared to those isolated from healthy placentae. PE sera induced an up-regulation of PCT production in macrophages and placental cells. The treatment of PBDM with PE sera in the presence of anti-TNF-α, completely abrogated the effect induced by pathologic sera.

Conclusions

Trophoblast cells are the main producer of PCT in PE placentae. TNF-α, in association with other circulating factors present in PE sera, up-regulates PCT production in macrophages and normal placental cells, thus contributing to the observed increased in circulating PCT in PE sera.

Key words: Adalimumab, CGRP, Placenta, Pre-eclampsia, Procalcitonin.

1. Introduction

Pre-eclampsia represents a multisystem pregnancy complication characterized by hypertension and proteinuria that occurs after 20 weeks of gestation in previously normotensive women [1]. This pathological condition accounts for up to 5% of all pregnancies worldwide and seems to be more frequent in woman's first gestation [2]. Despite an intensive research carried out in the field in the last decades, the pathogenic mechanisms leading to the onset of pre-eclampsia still remain elusive. Pre-eclampsia develops in two stages. The first, preclinical stage, is characterized by poor development of the early placenta and the maternal blood supply. The second stage is characterized by failure of spiral artery remodeling and hypoperfusion leading to placental hypoxia, oxidative stress, and subsequent release into the maternal circulation of several placental factors. The release of these factors is then responsible for endothelial dysfunction and for the excessive systemic inflammation that causes the clinical manifestations of the disease [3-6]. Women suffering of this condition exhibit exaggerated production of circulating, as wells as local, pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-8 [7,8]. Moreover there is increasing evidence that peptides of the calcitonin family are involved in the pathogenesis of pre-eclampsia [9]. Low amount of calcitonin gene-related peptide (CGRP) in both maternal and fetal circulation was shown to negatively influence the reactivity of the placental vascular system in pre-eclamptic (PE) patients. In fact, CGRP-dependent vascular relaxation appears to be compromised in PE pregnancies [10].

Adrenomedullin (AM) is another major player of the calcitonin family, whose role is fundamental for a successful pregnancy and its deregulation leads to the development of PE related features [11]. Plasma levels of this peptide are elevated during normal pregnancy, but are drastically blunted in severe pre-eclampsia [12]. AM promotes fetal vessel branching in the labyrinth, preserves the remodelling and the formation of the endothelial layer of maternal uterine spiral arteries and markedly reduces the number of maternal uterine NK cells present in the placenta [13]. Besides CGRP and AM, procalcitonin (PCT) is another key component of the calcitonin family members. PCT is the prohormone of calcitonin released into the circulation mainly by parafollicular cells of the

thyroid gland, and, to a lesser extent, by the bronchial Kulchitsky (K) cells, under physiological conditions [14,15]. We recently demonstrated, together with other groups, that PCT can also be produced by other cell types like monocytes [16], macrophages [17] and glial cells [18]. The fact that a wide variety of different tissues contribute to PCT production seems to suggest that this protein is involved in regulating various homeostatic processes throughout the body.

High levels of PCT in the bloodstream are associated with bacterial infections [19,20] and several studies are still ongoing in clinic to ascertain the reliability of PCT as diagnostic and prognostic marker of bacterial sepsis [21,22]. PCT is a suitable protein for routine laboratory analysis as displays both high stability in serum and responds better to inflammatory stimuli than other laboratory parameters, such as C-reactive protein and TNF-α [23]. The fact that PCT is genetically linked with pregnancy disorders, like pre-eclampsia, has recently emerged [24].

It is now widely accepted that PCT levels are higher in PE than in healthy pregnant women sera [25-28], further emphasizing the existence of a generalized inflammatory status in these patients and therefore routinely assessed in clinical practice together with the other markers of the disease.

Our study aimed at better understanding whether PCT can represent a reliable diagnostic marker in PE patients and assessed whether it could also be considered a valuable predictive marker of this condition. Furthermore, we sought to characterize the placental tissue cell types producing PCT during the first trimester and at the end of gestation in normal and PE pregnancies. Immunofluorescence and quantitative RT-PCR (RT-qPCR) analysis were applied to investigate the contribution of endothelial cells (DEC), stromal cells (DSC) and trophoblast cells (EVT), together with macrophages, for their PCT expression under normal and pathological settings. Then, we further explored the possibility to interfere with pathways sustaining inflammation in pre-eclampsia. We also identified Adalimumab (*Humira*), a commercially available TNF-α-inhibitory monoclonal antibody, as an effective drug able to dampen PCT levels in the placental milieu, making it a promising therapeutic candidate for controlling the disease.

2. Material and Methods

2.1. Reagents and antibodies

The human cytokines TNF-α was purchased from PeproTech EC Ltd. (DBA Italia S.r.l, Milan, Italy). Polyclonal rabbit anti-human PCT and monoclonal mouse anti-Cytokeratin 8+18 (clone 5D3) antibodies were purchased from Abcam (ProdottiGianni, Milan, Italy). Monoclonal mouse anti-human CD14, CD45, CD10, CD13, CD31, CD105 -FITC conjugated and control mouse IgG1-PE and -FITC conjugated were from Immunotools (Germany). CD68 (Macrosialin, monoclonal mouse anti-human antibody, clone EMB11), monoclonal mouse anti-human von Willebrand Factor (vWF, clone F8/86) and monoclonal mouse OV-TL 12/30 anti-cytokeratin 7 were purchased from Dako (Milan, Italy). Mouse monoclonal anti-human Vimentin (clone V9) was obtained from Sigma-Aldrich (Milan, Italy). Secondary goat anti-mouse Cy3-conjugated and anti-rabbit IgG FITC-conjugated were purchased from Jackson ImmunoResearch (LiStarFish, Milan, Italy). Adalimumab (Humira®) was purchased from AbbVie (Campoverde di Aprilia, Italy).

A pool of fresh sera from healthy blood donors kindly provided by the local blood bank (Immunotransfusional Department, Maggiore Hospital, Trieste, Italy) was used as normal human serum (NHS). The sera for the cell culture were heat inactivated at 56°C for 30 minutes before use. An informed consent was obtained from all patients participating in the study.

2.2. Subjects

PE patients and healthy women were recruited in the Department of Obstetrics & Gynaecology of IRCCS "Burlo Garofolo", (Trieste, Italy) and the Nuffield Department of Obstetrics & Gynaecology (NDOG), Oxford University (UK) as described by Agostinis et al [29].

Serum samples were collected from women with pre-eclampsia (n = 30) enrolled in Nuffield Department of Obstetrics & Gynaecology (NDOG), who were matched for age, parity, and gestational age to 30 normal pregnant women (Table I). Pre-eclampsia was defined as the new onset of a systolic blood pressure (BP) ≥140 mmHg or diastolic BP ≥90 mmHg on at least two occasions within 24 hours and new onset proteinuria ≥300 mg in a 24-hour urine collection, 50 mg/mmol

protein/creatinine ratio or at least 2+ on dipstick testing on two consecutive measurements. For measurement of blood pressure, the woman was rested and reclining at an angle of 45° and a digital device was used. All patients and healthy controls had singleton pregnancies with undetected fetal abnormality. Blood samples were collected, serum was isolated and samples stored at -80°C until analysis. These studies were approved by the Oxfordshire Research Ethics Committee, adhere to the principles of the Declaration of Helsinki, and written consent was obtained from each participant.

PE patients and control pregnant women were recruited in the Trieste's Institute for Maternal and Child Health IRCCS "Burlo Garofolo" following the same criteria described above. Serum samples of 13 women with pre-eclampsia were matched for age, parity, and gestational age to 13 healthy pregnant women (Table II).

The cohort of pregnant women for prospective studies was followed by the Prenatal Diagnosis and Gynaecologic Unit of the Institute for Maternal and Child Health, IRCCS "Burlo Garofolo" in Trieste, Italy. All women were recruited from October 2007 to April 2009 as described by Di Lorenzo and colleagues [30]. Patients were matched for maternal age (32±4) and parity (nulliparity 19/25). Patients were followed from first trimester ultrasound aneuploidy screening to delivery. The study was approved by the Research Ethics Committee of the Institute Burlo Garofolo and adhere to the principles of the Declaration of Helsinki. We enrolled singleton pregnancies between 11 and 13 weeks of gestation. All pregnancies were dated by last menstrual period if consistent with crown-rump length (CRL) measurements (±7 days). We selected from this serum bank all women (n = 25) who subsequently developed pre-eclampsia and 25 matched control, following the criteria for inclusion described above.

Since PCT is related to systemic infection, the diagnosed infectious diseases or conditions were excluded in our patient collective.

2.3. Human tissues.

Placenta and decidual biopsy specimens have been obtained from healthy women undergoing elective termination of pregnancy at 8-12 weeks' gestation or from PE and control patients

undergoing caesarean delivery. The study was approved by the institutional review board of The Maternal-Children's Hospital (RC 41/08, IRCCS "Burlo Garofolo", Trieste, Italy) and informed consent was obtained from all patients providing the tissue specimens.

2.4. Isolation and culture of human endothelial, stromal and trophoblast cells from placental tissues

Endothelial and stromal cells from decidual tissues were purified as previously described with some modifications[31]. Briefly, decidual tissues were digested overnight at 4°C with 0.25% trypsin (Sigma-Aldrich), 50 µg/mL DNase1 (Roche, Milan, Italy) in PBS and then treated with collagenase type I (3 mg/mL; Worthington Biochemical, DBA, Milano, Italy) for 30 minutes at 37°C. Following Ficoll-Paque Plus density gradient centrifugation (GE Healthcare, Euroclone, Milan, Italy), decidual human endothelial cells (DECs) were isolated by positive selection using Dynabeads M-450 (Dynal, Oslo, Norway) coated with lectin Ulex europaeus 1 (Sigma-Aldrich). DECs were cultured in flask pre-coated with 5µg/cm^2 fibronectin (Roche) using endothelial serum-free basal medium (GIBCO, LifeTechnologies, Milan, Italy) supplemented with 20 ng/mL bFGF, 10 ng/mL EGF, and penicillin (50 U/mL)/streptomycin (50 µg/mL).

Decidual stromal cells (DSC) were obtained by culturing the endothelial-negative cell fraction in RPMI plus 10% fetal calf serum (FCS) without adding growth factors. Non-adherent cells were removed by extensive washing, and adherent cells were used only when the resulting cell population was negative for CD14, CD45, CK8+18, vWF, or CD31. The purity of DEC and DSC isolated from first trimester decidua was routinely assessed by cytofluorimetric and qPCR analysis, as previously reported [31].

Extra Villous Trophoblasts (EVT) were purified from placental tissue incubated with Hanks' Balanced Salt Solution (HBSS) containing 0.25% trypsin and 0.2 mg/ml DNase I (Roche) for 20 min at 37°C, following a published procedure [32]. The cells were seeded on 25-cm^2 flask pre-coated with 5µg/cm^2 fibronectin (Roche) in RPMI (GIBCO, Lifetechnologies) supplemented with 10% foetal calf serum (FCS) to remove non-adherent leucocytes and syncitiotrophoblasts, and used

within 12 h. The purity of EVT isolated from first trimester placenta was routinely assessed by IF and qPCR analysis, as already previously reported [33].

2.5. Immunohistochemical analysis

Tissue samples were fixed in 10% buffered formalin and paraffin embedded. Four-micrometers-thick tissue sections were deparaffinized and rehydrated. The antigen unmasking technique was performed using Novocastra Epitope Retrieval Solutions pH6, pH 9, and pH 8 in a PT Link Dako pre-treatment module at 98°C for 10 minutes. The sections were then brought to room temperature and washed in PBS. After neutralization of the endogenous peroxidase with 3% H_2O_2 and Fc-blocking by a specific protein block (Novocastra UK), the samples were incubated overnight at 4°C with the primary antibodies. IgG from normal rabbit sera were used as negative control. Staining was revealed by the Horseradish Peroxidase (HRP) polymer detection kit (Novocastra, Code RE7280-K). The sections were counterstained with Harris hematoxylin (Novocastra) and analysed under a Leica DMD108 optical digital microscope (Leica Microsystems, Germany).

2.6. Immunofluorescence analysis

Seven-micrometer-thick tissue sections of snap-frozen placentae or deciduas, embedded in OCT medium (Bio-Optica, Milan, Italy), were fixed in acetone and stained for immunofluorescence analysis with rabbit anti-human PCT, overnight at 4°C, followed by incubation with anti-rabbit IgG-FITC (1:300) 1h at RT. Then the same sections were stained with mouse anti anti-vWF or anti-CK8-18 or anti-CD68 (all used 1:50), followed by anti mouse IgG-Cy3 conjugated (1:300), for 1h at RT. The nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) and the slides were mounted with the Mowiol based antifading medium (Sigma-Aldrich). Images were acquired with fluorescence microscope Leica DM2000 (Leica Microsystems, Germany) equipped with Leica DFC420 camera.

2.7. Characterization of DEC and EVT from PE patients

DECs, cultured at confluence in an 8-chamber slide (BD Falcon) or cyto-centrifuged EVT from PE specimens were fixed with FIX&PERM kit (Invitrogen, Life Technologies) for 15 min at room

temperature (RT). Incubation with primary antibodies (used antibodies are listed above) was performed for 1 hour at RT. Cells were then washed and incubated with secondary antibodies (1:300) for 45 min at RT. The nuclear were stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). The glass was mounted with a fluorescence-preserving mounting solution (Dako). Images were acquired by the fluorescence microscope Leica DM 3000 (Leica) using the Leica DFC320 (Leica) camera.

2.8. Flow cytometry

Stromal cells ($5x10^5$) isolated from PE deciduas were fixed with the fixation reagent FIX&PERM kit for 15 min at RT in the darkness and incubated with primary antibodies (used antibodies are listed above) for 1 h at 37°C in a thermomixer (Eppendorf) at 800 rpm. Antibodies directed against intracellular antigens were diluted in permeabilization reagent of the FIX&PERM kit, while antibodies for cell surface antigens were diluted in dPBS-BSA1%. Incubation with secondary antibodies anti-mouse-FITC F(ab)' (1:50) or anti-rabbit-FITC (1:50) was performed for 30 min on ice.

Cells were resuspended in 1% paraformaldehyde, the fluorescence was acquired with the FACScalibur (BD Bioscience) and data processed by the software CellQuest.

2.9. Isolation and differentiation of Human Peripheral Monocytes into macrophages and their in vitro polarization.

Peripheral blood mononuclear cells (PBMCs) from healthy blood donors were isolated from anonymous buffy coats, kindly provided by the local blood bank (Immunotrasfusional Department, Maggiore Hospital, Trieste, Italy) using Ficoll-Paque Plus density gradient (GE Healthcare, Euroclone). Residual T and B cells were removed from the monocyte fraction by plastic adherence, after incubation for 2 hours at 37°C and 5% CO2 in RPMI-1640 GlutaMAX (Life Technologies) supplemented with 10% NHS and 1% Penicillin/Streptomycin (Sigma-Aldrich). Fully differentiated macrophages were obtained by culturing 10^6 monocytes/ml for 7 days at 37°C and 5% CO_2 with the same medium above described and replaced twice a week.

2.10. Stimulation of the cells with PE or matched control sera.

The cells (PBDM, EVT, DEC and DSC) were incubated for 24 hours at 37°C and 5% CO_2 in medium supplemented with 10% of sera obtained from 5 controls or 5 PE women (Table III), or with a pool of PE serum added with anti-TNF-α (Adalimumab) 50ng/ml or with TNF-α 25ng/ml. After incubation the cells were lysated and total RNA was extracted. We repeated the experiment with at least two different cell populations, for each cell type.

2.11. RNA isolation, cDNA synthesis and Quantitative Real-Time PCR (qPCR)

RNA was purified from cells with EuroGOLDtrifast (Euroclone) according to the manufacturer's instructions. Total RNA was extracted and reverse transcribed as previously described[32]. Quantitative Real-Time PCR (qPCR) was carried out on a Rotor-Gene 6000 (Corbett, Qiangen, Ancona, Italy) using iQ SYBR Green Supermix (Applied Biosystems, Milan, Italy). Table IV shows the primer list used for qPCR. The melting curve was recorded between 55°C and 99°C with a hold every 2s. The relative amount of gene production in each sample was determined by the Comparative Quantification (CQ) method supplied as part of the Rotor Gene 1.7 software (Corbett Research) [34]. The relative amount of each gene was normalized with 18S and expressed as arbitrary units (AU) considering 1 AU obtained from fully differentiated macrophage used as calibrator.

2.12. Quantification of PCT and CGRP amount on the sera

The amount of PCT in PE or matched control sera was quantified automatically by the Modular® Analytics E170 Module (Roche Diagnostics, Milan, Italy) using BRAHMS PCT reagent (Roche, Mannheim, Germany). CGRP in PE or matched control sera was measured with an ELISA kit (Abnova, Prodotti Gianni, Milan, Italy), following the manufacturer´s instructions.

2.13. Statistical Analysis

Statistical analysis was performed with Microsoft Office Excel 2003 (Microsoft Corporation, Redmond, CA, USA). Data were reported as mean ± S.E.M. Mann-Whitney test was used to compare two groups of data and p value of < 0.05 was considered significant.

3. Results

3.1. Analysis of PCT and CGRP in PE and control sera.

PCT expression levels were analysed in early onset PE patients and in healthy women with normal, ongoing pregnancies (CTRL) matched for age, parity, and gestational stage. These patients were enrolled in the Department of Obstetrics & Gynaecology of IRCCS Institute for Maternal and Child Health "Burlo Garofolo" (Trieste, Italy; Figure 1A). Since both PCT and CGRP originate from calcitonin/calcitonin gene-related peptide gene (CALC-I gene) upon alternative splicing [20], both genes were included into the analysis. Interestingly, a significant increase of the PCT levels, associated with a reduction of the CGRP expression (Fig. 1A and B), was detected in PE patients as compared to control pregnant women in the third trimester of gestation (Term). These data were validated by similar analysis performed on plasma from patients enrolled in the Nuffield Department of Obstetrics & Gynaecology (NDOG), Oxford University, UK (Supplemental Figure 1).

We then quantified PCT and CGRP in sera of pregnant women collected between 11° and 13° gestational week who subsequently developed pre-eclampsia (Fig. 1B and 1D). These serum samples were obtained from a bank of sera collected for prospective studies from pregnant women followed by the Prenatal Diagnosis and Gynaecologic Unit of the Institute for Maternal and Child Health e IRCCS "Burlo Garofolo" in Trieste.

No significant differences in the expression level of PCT were detected between PE patients and control pregnant women during the first trimester of gestation, further supporting the notion that PCT cannot be considered a predictive risk markers for the development of pre-eclampsia (Figure 1C and D)[35].

3.2. Localization of PCT in first trimester placenta.

To understand whether placenta represents a source of PCT and therefore contributes to its release into the maternal circulation, we performed immune-histochemical analysis of PCT distribution in human placental tissues derived from the first trimester of gestation. We observed that PCT is

abundantly and widely expressed in the placental tissue, mostly intracellular, and mainly associated with the decidual glands (Fig. 2C) and cells localized in the decidual stroma (A, B). No staining was present on the surface of endothelial cells (Fig. 2A, red arrows) and on the syncytiotrophoblast layer (Fig. 2D), nor on non-pregnant uterus (Fig. 2E), being all comparable with deciduas incubated only with secondary antibodies (Fig. 2F).

To further investigate if trophoblast cells were responsible for PCT production in the decidual stroma we performed double immunofluorescence experiments on healthy first trimester gestation human placenta sections using antibodies recognizing PCT protein and cytokeratin (CK) 8+18, a specific markers for trophoblast cells. As shown in Figure 3A PCT strongly co-localizes with CK8+18 marker in interstitial decidua indicating that trophoblast cells are involved in PCT synthesis. This observation was additionally confirmed by real-time quantitative PCR (RT-qPCR) on mRNA purified from normal placental tissue derived from the first and third trimester of pregnancy. Interestingly, the expression of this pro-hormone resulted significantly higher in the third trimester of gestation as compared to the first trimester (Fig. 3B), probably caused by a physiological increase of the inflammatory state during pregnancy[36]. PCT expression by qPCR was also performed on mRNA derived from decidual endothelial cells (DECs), decidual stromal cells (DSCs) and extravillous trophoblast cells (EVT) isolated from normal first trimester decidua (obtained from voluntary abortion). In line with the immune-histochemical data, all cell types investigated were able to express PCT pro-hormone even thought to a lesser extent as compared to peripheral blood derived macrophages (PDBM) used as calibrator (Fig. 3C).

3.3. Local expression of PCT in PE placental tissues.

Since we demonstrated that PCT levels are much higher in PE sera we wanted to test whether the placental expression of this pro-hormone reflects the circulating levels of the protein. To this end, total mRNA was isolated from placental tissues derived from caesarean delivery of PE and healthy women at the same gestational age and PCT expression was analysed by RT-qPCR. As showed in Figure 4A, we confirmed the higher expression of PCT in PE derived placental tissue.

We then isolated trophoblast, stromal and endothelial cells from PE and normal placentae (at the same gestational age), that were extensively characterized to confirm their purity once seeded on fibronectin (Supplemental figures 2, 3 and 4). In this setting PCT mRNA expression levels were normalized to those of the human housekeeping gene 18S. Results were expressed as AUs, in which 1AU represents the value obtained with macrophages cultured in NHS (positive control). qPCR analysis revealed that all cell types isolated from PE patients are characterized by a higher expression on this pro-hormone as compared to control placenta, even though this increment did not reach the statistical significance only for DSC (Fig. 4B, C and D). It is interesting to note that trophoblast cells (EVT) showed a 5-fold increase in PCT expression as compare to mRNA produced by the calibrator PBDM, thus indicating that they are the major producer of PCT in pre-eclamptic placenta.

3.4. Sera derived from PE women affect PCT expression by placental cells.

To explain the mechanisms that lead placental cells to the increased PCT production in pre-eclampsia, we stimulated DECs, DSCs and EVT, with PE or control sera (10%) for 24 hours and we analysed their PCT mRNA expression. The graphs reported in Figure 5 showed that the PE serum induced a significant increase in the PCT expression by EVT and to a lesser extent by DSC. Only DECs were unable to modulate their PCT mRNA expression.

3.5. Macrophages contribute to the placental production of PCT.

Macrophages are known producers of PCT [17]. We, therefore, investigated their contribution to placental production of this molecule. A double staining with antibodies against PCT and CD68 was performed on first trimester placental sections. As shown in Fig. 6A, PCT expressing cells were also positive for CD68 only in PE, but not in normal, placentae thus indicating that macrophages represent a source of this molecule under pathological conditions.

This observation prompted us to assess the effect of PE sera on the macrophage PCT production. To this aim we first incubated for 24 hours PBDM with a pool of PE or control matched women-sera and PCT gene expression was then analysed by RT-qPCR. These experiments were performed in

duplicate, analysing a pool of 5 PE sera, or 5 matched control, on three different macrophage populations. As shown in Fig. 6B treatment with PE sera significantly increased the PCT expression by PBDM.

Since it is well documented that the circulating levels of pro-inflammatory cytokines, like TNF-α, are higher in PE patients as compared to normal pregnancy [7], we tried to block the up-regulation of PCT production induced by PE sera in PBDM. To this aim PE sera were incubated in the presence of Adalimumab, a TNF-α-inhibitory monoclonal antibody widely used for the treatment of several autoimmune diseases. As showed in figure 6C, Adalimumab (anti-TNF-α) was able to completely abolish the expression of PCT induced by PE sera. Surprisingly, we failed to detect an increase of PCT expression by the sole incubation with TNF-α, suggesting that the stimulatory effect of TNF-α, although sufficient to induce IL-1β expression (Fig. 6D), is necessary but not sufficient to increase the expression of PCT.

4. Discussion

Despite decades of research, the mechanisms underlying the cause and progression of pre-eclampsia are still poorly understood. In order to develop effective pharmacological approaches to prevent the onset of this disorder, it is important to discover new molecular target involved in the pathogenesis of this syndrome [37]. In this study, we demonstrated that PCT can play a detrimental role in pre-eclampsia, and that an anti-TNF-α antibody-based immunotherapy could represent a successful strategy to restore the physiological immune-endocrine crosstalk in placenta.

We initially analysed the circulating levels of PCT in two cohorts of PE patients, one enrolled in Nuffield Department of Obstetrics & Gynaecology (NDOG), Oxford University (UK) and the other in the Department of Obstetrics & Gynaecology of Institute for Maternal and Child Health - IRCCS "Burlo Garofolo"- Trieste (Italy). The results obtained are in agreement with published data showing an increase in the PCT levels associated with a reduction in the CGRP levels in PE patients [25,26,38,39] compared to women with normal, ongoing pregnancies (CTRL), matched for age, parity, and gestational age. These observations assigned to maternal PCT an important role in the pathophysiology of pre-eclampsia making it an attractive candidate as a novel diagnostic marker of the syndrome. We did not observed a statistical difference in the amount of circulating PCT in first trimester sera obtained from normal pregnant women and women that subsequently developed pre-eclampsia, as published by Birdir and coworkers [35]. This would further indicate that PCT cannot be a predictive marker of this pregnancy disorder but a biomarker for women with suspected pre-eclampsia [28]. One interesting aspect we unveiled is the inverse correlation between the expression of PCT and CGRP, a peptide with potent vasodilatory effect on vascular tone [40] and sought to be involved in placental development and fetal growth [10]. One possible explanation for this phenotype rely on an unbalanced alternative splicing leading to a low production of this peptide and a concomitant increase of PCT expression [23].

It is widely proven that almost all peripheral tissues produce PCT upon bacterial infection [20], although human placenta was never investigated. Our data clearly showed that placenta is involved

in PCT synthesis. First, immunohistochemical analysis and double immunofluorescence confirmed the presence of this pro-hormone at the protein level, mainly associated with trophoblast cells and macrophages. Second, RT-qPCR showed an progressive increase in PCT mRNA expression from the first to the third trimester of gestation, probably determined by the physiological increase of the inflammatory state [36]. We observed that DECs, DSCs and EVTs can all synthesize PCT, even if to a lower extent as compared to macrophages. It is interesting to note that Hu and co-workers demonstrated an increase of circulating PCT during the first and third trimester of pregnancy[41]. It is tempting to speculate that such increase could be correlated to the placental increase of PCT production.

These observations prompted us to investigated whether the systemic inflammatory condition of PE patients was able to influence the local placental PCT expression. Based on RT-qPCR we detected a strong increase in PCT mRNA expression in PE compared to normal placenta. Even though trophoblast and endothelial cells clearly enhanced their PCT expression levels, they cannot be considered the unique cell types responsible for the observed phenotype. Moreover stromal cells, which represent the more abundant cell type present in the tissue, didn't show a significant increase in the PCT mRNA level. Also tissue macrophages are certainly involved in PCT production in PE decidua, as demonstrated by co-localization of CD68 immunoreactivity with PCT. Since macrophages are well-known producer of PCT, but it is technically challenging their isolation from the decidua, we decided to mimic the pathological placental microenvironment by incubating PBDM with PE sera. Indeed it has been demonstrated by Kalkunte and colleagues that the sole injection of pre-eclamptic sera in animal models [42] is sufficient to induce all symptoms of the syndrome in pregnant mice. This treatment was also performed on DEC, DSC and EVT obtained from healthy placentae.

While macrophages, trophoblasts and also stromal cells all up-regulated, even though to a different extent, their PCT mRNA levels, DEC where poorly responsive, showing an opposite behaviour in respect to what we observed in PE decidua. One possible explanation for this latest observation may

rely on the fact that DEC are in direct contact with blood circulating factors and therefore they may require higher concentration of stimuli to fully respond.

Since a strong difference was observed in macrophage PCT expression under PE sera stimulation and since they seemed to be the major producers of PCT in pre-eclamptic placentae at protein level, in an attempt to identify the molecules involved in enhancing PCT production we sought to test the putative role of TNF-α, the main pro-inflammatory cytokine whose levels have been shown to increase in PE patients [7]. In particular we took advantage of an anti-TNF-α antibody, the Adalimumab, to hamper the activity of TNF-α. Sera incubated with this antibody completely lost their ability to promote PCT up-regulation but, unexpectedly, TNF-α alone, used at the concentration found in PE sera, was completely unable to induce PCT production by human macrophages although able to increase the expression of IL-1β [7]. Similar findings were provided by Balog et coworkers [43] based on the observation that the anti-TNF-α antibody significantly decreased intracellular PCT production by leukocytes stimulated with *S. aureus*. These data altogether suggest that TNF-α is necessary for the up-regulation of PCT but probably it requires the cooperation with additional factors still to be identified (see graphical abstract). It is known that PE sera contains several others pro-inflammatory cytokines such as IL-8, IL-1β and interferon (IFN)-γ[44] or protein aggregates[45], that could be responsible for the modulation of the synthesis and production of PCT.

5. Conclusions

In conclusion we confirmed that PCT can be considered a diagnostic but not a predictive marker of pre-eclampsia. This pro-hormone is locally expressed in placenta in physiological conditions and its expression is higher in PE placentae compared to normal healthy control. We verified PCT expression at both mRNA and protein level in normal first trimester EVT and DEC cells and demonstrated that PCT is up-regulated upon stimulation with PE sera. Decidual macrophages were identified as another important source of PCT production in pathological pregnancies, as confirmed by *in vitro* stimulation assays using PE sera. Interestingly an anti-TNF-α monoclonal antibody, Adalimumab, used in treatment of several autoimmune diseases, was able to block the up-regulation

of PCT expression induced by PE sera on PBDM, making it a potential therapeutic tool for the treatment of pre-eclampsia.

Acknowledgments

The contribution of Leila Lo Bello, Maddalena Tonon, Berkan Vural, Dionne Tannetta, Lorenzo Monasta, is gratefully acknowledged. This work was supported by grants from the Institute for Maternal and Child Health, IRCCS "Burlo Garofolo", Trieste, Italy (RC41/08, RC 01/09, RC 34/11), and Fondazione Cassa di Risparmio, Trieste to Roberta Bulla.

Conflict of interest

All authors state explicitly that potential conflict of interests does not exist. All authors deny any financial relationship with biotechnology manufacturers, pharmaceutical companies, and other commercial entities in relation to this original research.

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Table I. Characteristics of the cohort from Nuffield Department of Obstetrics & Gynaecology (NDOG), Oxford University (UK), (n = 60). Data are expressed as mean values (+/- standard deviation), n.s. not significant.

Plama samples	**Normal Pregnant (n=30)**	**PE (n=30)**	***p***
Age (years)	29.5 (±5.5)	30.7 (±5.8)	n.s.
Nulliparity	22/30	22/30	n.s.
Gestation at sample (days)	238.2 (±35.6)	241.5 (±32.5)	n.s.
Booking systolic BP (mmHg)	109.5 (±13.0)	140.5 (±14.4)	p<0.05
Booking diastolic BP (mmHg)	65.9 (±9.9)	88.5 (±14.9)	p<0.05
BMI	23.9 (±0.9)	28.0 (±1.8)	p<0.05
Maximum 24 hr proteinuria (mg)	< 300	4672.7(± 4721)	p<0.05
data are expressed as mean ± SD			

Table II. Characteristics of the cohort from Department of Obstetrics & Gynaecology of IRCCS "Burlo Garofolo", (n = 26). Data are expressed as mean values (+/- standard deviation). n.s. not significant, n.d. not defined.

Serum samples	**Normal Pregnant (n=13)**	**PE (n=13)**	***p***
Age (years)	29.5 (±3.1)	36.8 (±2.2)	n.s.
Nulliparity	7/13	8/13	n.s.
Gestation at sample (days)	203 (±3.7)	231 (±1.9)	n.s.
Booking systolic BP (mmHg)	110	179 (±19.1)	p<0.05
Booking diastolic BP (mmHg)	70	104 (±9.4)	p<0.05
BMI	n.d.	26.2 (±6.4)	n.d.
Maximum 24 hr proteinuria (mg)	< 300	2588.1 (±1648.1)	p<0.05
data are expressed as mean ± SD			

Table III. Patient details for normal pregnant (n = 5) and PE women (n = 5) sera used for the cell stimulation. Data are expressed as mean values (+/- standard deviation). n.s. not significant.

	Normal Pregnant (n=5)	PE (n=5)	*p*
Age (years)	33 (±1.8)	36.8 (±4)	n.s.
Nulliparity	1/5	1/5	n.s.
Gestation at sample (days)	273 (±0.6)	243.5 (±0.9)	n.s.
Booking systolic BP (mmHg)	110 (±1.8)	152.7 (±7.8)	p<0.05
Booking diastolic BP (mmHg)	60 (±2.3)	93.6 (±14.1)	p<0.05
BMI	27.2 (±1.5)	23.4 (±3.3)	n.s.
Maximum 24 hr proteinuria (mg)	< 300 mg	1667 (±152)	p<0.05
data are expressed as mean ± SD			

Table IV. Primer used for qPCR analysis.

Gene	Primers	Sequence 5'>3'	Annealing Temperature (°C)	Amplicon Size (bp)	Gene Bank Accession Number
18S	For	ATCCCTGAAAAGTTCCAGCA	60	154	NM_022551
	Rev	CCCTCTTGGTGAGGTCAATG			
PCT	For	TCTAAGCGGTGCGGTAATCTG	60	85	NM_001741
	Rev	CAGTTTGGGGGAACGTGTGA			

Figure Legends:

Figure 1. Measurement of PCT (**A**) and CGRP (**B**) in PE patients (indicated with 'PE') and matched control pregnant women (CTRL) enrolled at Trieste's Hospital. PCT was significantly increased in PE women versus controls (*paired t test,* $p < 0.05$; *** $p < 0.005$*). Serum levels of PCT (**C**) and CGRP (**D**) measured at first trimester of pregnancy in women with a normal pregnancy (n = 25) and women who subsequently developed pre-eclampsia (n = 25). No statistical differences were observed between these groups of samples (*paired t test*).

The results are expressed as box plot graphs, in which the line in the middle of the box represents the median; the lower and the upper edges of the box are the 1st and 3rd quartile, respectively.

Figure 2. Immunohistochemical analysis of normal human placental tissue and uterus. PCT was analysed with rabbit polyclonal antibodies and revealed by the streptavidin-biotin-peroxidase complex method using DAB as chromogenic substrate (brown signal). Arrows indicate blood vessels. Scale bars 30μm.

Figure 3. (**A**) Double immunofluorescence analysis of frozen sections of normal first trimester placentae stained with rabbit anti-human PCT (in green fluorescence) and anti-CK8+18 (in red fluorescence) to investigate the PCT co-localization in trophoblast cells. The nuclei were stained with DAPI (Sigma-Aldrich) in blue. Images were acquired with fluorescence microscope Leica DM2000 (Leica Microsystems, Germany) equipped with Leica DFC420 camera.

(**B**) Analysis of mRNA expression of PCT gene in placental tissue derived from healthy placentae: 5 from first trimester and 5 from third trimester placentae. The PCT expression was also investigated in trophoblast (EVT), endothelial (DEC) and stromal (DSC) cells isolated from normal first trimester deciduas (**C**). Results were expressed as AUs, in which 1AU represents the value

obtained with macrophages cultured in NHS used as a positive control. Data represent the mean ± S.E.M. (* $p < 0.05$, Mann-Whitney test).

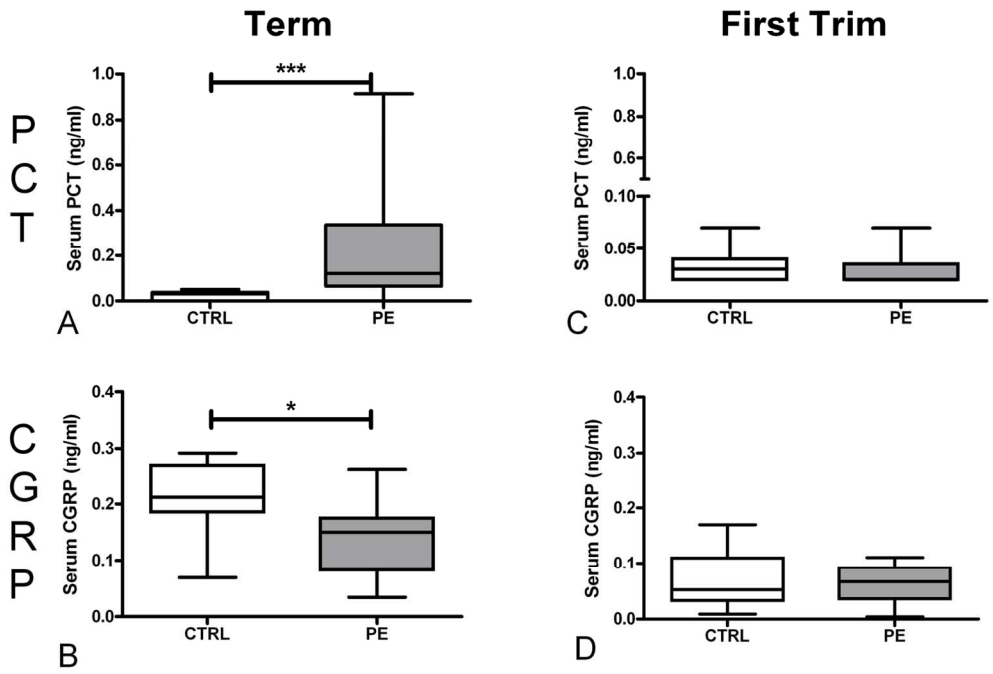
Figure 4. (A) Analysis of mRNA expression of PCT in placental tissue derived from 7 PE patients and 7 healthy matched control placentae. Results were expressed as AUs, in which 1AU represents the value obtained with a pool of healthy placenta cDNA as positive control. Barr represents the mean ± S.E.M. (* $p < 0.05$, Mann-Whitney test). **(B-D)** Analysis of mRNA expression of PCT gene in endothelial (DEC), stromal cells (DSC) and trophoblasts (EVT) isolated from term PE (PE) and control (CTRL) placental tissues. We analysed the cells isolated from 5 different patients and compared the results to cells isolated from 5 healthy placentae. Data represent the mean ± S.E.M. (* $p < 0.05$, Mann-Whitney test).

Figure 5. RNA prepared from EVT, DEC or DSC stimulated with 10% of PE or control sera for 24 hours, were quantified for PCT and 18S expression by qPCR. The sera obtained from PE patients is able to up-regulate the PCT expression compared to control sera. The experiments were performed in double analysing 5 different PE and 5 control sera on two populations of each cell type. Data represent the mean ± S.E.M. (* $p < 0.05$, ** $p < 0.01$, Mann-Whitney test).

Figure 6. (A) Double immunofluorescence analysis of frozen sections of normal first trimester or term PE placentae stained with rabbit anti-human PCT (in green) and anti-CD68 (in red) to investigate the PCT co-localization on tissue macrophages. The nuclei were stained with DAPI (Sigma-Aldrich) in blue. Images were acquired with fluorescence microscope Leica DM2000 (Leica Microsystems, Germany) equipped with Leica DFC420 camera. Original magnification 200X. (B) PBMC were stimulated with 10% of PE or control sera, total RNA of these cells was isolated and the expression of PCT was evaluated by qPCR. **(C)** PBMC were incubated for 24h with TNFα or 10% of PE sera, alone or in association with anti-TNFα. The total RNA of these cells

was isolated and the expression of PCT (**C**) and IL1-β (**D**) was evaluated by qPCR. Results were expressed as AUs, in which 1AU represents the value obtained with macrophages cultured in NHS used as a positive control. Data represent the mean ± S.E.M. (** $p < 0.01$, Mann-Whitney test).

Extravillous Trophoblast and Macrophages as a Potential Source of Circulating Procalcitonin in Pre-Eclampsia. Agostinis et al.
Figure 1.



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The results are expressed as box plot graphs, in which the line in the middle of the box represents the median; the lower and the upper edges of the box are the 1st and 3rd quartile, respectively.

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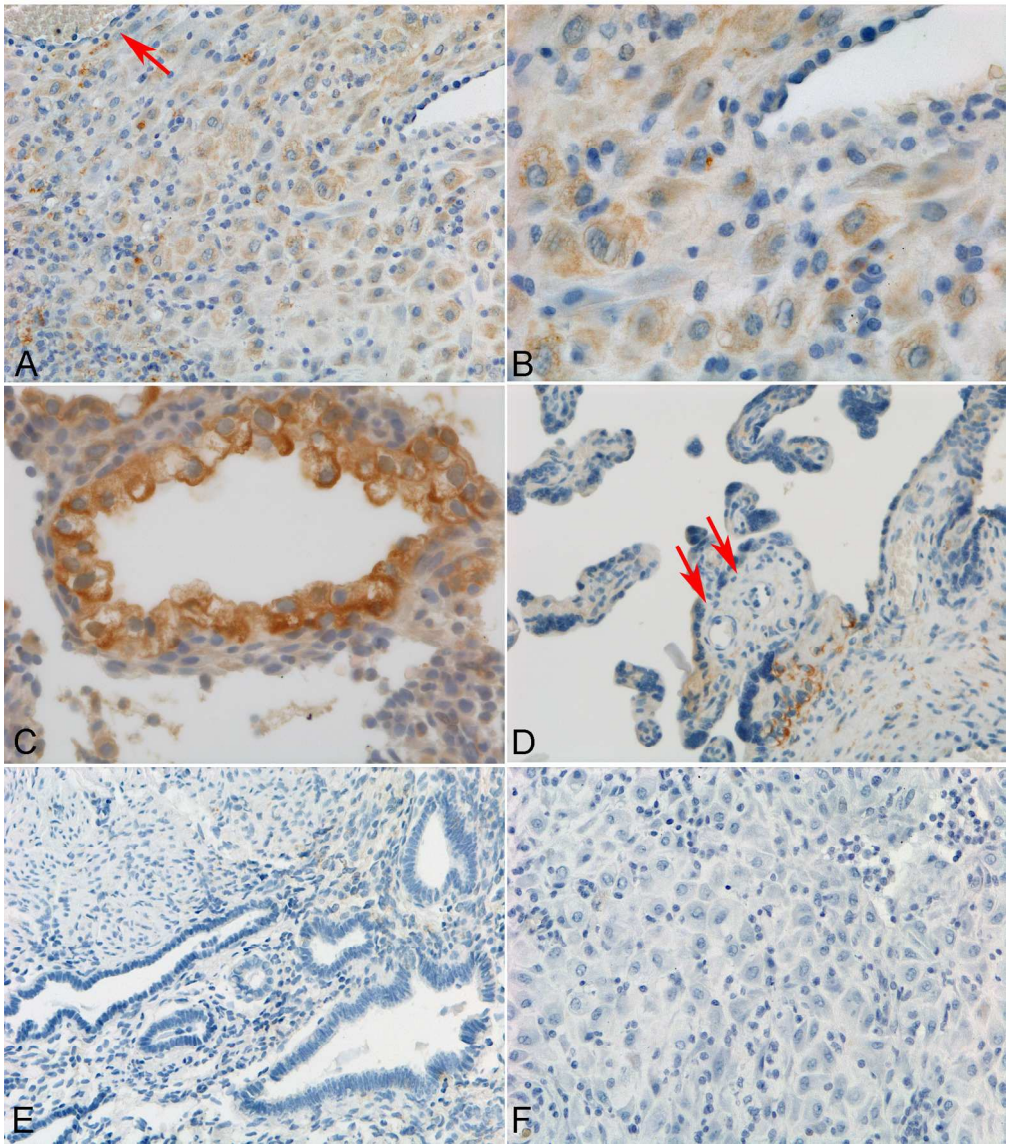
Extravillous Trophoblast and Macrophages as a Potential Source of Circulating Procalcitonin in Pre-Eclampsia. Agostinis et al.
Figure 2.



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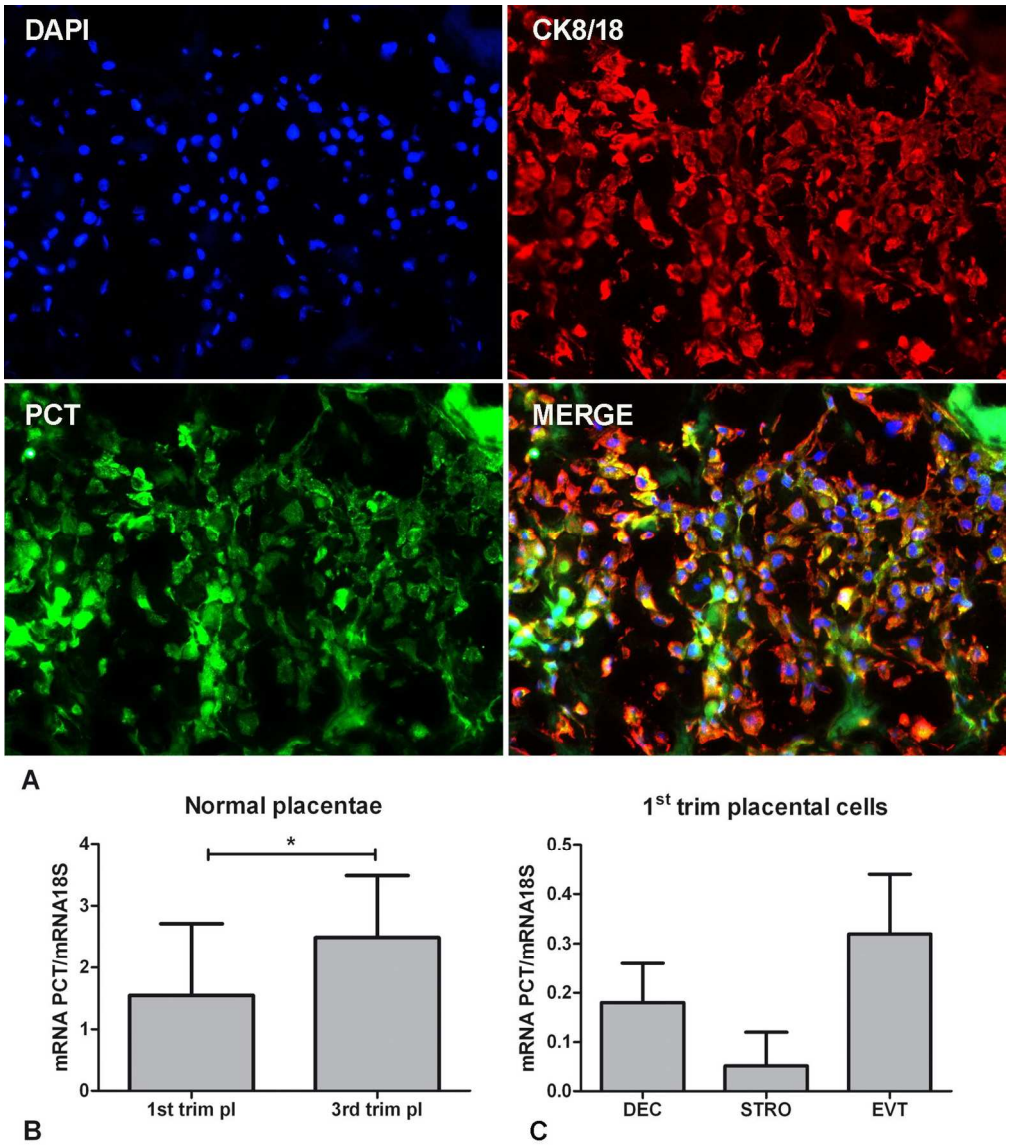

Figure 3. (A) Double immunofluorescence analysis of frozen sections of normal first trimester placentae stained with rabbit anti-human PCT (in green fluorescence) and anti- CK8/18 (in red fluorescence) to investigate the PCT co-localization in trophoblast cells. The nuclei were stained with DAPI (Sigma-Aldrich) in blue. Images were acquired with fluorescence microscope Leica DM2000 (Leica Microsystems, Germany) equipped with Leica DFC420 camera.
(B) Analysis of mRNA expression of PCT gene in placental tissue derived from healthy placentae: 5 from first trimester and 5 from third trimester placentae. The PCT expression was also investigated in trophoblast (EVT), endothelial (DEC) and stromal (DSC) cells isolated from normal first trimester deciduas (C). Results were expressed as AUs, in which 1AU represents the value obtained with macrophages cultured in NHS used as a positive control. Data represent the mean ± S.E.M. (* $p < 0.05$, Mann-Whitney test).

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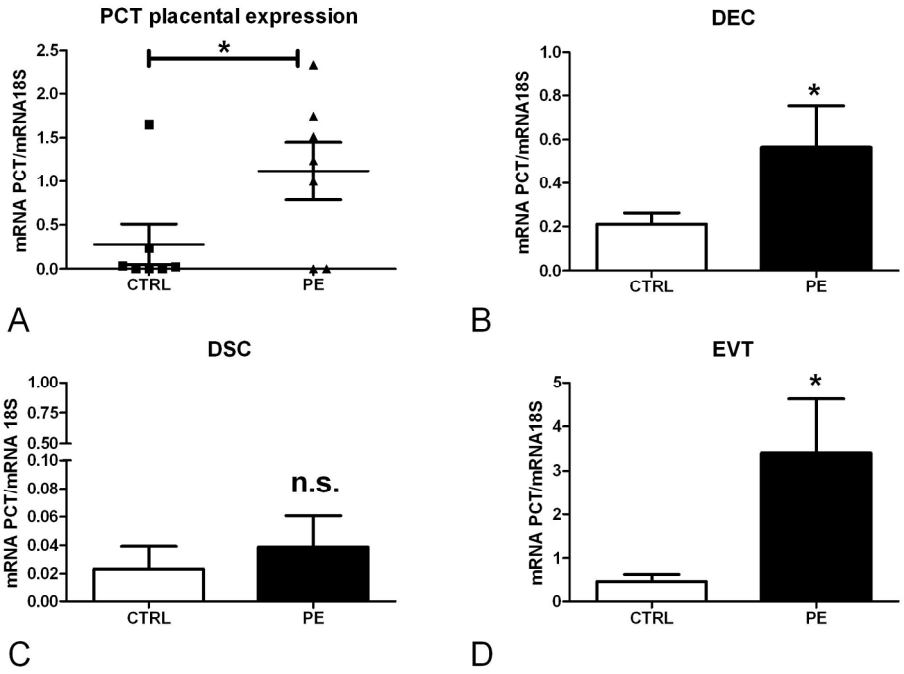

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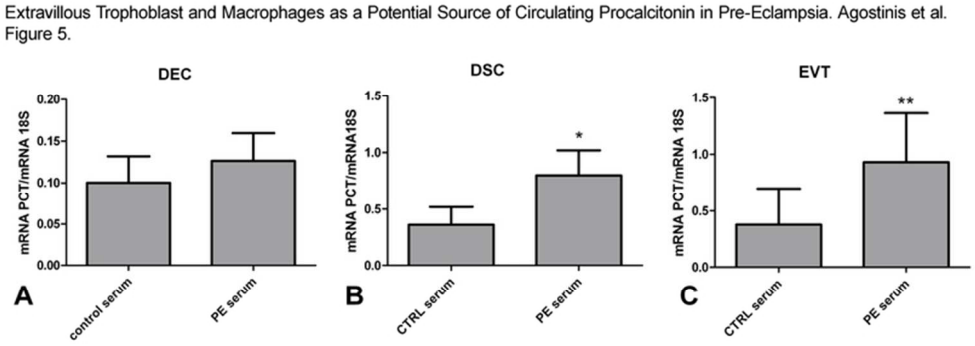

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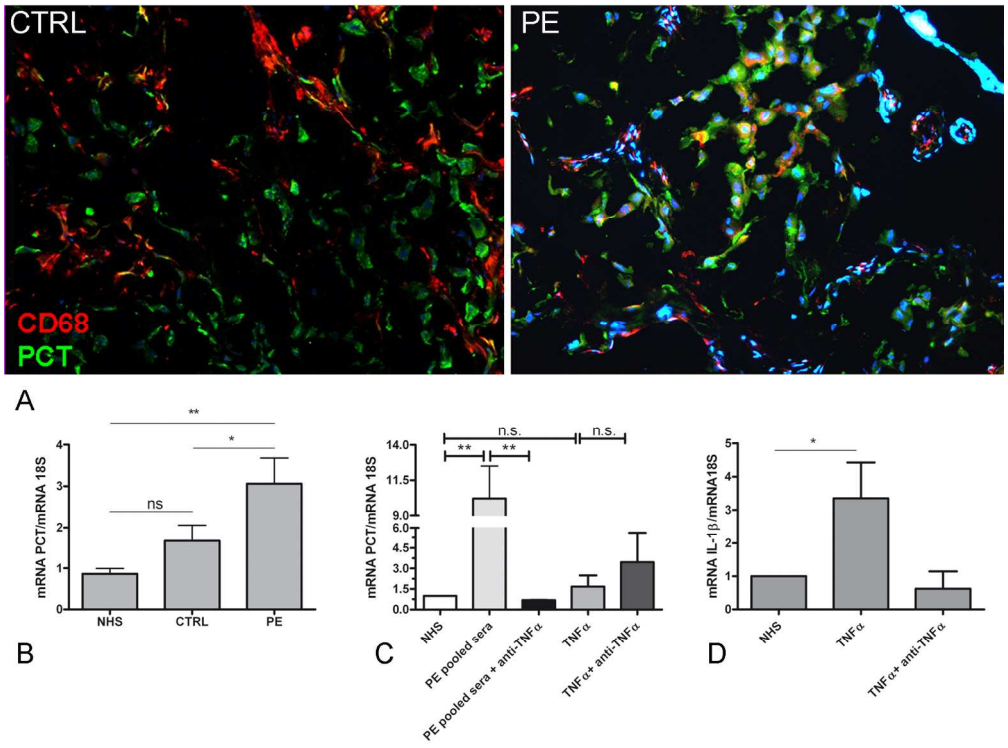
Extravillous Trophoblast and Macrophages as a Potential Source of Circulating Procalcitonin in Pre-Eclampsia. Agostinis et al.
Figure 6.



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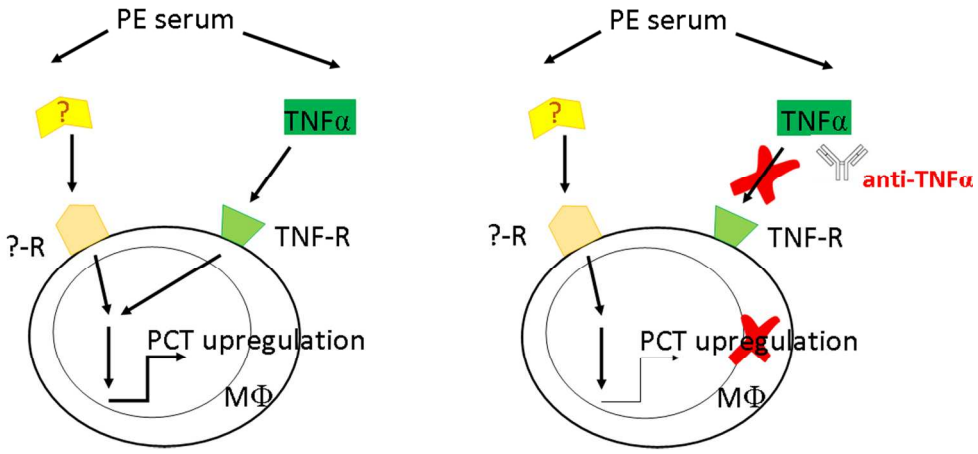
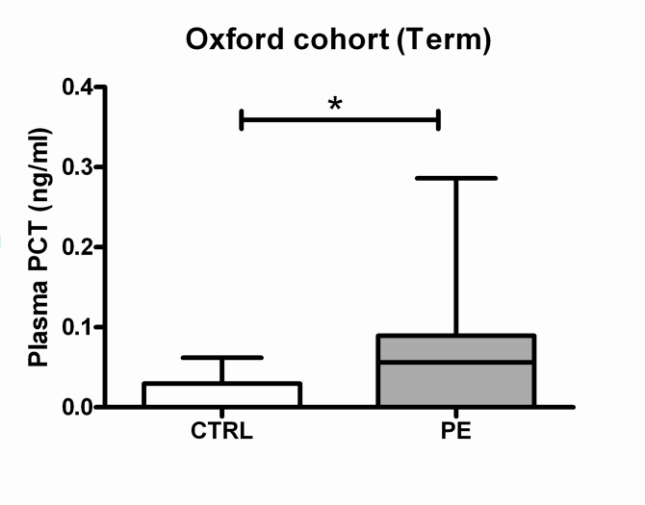


Figure 7. Graphical abstract

149x67mm (300 x 300 DPI)

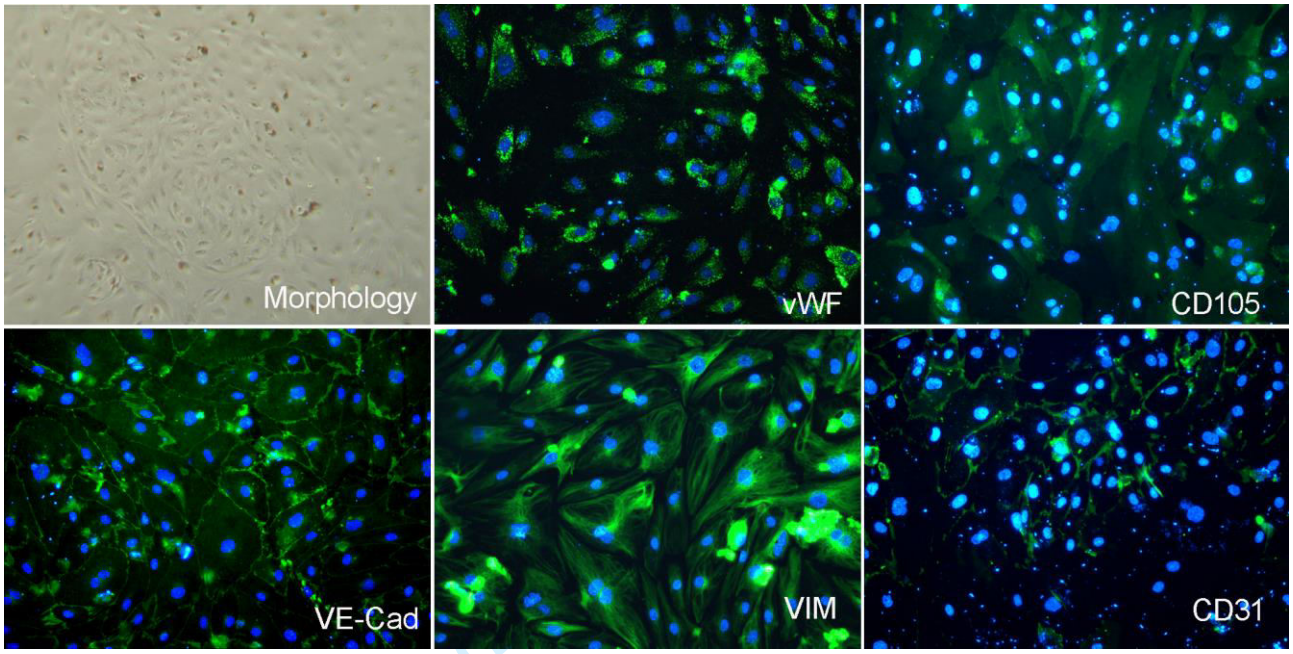
Supplemental Material Agostinis et al.

Supplementary Figure 1



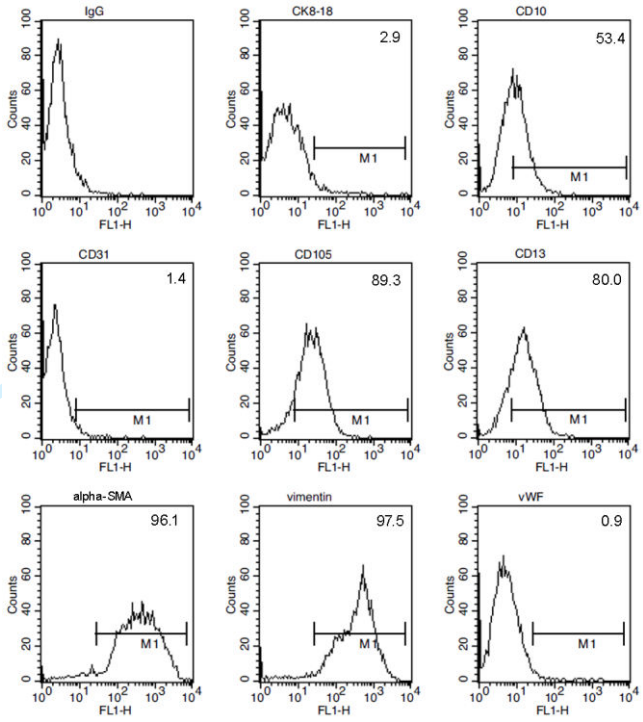
Supplementary figure 1. Measurement of PCT in pre-eclamptic patients (indicated with 'PE') and matched control pregnant women (indicated with 'C') enrolled at Oxford's Hospital (NDOG). PCT was significantly increased in pre-eclamptic women versus controls (*paired t test*). The results are expressed as box plot graphs, in which the line in the middle of the box represents the median; the lower and the upper edges of the box are the 1st and 3rd quartile, respectively. (*paired t test,* $p < 0.05$*).

Supplementary Figure 2



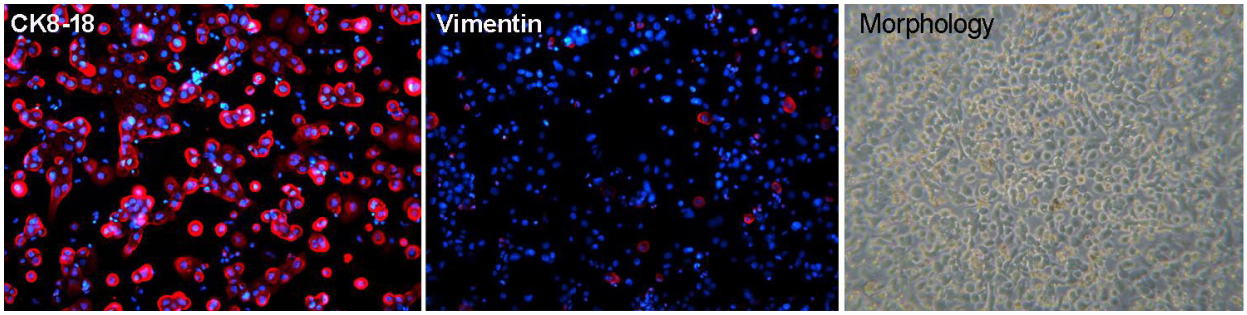
Supplementary figure 2. Immunofluorescence characterization of DEC isolated from pre-eclamptic deciduas. Endothelial cells were grown to confluence in 8-chamber culture slides. After fixation and permeabilization the cells were stained with mAb anti-human vWF, CD105, VE-Cadherin, vimentin, and CD31 followed by anti-mouse-FITC F(ab)' secondary antibodies. Nuclei were stained in blue by DAPI: Images were acquired with fluorescence microscope Leica DM2000 (Leica Microsystems, Germany) equipped with Leica DFC420 camera. Original magnification 200X.

Supplementary Figure 3



Supplementary figure 3. Characterization of the purity of decidual stromal cells isolated from pre-eclamptic tissue. DSCs were characterized by cytofluorimetric analysis for the expression of CK8-18, CD10, CD105, CD31, CD13, alpha-SMA, vimentin, and vWF and the expression of these markers (green lines) was compared with correlated control antibodies (black lines).

Supplementary Figure 4



Supplementary figure 4. Immunofluorescence characterization of EVT isolated from pre-eclamptic villi. Trophoblast were cyto-centrifuged and, after fixation and permeabilization, were stained with mAb anti-human CK8-18 or anti-human vimentin, followed by anti-mouse-Cy3 F(ab)' secondary antibodies. Nuclei were stained in blue by DAPI: Images were acquired with fluorescence microscope Leica DM2000 (Leica Microsystems, Germany) equipped with Leica DFC420 camera. Original magnification 200X.