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Increased levels of cell-free hemoglobin, oxidation markers, and the antioxidative heme scavenger α_1-microglobulin in preeclampsia

Magnus G. Olsson[1], Magnus Centlow[2], Sigurbjörg Rutardóttir[1], Iréne Stenfors[1], Jörgen Larsson[1], Bahram Hosseini-Maaf [3], Martin L. Olsson[3], Stefan R. Hansson[2*] and Bo Åkerström[1]

Divisions of [1]Infection Medicine, [2]Obstetrics and Gynecology, [3]Hematology & Transfusion Medicine, Lund University, Lund, Sweden

[*]To whom correspondence should be addressed:

Dr Stefan Hansson

Lund University, Tornavägen 10, 221 84 Lund

E-mail: stefan.hansson@med.lu.se

Tel. Nr: 046-2223011

Fax Nr: 046-157868

ABSTRACT

Preeclampsia is a major cause of morbidity and mortality during pregnancy. To date, the pathogenesis of the disease is not fully understood. Recent studies show that preeclampsia is associated with overexpression of hemoglobin genes α2 and γ and accumulation of the protein in the vascular lumen of the placenta. Hypothesizing that cell-free hemoglobin leaks from the placenta into the maternal circulation and contributes to the endothelial damage and symptoms by inducing oxidative stress, we analysed fetal and adult hemoglobin (HbF, HbA), haptoglobin, oxidation markers and the heme scavenger and antioxidant α_1-microglobulin in plasma, urine and placenta in preeclamptic women (n=28) and normal pregnancies (n=27). The mean plasma concentrations of HbF, HbA, protein carbonyl groups, membrane peroxidation capacity and α_1-microglobulin were significantly increased in preeclamptic women. The levels of total plasma Hb correlated strongly with the systolic blood pressure. The plasma haptoglobin concentrations of women with preeclampsia were significantly depressed. Increased amounts of α_1-microglobulin-mRNA and protein were found in placenta from preeclamptic women and the levels of plasma and placenta α_1-microglobulin correlated to plasma Hb-concentrations. The heme-degrading form t-α_1-microglobulin was significantly increased in urine in preeclampsia. These results support that hemoglobin-induced oxidative stress is a pathogenic factor in preeclampsia.

Keywords: Hemoglobin, oxidative stress, oxidation markers, ROS, α_1-microglobulin, preeclampsia

INTRODUCTION

Preeclampsia (PE) is a disease affecting approximately 5-10 % of all pregnancies, causing multiorgan damage that manifests as hypertension, proteinuria, and peripheral oedema [1]. Due to placental insufficiency, intra-uterine fetal growth restriction is seen in 25 % of the cases. The only effective cure known for PE is delivery of the fetus and thereby removal of the placenta. Although the underlying mechanisms of PE are not fully understood, a generally accepted view is that the disease progresses in two stages; First, a defective placentation due to impaired trophoblast remodeling of the spiral arteries leads to insufficient utero-placental blood flow [2]. Uneven perfusion of the placenta results in local ischemia and oxidative stress with formation of free radicals and reactive oxygen species (ROS). The second stage of PE is characterized by the maternal response to a yet unknown placental factor. A general vascular endothelial damage is typical, eventually affecting all maternal organs and giving rise to the clinical symptoms of the disease [3].

By gene expression microarray technology, it was recently shown that PE is associated with overexpression of the hemoglobin (Hb) subunits α2 and γ in the placenta [4]. Hb, the oxygen-carrying molecule in red blood cells, consists of four globin chains, each binding an iron-containing heme group. Fetal Hb (HbF) is a tetramer, formed by two α- and two γ-globin subunits, whilst adult Hb (HbA) consists of two α- and two β-subunits [5]. Cell-free Hb and its metabolites are known to be harmful due to their oxidative properties. OxyHb, i.e. ferrous (Fe^{2+}) Hb binding oxygen (O_2), is known to undergo spontaneous intramolecular oxidation-reduction reactions, in which the iron is oxidized to the ferric (Fe^{3+}) form (metHb) and the oxygen is reduced to superoxide. Further reactions lead to formation of ferryl (Fe^{4+}) Hb, free heme and various ROS [6]. ROS include hydrogen peroxide and the free radicals superoxide and hydroxyl radicals. All these compounds are toxic since they can cause oxidative damage on DNA, matrix molecules, cell membranes and other tissue components [7, 8]. The pro-

oxidative and pro-inflammatory properties of cell-free (extracellular) Hb are normally counteracted by different Hb- and heme detoxification mechanisms [9]. For instance, haptoglobin (Hp) and hemopexin are well-described binders of Hb and heme, respectively, in blood [10, 11]. CD163 on macrophages scavenges the Hp-Hb complex [12] whilst CD91 on several cell types including macrophages and syncytiotropho-blasts scavenges the hemopexin-heme complex [13]. In addition, heme oxygenase is an important intracellular heme degradation system [14].

α_1-microglobulin (α_1m), a 26 kDa ubiquitous plasma and tissue protein, has recently been shown to be involved in the defence against cell-free Hb and heme [15-17]. α_1m, also known as protein HC [18], is mainly synthesized in the liver. After secretion, α_1m is rapidly distributed to different tissues where it is found in the extravascular compartments in a free form and as high molecular weight complexes bound to IgA [19, 20], albumin and prothrombin [21]. α_1m binds the heme group [16, 22] and a heme-degrading truncated variant (t-α_1m), lacking the C-terminal tetrapeptide LIPR, is induced by a reaction with Hb [16]. The protein is also a radical scavenger [23], and has reductase [24] and anti-oxidant properties [17]. Its synthesis in liver and blood cells is up-regulated by cell-free Hb and ROS [25].

In this study, we hypothized that cell-free Hb, originating from the placenta, is involved in the development of PE by inducing oxidative stress. Concentrations of cell-free HbA and HbF were measured in term maternal plasma and urine from normal uncomplicated pregnancies and from women diagnosed with PE. Plasma levels of Hp, protein carbonyl groups, a marker of oxidative stress, the membrane peroxidation capacity and α_1m were measured. Furthermore, α_1m and the heme-degrading form t-α_1m were also measured in the urine samples. Finally, the concentrations of α_1m-protein and gene expression (mRNA) were analysed in placenta tissue from the study groups.

MATERIALS AND METHODS

Sample collection

Blood and urine samples were collected just prior to delivery from 28 preeclamptic patients and 27 normal uncomplicated pregnancies (Table 1) admitted at the Department of Obstetrics and Gynecology, Lund University Hospital, Sweden. The sampling was performed following written consent and the study has been approved by the ethical committee review board for studies in human subjects at Lund University. Women with gestational or essential hypertension and/or women with systemic diseases were excluded. The two groups were matched based on parameters listed in Table 1. Late onset preeclampsia (>35 gestational weeks), was defined as blood pressure >140/90 mmHg and proteinurea >0.3 g/l. Blood pressure was controlled after patient admission. Proteinuria was screened for with Albustix® and when positive further quantified using radioimmunoassay. For plasma Hb (HbF and HbA), Hp and α_1m analysis, 6 ml blood were collected into EDTAVacuette© plasma tubes (Greiner Bio-One GmbH, Kremsmünster, Austria). Samples were collected without a tourniquet in order to avoid sampling-related hemolysis and subsequently centrifuged at 2000 g for 20 minutes after which the plasma was transferred into cryo tubes. Ten ml urine were sampled in 15 ml tubes (Sarstedt AB, Landskrona, Sweden) containing 300 µl urine conservation solution (2 M tris- hydroxymethyl aminomethane, 0.9 M disodium-EDTA, 0.2 M benzamidine chloride and 0.5 M sodium azide). Urine was then transferred into cryo tubes. A 10x10x10 mm cube of placental villous tissue from one cotyledon was removed after delivery and immediately put on dry ice. Frozen human placental tissues were pulverized, and the placental powder (25 mg frozen weight) was homogenized using 3 ml 8 M urea and 2% CHAPS supplemented with a protease cocktail (Complete mini plus EDTA, Roche, Mannheim, Germany). Samples were stirred for 1 hour followed by centrifugation at 43000 g for 2 hours. All samples were stored in -80ºC until further analysis.

Reagents and proteins

Adult oxyHb (HbA) and fetal oxyHb (HbF) were purified as previously described [26] from whole blood, freshly drawn from healthy subjects, or umbilical cord blood, respectively. Human γ-chains were prepared by dissociation of purified HbF with p-mercuribenzoate Sigma-Aldrich, St-Louis, MO, USA) and acidic precipitation as described by Kajita et al. [27] with modifications by Noble [28]. The absolute purity of HbF (from contamination with HbA), and of γ-chains (from contamination with α- and β-chains), was determined by non-denaturing PAGE followed by blotting with mouse antibodies specific to a peptide derived from human γ-chains (Santa Cruz Biotechnology, Santa Cruz, CA, USA). HbA, used in the ELISA, extravidin-alkaline phosphatase, 2,4-dinitrophenylhydrazine (DNP-hydrazine), 2-thiobarbituric acid and O-phenylenediamine were purchased from Sigma. Human α_1m was purified from urine as described by Åkerström [29]. Rabbit polyclonal antibodies were prepared against human α_1m [30] and the C-terminal tetrapeptide (LIPR) of α_1m [16], mouse monoclonal antibodies against human α_1m (BN11.2) [31], goat anti-human α_1m and goat anti-rabbit immunoglobulin were prepared as previously described [32]. Rabbit antibodies to human γ-chains, and hence specific for HbF, were produced by AgriSera AB (Vännäs, Sweden) and purified by protein A-Sepharose (Sigma) chromatography followed by affinity chromatography using a column with HbF coupled to cyanogen bromide-activated Sepharose CL-4B (GE Healthcare, Uppsala, Sweden). Rabbit antibodies against HbA (IgG purified) commercially available from Dako A/S (Glostrup, Denmark), were affinity purified using a column with human HbA, immobilized on cyanogen bromide-activated Sepharose CL-4B, and biotinylated. Anti-DNP-keyhole limpet hemocyanin (KLH) was from Invitrogen (Eugene, Oregon, USA).

Enzyme-linked immunoassay (ELISA)

A competitive ELISA was employed for quantification of HbA. Briefly, 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated with HbA (Sigma; 5 µg/ml in PBS) overnight at room temperature (RT), rinsed with PBS + 0.05% Tween-20, and then incubated 2 h at RT with a mixture of 50 µl standard HbA (purified in the lab) or the patient samples, and 50 µl rabbit anti-HbA (5 µg/ml in PBS + 0.05% Tween-20 + 1% BSA, incubation buffer). After rinsing, the wells were incubated for 1 h at RT with alkaline phosphatase-conjugated porcine anti-rabbit IgG, at a 1:2500 dilution in incubation buffer. Finally, after rinsing, a substrate solution containing p-nitrophenyl phosphate (1 mg/ml) in 1 M diethanolamine + 0.5 mM $MgCl_2$, pH 9.8, was added, and the absorbance was read at 415 nm at the onset of the reaction until a peak absorbance was obtained.

A sandwich-ELISA was used for quantification of HbF. Ninety six-well microtiter plates were coated with affinity-purified rabbit anti-HbF (5 µg/ml in PBS) overnight at RT. In the second step, a standard series of HbF or the patient samples, diluted in incubation buffer, were incubated for 2 h at RT. In the third step, biotinylated affinity-purified rabbit anti-HbA antibodies, in a 1:1000 dilution, were added and incubated for additional 2 h at RT. In the fourth step, extravidin-alkaline phosphatase, 1:10000 dilution, was added and incubated for 1 h at RT. Finally, a substrate solution containing p-nitrophenyl phosphate (1 mg/ml) in 1 M diethanolamine with 0.5 mM $MgCl_2$, pH 9.8, was added, and the absorbance was read at 415 nm at the onset of the reaction until a peak absorbance was obtained.

Radio-immunoassay (RIA)

Radiolabelling of α_1m with ^{125}I was done using the chloramine T method. Protein-bound iodine was separated from free iodide by gel-chromatography on a Sephadex G-25 column (PD10, GE Healthcare). A specific activity of around 0.1-0.2 MBq/µg protein was obtained.

RIA was performed by mixing goat antiserum against human α_1m (0.2 ml, dil. 1:6000) with ^{125}I-labelled α_1m (0.1 ml, app. 0.05 pg/ml) and unknown patient samples or standard α_1m-concentrations (0.2 ml). The dilutions were done in 0.1 M sodium phosphate, pH 7.5 + 0.1 % BSA (RIA-buffer). After incubating overnight at RT, antibody-bound antigen was precipitated by adding 0.3 ml bovine serum and 1.6 ml 15 % polyethylene glycol in the RIA-buffer, centrifuged at 2500 g for 40 min, after which the ^{125}I activity of the pellets was measured in a Wallac Wizard 1470 gamma counter (Perkin Elmer Life Sciences).

RNA isolation and Real-Time PCR

RNA was extracted with Trizol® according to the manufacturer's instructions (Invitrogen). Briefly, 120-150 mg of placental tissue was homogenized in 2-3 ml Trizol on ice. Cellular debris was removed by spinning the samples at 12000g for 10 minutes after which chloroform was added. The aqueous phase containing the RNA was isolated. The pellet was dissolved in RNAse-free water and RNA integrity was assessed using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, USA) and a 1% (w/v) agarose gel to confirm a high RNA quality. Reverse transcription was performed on 1 μg total RNA at 42°C for 60 minutes in the presence of 0.5 μg oligo(dT)$_{18}$ primer, 200 U reverse transcriptase and 20 U RiboLock™ Ribonuclease inhibitor in reaction buffer (RevertAid™ H Minus First Strand cDNA Synthesis Kit, Fermentas GMBH, St. Leon-Rot, Germany). Real-Time PCR was then used to quantify α_1m-specific reverse transcriptase-formed cDNA. Human β-actin was used to standardize the expression of α_1m. Primers were designed accordingly: α_1m forward primer 5′-CACTCGTTGGCGGAAAGG-3′, reverse primer 5′-ACTCATCATAGTTGGTGTGGAC-3′; β-actin forward primer 5′-GAGCAAGAGAGGCATCCTCACCC-3′, reverse primer 5′-CCAGCCAGGTCCAGACGCAGG-3′. The expression was analyzed using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). Amplification was performed at 55°C for 40 cycles

in iCycler Thermal Cycler (Bio-Rad) and data analyzed using iCycler iQ Optical System Software (Bio-Rad).

SDS-PAGE and Western blot

SDS-PAGE (*T*=12%, *C*=3.3%) was performed as described by Laemmli [33]. The gels were run under reducing and non-reducing conditions using high molecular weight standard (rainbow markers, GE Healthcare). The separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon, Millipore, Bedford, MA, USA). The membranes were then incubated with polyclonal antibodies and Western blot was performed using ^{125}I-labeled secondary antibodies and developing the membranes using Fuji FLA 3000 phosphoimaging system (Fujifilm Sweden AB). The protein bands and calculations of the ratio t-α_1m/α_1m were quantified by densitometry using Adobe Photoshop CS3 software (Adobe systems inc. Mountain View, CA,USA) as described previously [34].

Immunohistochemistry

Immunohistochemistry was performed using EnVisionTM+System-HRP (Carpinteria, CA, USA) on 4 samples from the PE group and 4 from the control group respectively according to the manufacturer's instructions. Briefly, fresh frozen sections, 12 μm thick, of the placenta samples were fixed by immersion in 4% PBS buffered formaldehyde for 15 min at RT. Sections were then incubated in a blocking solution containing 3% H_2O_2 for 10 minutes at RT. The mouse anti-α_1m primary antibody (BN11.2) was diluted 1:000 in the diluent containing 1x PBS with 0.25% Triton X100 and 1.5% normal goat serum. Sections were incubated over night at 4ºC after which the sections were rinsed. Enough peroxidase labelled polymer were applied to cover the sections and then incubated for 30 min. After rinsing, LiquidDAB substrate chromogen solution were applied to cover the tissue sections

and incubated for 8 min. The sections were then rinsed and stained with Mayers hematoxylin after which sections were mounted with Mountex (Histolab, Göteborg, Sweden) and coverslipped. Sections were viewed under an Olympus BX-60 microscope and images captured using an Olympus DP50CU digital camera.

Measurement of plasma Hp

Hp in plasma was measured at the Department of Clinical Chemistry and Pharmacology, University and Regional Laboratories, Lund, Sweden, using an automated immuno-turbidimetric assay.

Membrane peroxidation capacity of plasma

The capacity of plasma to induce membrane peroxidation was measured by the thiobarbituric acid (TBA) method described by Gutteridge [35]. Briefly, erythrocyte membranes, purified as described earlier [16] were incubated with 10 µl plasma in a total volume of 150 µl PBS at 37°C for 4 h. TBA-reagent (0.5% 2-thiobarbituric acid and 0.5% SDS in H_2O) and 0.2 M glycine-HCl, pH 3.6 were then added and the mixture heated to 100°C for 15 min. After cooling to RT the absorbance at 532 nm was read.

Determination of protein carbonyl groups

Formation of protein carbonyl groups in plasma was determined as described [36]. Briefly, samples were mixed with DNP-hydrazine for 45 minutes in RT. The DNP-hydrazine derivatized samples were then diluted in PBS and coated on a 96-well microtiter plate for 2 hours in RT. After rinsing, the plate was incubated with rabbit anti-DNP-KLH (Invitrogen; diluted 1:2000 in PBS, 0.1% BSA, 0.25% tween 20; incubation buffer) for 2 hours in RT, followed by rinsing and incubation with horse radish peroxidase-conjugated porcine anti-

rabbit IgG (Dako A/S; diluted 1:2000 in incubation buffer) for 1 hour in RT. Finally the plate was incubated with substrate solution (1 tablet O-phenylenediamine dissolved in 60 mM Tris-HCl, pH 8.5, 50 mM Na_2HPO_4 and hydrogen peroxide) and absorbance was read at 450 nm at the onset of the reaction until a peak absorbance was obtained.

Statistical analysis

Statistical analysis was performed using Origin 8 software (Microcal, Northampton, MA, USA). The significance of differences between groups was evaluated using both Student´s t-test and Mann-Whitney U-test. $P<0.05$ was considered statistically significant. Associations between variables were assessed with the correlation coefficient according to Pearson.

RESULTS

HbF and HbA in plasma and urine

The concentrations of HbF and HbA were measured in all plasma samples from women with PE and normal control pregnancies (Fig. 1A, C). A nine-fold and highly significant increase of the HbF concentration was seen in PE patients (mean value 1.09 μg/ml, p-value 3.8×10^{-14}) as compared to the controls (mean value 0.126 μg/ml). The concentration of HbA was also significantly elevated in PE (mean value 4.4 μg/ml) compared to the control group (mean value 2.97 μg/ml). Although the increase was less pronounced (1.5-fold) than that of HbF, the small individual variation within the two groups yielded a high significance of the difference (p-value 1.7×10^{-9}). The concentrations of HbF and HbA were also determined in urine from the two study groups (Fig. 1B, D). The mean HbF concentration was increased in women with PE (PE, mean value 4.7 μg/ml; C, mean value 0.095 μg/ml), but did not reach significance due to the large sample variation (p-value 0.15). Furthermore, the mean concentration of urinary HbA was higher in the PE patients compared to controls (PE, mean value 30.9 and C, median value 12.8 μg/ml) but not significant (p-value 0.73).

To investigate the correlation between Hb and the severity of the disease, we plotted total Hb (HbF + HbA) concentrations in plasma against the systolic blood pressure in both groups (Fig. 2). A strong positive correlation was found ($r = 0.72$, p-value 7.1×10^{-10}).

Hp, oxidation capacity and oxidation markers in plasma

In order to determine whether the increased Hb concentration in the PE patients is paralleled by a lower Hp concentration resulting from Hb-Hp complex formation and CD163-mediated elimination of the complex from plasma, plasma Hp levels were determined (Fig. 3A). The results display a significant decrease (p-value 0.05) of the Hp concentrations in PE samples as compared to controls (PE, mean value 0.55 g/L; C, mean value 0.77 g/L). We

hypothesized that the increased cell-free Hb-concentrations results in an increased oxidation capacity of plasma. This was investigated by examining the ability of plasma to drive membrane lipid peroxidation (Fig. 3B) [35]. A small but significant increase in peroxidation capacity was observed in plasma from women with PE (p-value 0.05) indicating increased oxidation in PE. The involvement of oxidation in PE was further investigated by analyzing the levels of protein carbonyl groups, a marker of oxidation events, in plasma of all samples (Fig. 3C). Results displayed a highly significant increase in the PE samples as compared to the control samples (p-value 1.7×10^{-6}).

α_1m in plasma and urine

The concentration of α_1m was measured in all plasma and urine samples (Fig. 4). The plasma concentration of α_1m was significantly increased in preeclampsia (mean value 17.0 µg/ml, p-value 0.05) compared to controls (mean value 14.5 µg/ml). The urine concentration of α_1m was also significantly increased (p-value 0.0004) in women with PE (PE, mean value 22.9 µg/ml; C, mean value 5.3 µg/ml). The heme-degrading form of α_1m, t-α_1m [16, 37], lacking the C-terminal peptide LIPR was investigated by quantifying the α_1m- and t-α_1m-bands on Western blots, using anti-α_1m and anti-LIPR antibodies, and calculating the ratio α_1m/t-α_1m (Fig. 5). A significant increase of the ratio was found in women with PE as compared to control women (p-value 0.03). No t-α_1m was seen in plasma, in agreement with previous findings [34].

α_1m in placenta

The α_1m protein concentrations in the placenta samples (Fig. 6A) were more than doubled in women with PE (mean value 0.58 µg/ml) compared to controls (mean value 0.235 µg/ml; p-value 4.13×10^{-6}). Furthermore, a strong positive correlation was found between

total plasma Hb (HbF + HbA) and placental α_1m (r = 0.69, p-value 7.8 x 10^{-8}) (Fig. 6C) and a positive, but less pronounced correlation was found between total plasma Hb (HbF + HbA) and plasma α_1m (r = 0.30, p-value 0.03) (Fig. 6D). To estimate the expression of the α_1m-producing *AMBP* gene in placental cells, the amount of α_1m-mRNA in the placenta samples was determined by real-time-PCR normalized to β-actin mRNA (Fig. 6B). A lower mean Ct-value was seen in women with PE compared to controls, indicating a higher mean α_1m mRNA in PE. However, a large variation was observed and the difference was not significant (p-value 0.16). The localization of α_1m in PE- and control placenta tissue was investigated by immunohistochemistry. Fig. 7 shows the result in a representative PE placenta. A ubiquitous staining pattern was seen, although more intense in the syncytiotrophoblast layer and the vascular endothelium. Occasional areas of stroma cells were also positive. No qualitative difference of immunohistochemical staining was seen between PE and control placentas (not shown).

DISCUSSION

In this study we report increased maternal plasma levels of HbF and HbA, oxidation capacity and oxidation markers. Furthermore, plasma levels of Hp were depressed in PE, while concentrations of the antioxidant and radical scavenger α_1m were increased. α_1m expression was also increased in placenta tissue from women with PE. The results indicate that cell-free fetal Hb may have a role in the etiology of the disease by induction of oxidative stress in the placenta (stage 1), and by leakage over the placenta barrier to induce systemic oxidative stress (stage 2). Figure 8 is a schematic illustration based on our results, showing how cell-free fetal Hb may at least partially constitute the missing link between stage 1 and 2.

The early stage of PE development is characterized by uneven and insufficient perfusion of the placenta leading to hypoxia [3, 38]. Hypoxia has been reported to increase erythropoiesis, via HIF-1-induced synthesis of erythropoietin (reviewed in [39]), and induce a switch from HbA to HbF in cord blood cells and bone marrow cells [40, 41]. Thus, the up-regulation of HbF gene expression and placental HbF accumulation reported by Centlow et al. [4] may be induced in early pregnancy as a result of the local hypoxia. Cell-free HbF may then participate in oxidative damage to placental tissues and thereby initiate its own leakage through the blood-placenta barrier. Evidence of Hb-induced oxidative stress is provided by the elevated mRNA levels of α_1m and protein observed in the PE placenta. Previous in-vitro studies have shown that hepatocytes and blood cells exposed to cell-free Hb, heme and ROS, upregulate the α_1m mRNA expression, protein synthesis and secretion [25]. Therefore, it can be speculated that the increased concentration of placental α_1m is a local response towards the elevated HbF-concentrations and increased ROS activities in placenta, as illustrated in Fig. 8.

The elevated HbF concentrations in maternal blood may be the result of ROS-induced leakage through the blood-placental barrier, which causes subsequent oxidative damage in the maternal circulation, including hemolysis of adult erythrocytes and elevation of cell-free

HbA, as shown in Fig 8. As the disease progresses the systemic concentrations of cell-free Hb will increase, followed by upregulation of α_1m synthesis and secretion from the liver as previously described [25], leading to increased maternal α_1m concentrations. The liver is the major site of α_1m synthesis and, assuming that the placenta barrier is compromised in PE, part of placenta α_1m may therefore originate from maternal liver. This is supported by the strong correlation between Hb- and α_1m-concentrations on both sides of the placental barrier (Fig. 6C and D).

The association between PE and oxidative stress is well-documented [42, 43]. The results from this study support the involvement of oxidative factors in the etiology of the disease and suggest that Hb may contribute to this. Cell-free Hb is well known to have pro-inflammatory and pro-oxidative properties [7, 8]. The molecule can undergo auto-oxidation with formation of ROS and release of the reactive heme group. Heme and ROS may cause further oxidation and membrane damage [7-9, 26]. Indeed, we found evidence of higher oxidation capacity (Fig. 3B) as well as higher levels of oxidatively modified proteins in plasma from the PE patients (Fig. 3C). The elevated levels of cell-free circulating Hb in PE may therefore cause oxidative stress, and subsequent endothelial and vascular dysregulation/damage as well as the kidney impairment which is associated with PE.

Cell-free Hb is also a scavenger of nitric oxide (NO) [44], and may thus contribute to some of the vasoconstriction and hypertension observed in PE. In fact, a strong correlation between the total plasma Hb concentration and the systolic blood pressure was observed ($r=0.72$, Fig. 2). The HbA + HbF concentrations in the PE patients (3-10 mg/L, equivalent to 0.2-0.6 μM heme) are lower than the Hb-ranges associated with hypertension in for instance sickle cell disease [44], during infusion of blood substitutes based on cell-free Hb molecules [45] or intravascular hemolytic blood transfusion reactions [46], but heme concentrations as low as 1 μM have been described to significantly bind NO [47] and therefore it cannot be

excluded that NO-scavenging by Hb contributes to the hypertension associated with PE. However, it remains to be shown whether the cell-free Hb in plasma of women with PE indeed scavenges physiologically significant amounts of NO.

As expected, the elevated Hb concentrations of the PE patients were accompanied by a significant decrease of the Hp concentrations (Fig. 3A), suggesting a depletion of Hp after complex-binding to Hb and clearance of the Hb-Hp complex from the circulation by binding to the receptor CD163 [12]. This supports that the elevated levels of Hb are a result from an in vivo-process and not connected with the sample handling. Interestingly, the Hb levels are increased in most of the patients in spite of the fact that Hp is not completely depleted, i.e. the Hp-concentrations are mostly above zero. This suggests that a certain amount of the Hb-Hp complex in plasma has not been cleared from plasma at the time of sample-collection. A possible explanation for this is that PE is characterized by a prolonged continuous production of cell-free Hb (i.e. hemolysis and leakage from placenta), at a rate which yields an elevated steady-state concentration of cell-free Hb. These results, with relatively low but clearly elevated concentrations of cell-free Hb, suggests that PE is a disease with a prolonged, early phase of moderate oxidative stress and gradual accumulation of tissue damage which finally leads to the severe symptoms including systemic hypertension and kidney failure.

Kidney damage and proteinuria are hallmarks of PE [48, 49] and significantly increased concentrations of total protein (Table 1), as well as the specific proteins albumin and α_1-acid glycoprotein (not shown) were found in urine from the PE group. Although the pathophysiological mechanisms are very complex, our results suggest that increased cell-free plasma Hb levels may contribute to the renal injury. Overload of filtration and tubular re-absorption may lead to formation of precipitates called hemosiderin and cause oxidative damage including glomerular and tubular necrosis [7, 48]. In analogy, hemoglobinemia during intravascular hemolysis is associated with renal failure [46].

A pronounced increase of the α_1m concentration in urine was also seen in the PE samples (Fig. 4). Impaired kidney functions contribute to this but it may also be speculated that a local α_1m expression and excretion is induced in the tubular cells in response to the Hb overload. Kidney epithelial cells have been shown to have a regulated expression of the α_1m (*AMBP*) gene [49] and cell-free Hb and free radicals have been shown to up-regulate α_1m expression in other cells [25]. Interestingly, the heme-degrading form of α_1m, t-α_1m, was significantly increased in urine of women with PE (Fig. 4). This carboxy-terminally truncated form is generated by reactions with Hb and degrades the heme group into a yellow-brown chromophore linked to the protein and has previously been found in urine [16, 34]. T-α_1m is therefore an indicator of increased cell-free Hb-exposure. Consistent with Hb/hemosiderin depositions in tubuli, a possible site of formation of urinary t-α_1m in the PE patients may be the proximal tubules of the kidney.

To this day, diagnosis of PE is still based on clinical evaluation of symptoms in combination with markers reflecting liver and kidney function such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT), creatinine, urate and low molecular weight plasma proteins cystatin C and β_2-microglobulin [50]. Although various plasma factor concentrations have been shown to be associated with PE (PP13, sFlt1, sEndogline and VCAM [51, 52]), none so far has proven clinically useful. The results presented here indicate that cell-free HbA and HbF levels could be useful as diagnostic support for PE.

In conclusion, we have demonstrated significantly increased levels of fetal and adult Hb in plasma from women diagnosed with PE. Also, it was shown that the antioxidant and heme scavenger α_1m was upregulated in placenta and its concentrations increased in plasma and urine in PE. The results indicate that Hb may be an etiological factor involved in the

pathogenesis of the disease and that the increased levels of α_1m represent a physiological feed-back defence mechanism which is insufficient in women with the disease.

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LIST OF ABBREVIATIONS

Hb, hemoglobin; α_1m, α_1-microglobulin; PE, preeclampsia; ROS, reactive oxygen species; HbF, fetal hemoglobin; HbA, adult hemoglobin; t-α_1m, truncated α_1-microglobulin; β_2m, β_2-microglobulin; ELISA, enzyme linked immunoassay; RIA, radio immunoassay; PVDF membrane, polyvinylidene difluoride membrane; AMBP, α_1-microglobulin/bikunin precursor.

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LEGENDS TO FIGURES

Figure 1. Quantification of fetal and adult hemoglobin in plasma and urine. Samples were from uncomplicated normal pregnancies (C), and women diagnosed with PE (PE). The HbF and HbA concentrations in plasma **(A, C)** and urine **(B, D)** were measured by sandwich–ELISA and competitive ELISA, respectively. The results from the analysis are plotted as a scatter of individual patient data and as mean ± SEM. *** P<0.001.

Figure 2. Correlation between total plasma Hb and systolic blood pressure. Samples were from uncomplicated normal pregnancies and women diagnosed with PE. The total Hb (HbF + HbA) plasma concentration of each patient sample was plotted against the systolic blood pressure.

Figure 3. Measurement of haptoglobin, membrane peroxidation capacity and protein carbonyl groups in plasma. Samples were from uncomplicated normal pregnancies (C), and women diagnosed with preeclampsia (PE). **(A)** Plasma Hp concentrations were measured as described in Materials and Methods. Only 18 samples of the control group contained enough material required for Hp determination. **(B)** The capacity of plasma to induce membrane peroxidation was measured using the TBA method as described in Materials and Methods. **(C)** The levels of the oxidation marker carbonyl groups were measured in plasma using an ELISA method as described in materials and methods. The results from the analyses are plotted as a scatter of individual patient data and as mean ± SEM. *** P<0.001.

Figure 4. Quantification of α_1-microglobulin in plasma and urine. Samples were from uncomplicated normal pregnancies (C), and women diagnosed with preeclampsia (PE). The α_1m concentrations in plasma **(A)** and urine **(B)** were determined by RIA. The results from

the analysis are plotted as a scatter of individual patient data and as mean ± SEM. *** P<0.001, * P<0.05.

Figure 5. Quantification of t-α_1-microglobulin in urine. Samples from urine of uncomplicated normal pregnancies (C), and women diagnosed with preeclampsia (PE) were separated on SDS-PAGE and immunoblotted using polyclonal antibodies against α_1m. The bands corresponding to full length α_1m (33 kDa) and t-α_1m (30 kDa) were quantified by densitometry using Adobe Photoshop CS3 software. The ratio of free α_1m/t-α_1m was calculated and the results from the analysis are plotted as a scatter of individual patient data and as mean ± SEM. * P<0.05.

Figure 6. α_1-microglobulin in placenta and its correlation with total Hb. Samples were from uncomplicated normal pregnancies **(C, ○)** and women diagnosed with preeclampsia **(PE, ●)**. The α_1m protein concentration in placenta **(A)** was determined by RIA. Total RNA was extracted from homogenized cells, cDNA was prepared using reverse transcription and mRNA expression of α_1m was analysed using Real-Time PCR **(B)**. Amplification was performed as described in materials and methods. The mean normalized Ct values are shown for each group. To investigate the correlation between placental/plasma α_1m and total plasma Hb (HbF + HbA), placental α_1m **(C)** and plasma α_1m **(D)** concentration of each patient sample was plotted against the total plasma Hb concentration (determined as described in materials and methods). The results from the analysis are plotted as a scatter of individual patient data and as mean ± SEM. * P<0.05.

Figure 7. Analysis of placental α_1-microglobulin. Immunohistochemistry of α_1m in the preeclamptic placenta was performed as described in materials and methods. α_1m staining is seen in the syncytiotrophoblast cells of the villi (arrow marked "st") and in the vascular epithelium (arrow marked "ve"). Note the uneven staining, some villi have more intense staining than others. Scale bar: 100 μm.

Figure 8. A tentative chain of events involving the parameters reported in this paper and Centlow et al. [5]. The figure shows a schematic placenta villus with impaired feto-maternal barrier function causing leakage of placenta factors. 1: Unknown early events induce local hypoxia. 2: The hypoxia induces an upregulation of the placenta HbF genes and protein [5], ROS and α_1m gene expression and protein concentration. 3: Oxidative damage and leakage of the feto-maternal barrier results in increased maternal plasma concentrations of HbF and induction of ROS, hemolysis and increased maternal plasma concentrations of HbA. HbA and ROS will induce an upregulation of α_1m in placenta and maternal cells.

Figure 1

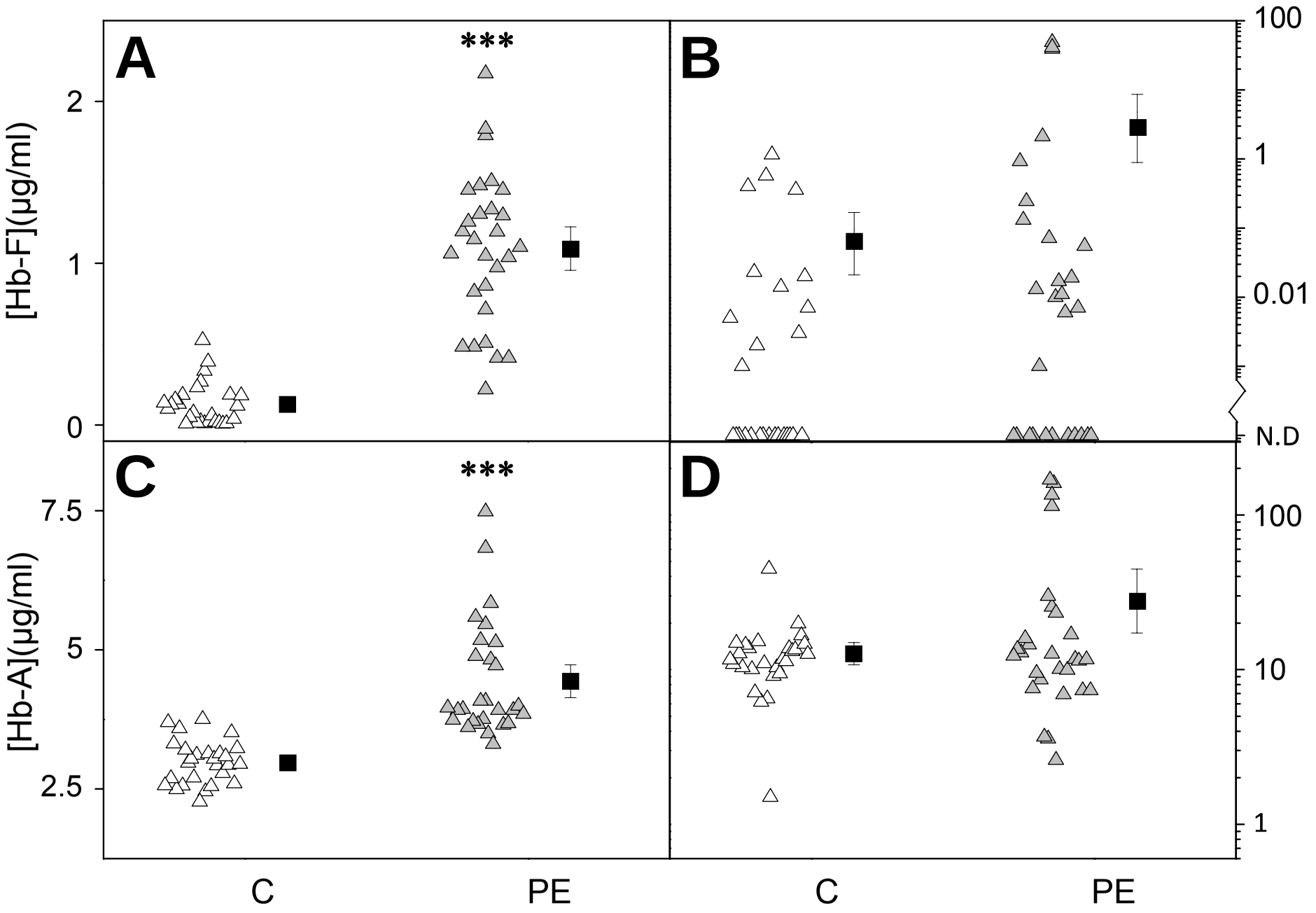
Plasma
Urine
A
B
C
D
***
***
[Hb-F](µg/ml)
[Hb-A](µg/ml)
2
1
0
7.5
5
2.5
100
1
0.01
N.D
100
10
1
C
PE
C
PE

Figure 2

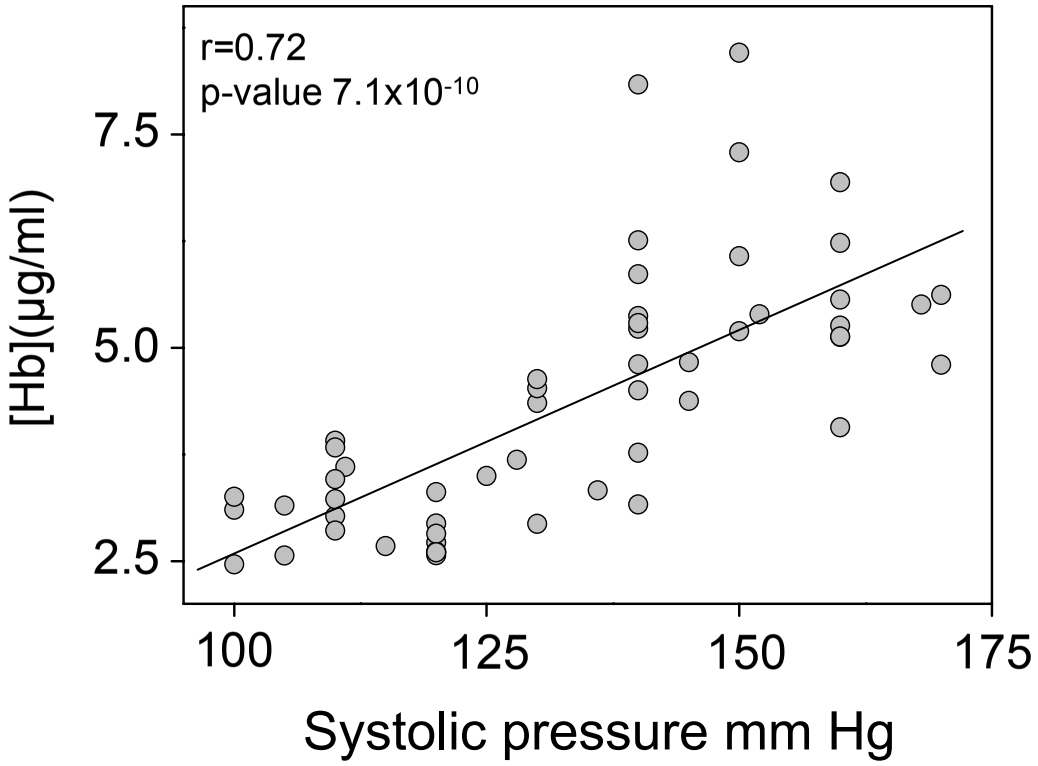
r=0.72
p-value 7.1x10-10
7.5
5.0
2.5
[Hb](µg/ml)
100
125
150
175
Systolic pressure mm Hg

Figure 3

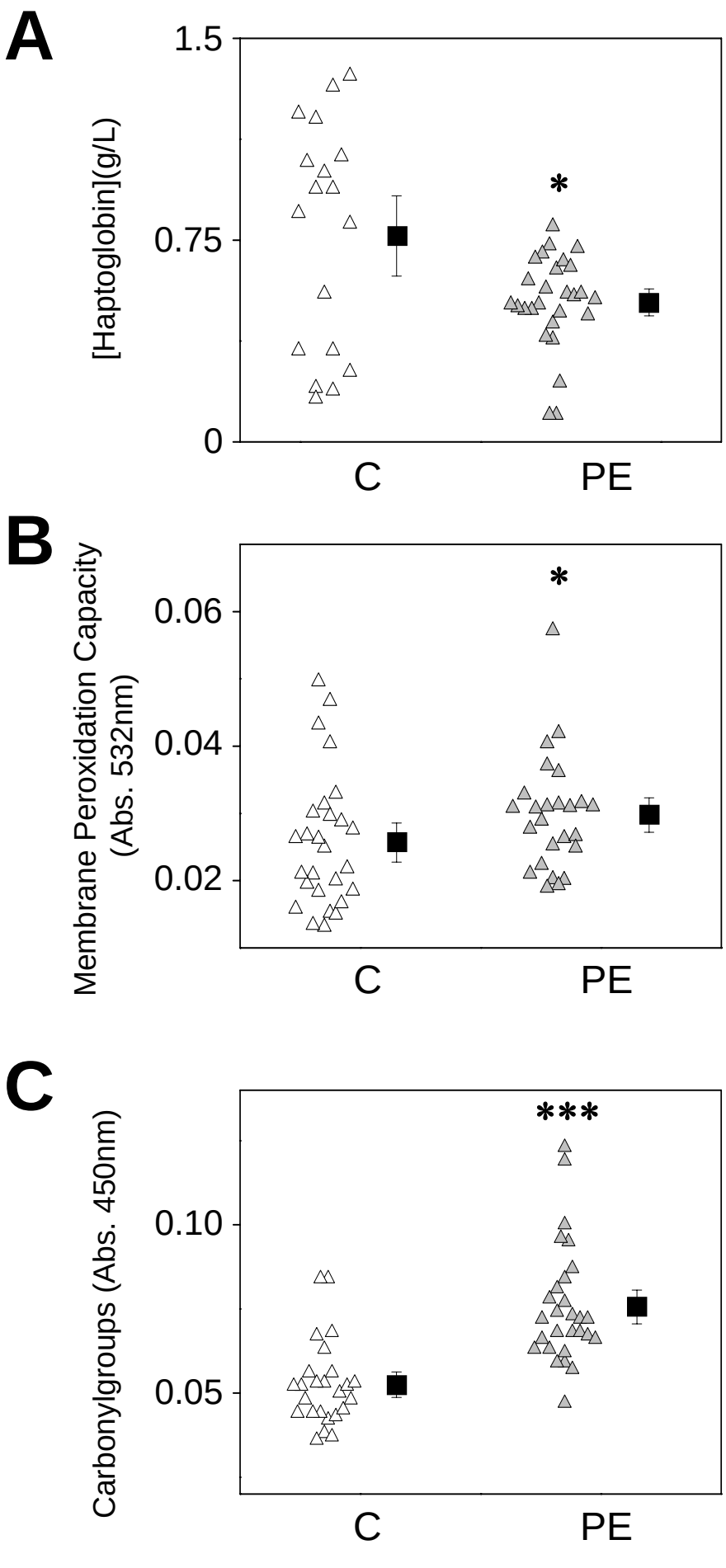
A
1.5
0.75
0
[Haptoglobin](g/L)
*
C
PE
B
Membrane Peroxidation Capacity
(Abs. 532nm)
0.06
0.04
0.02
*
C
PE
C
Carbonylgroups (Abs. 450nm)
0.10
0.05
***
C
PE

Figure 4

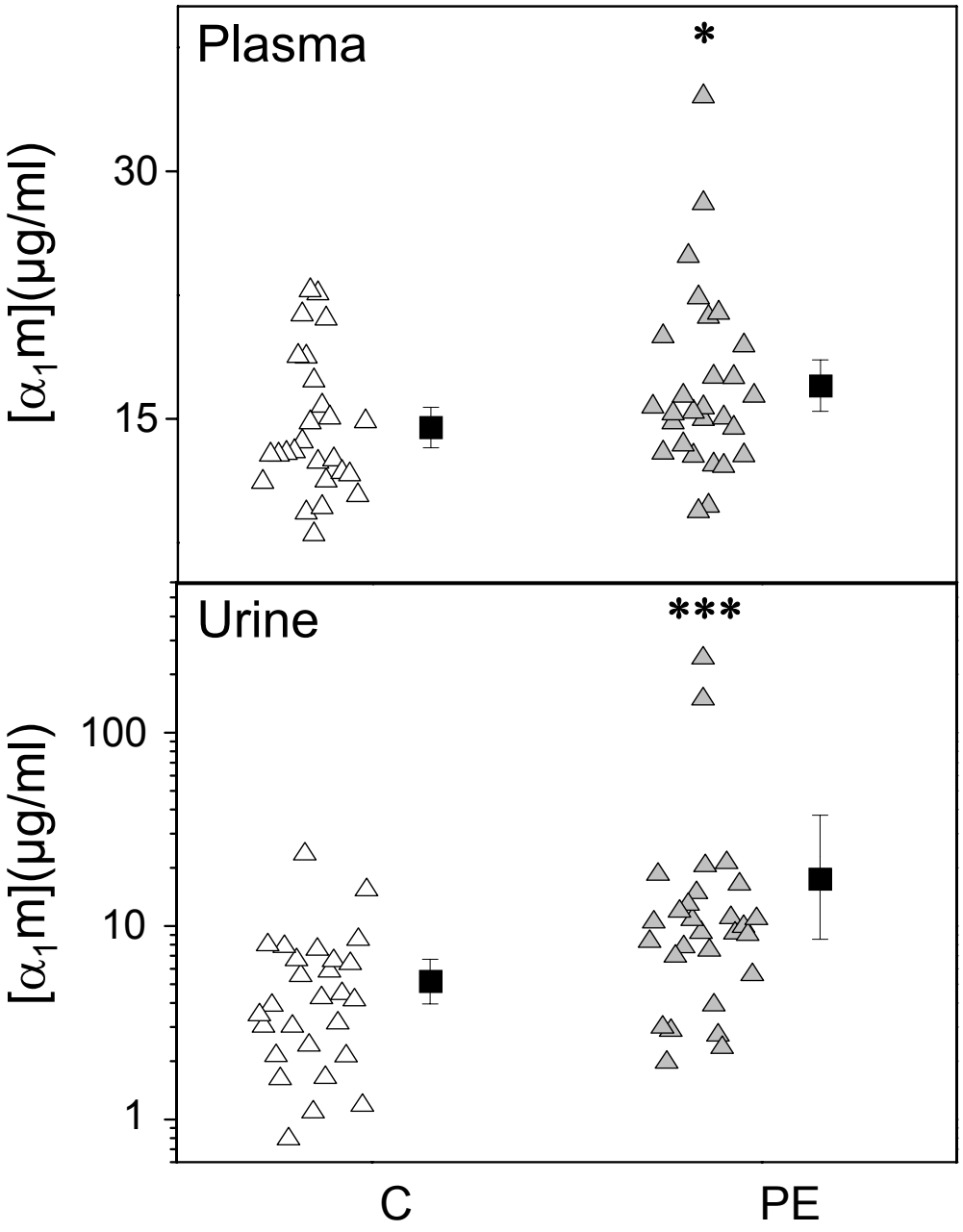

Figure 5

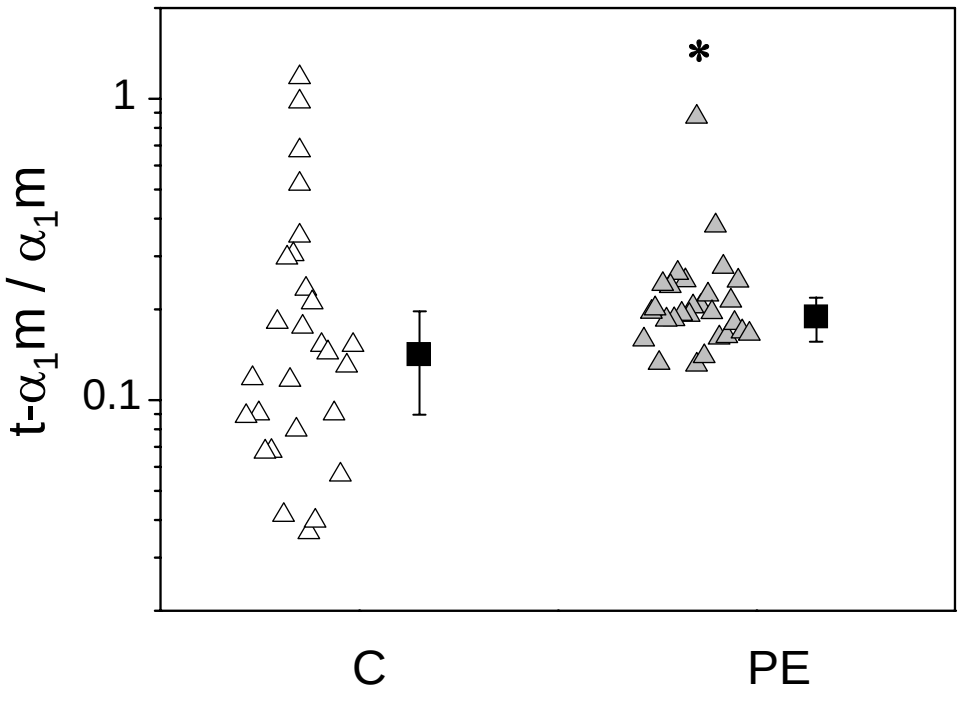

Figure 6

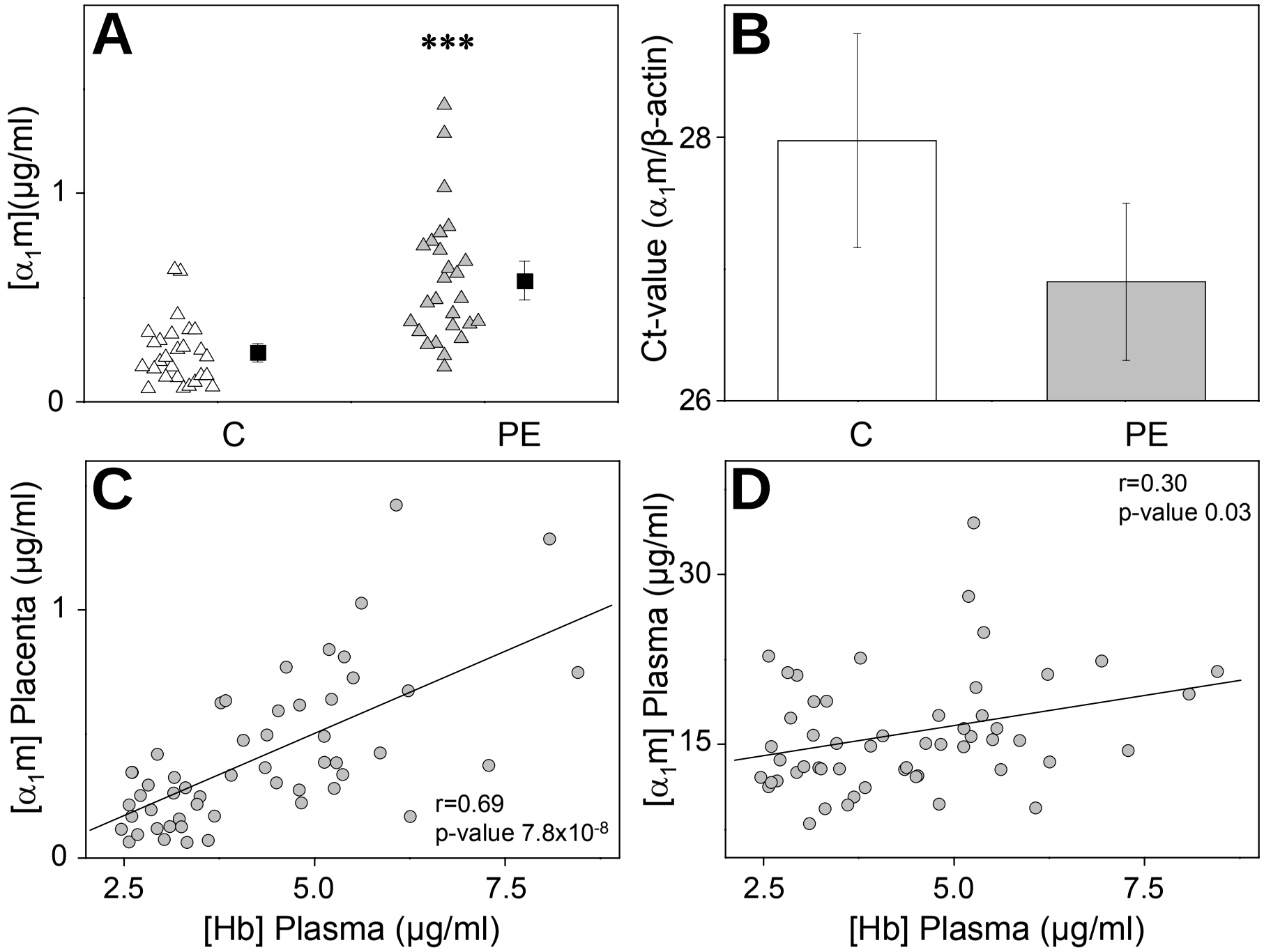
A
***
[α1m](µg/ml)
1
0
C
PE
B
Ct-value (α1m/β-actin)
28
26
C
PE
C
[α1m] Placenta (µg/ml)
1
0
r=0.69
p-value 7.8x10-8
2.5
5.0
7.5
[Hb] Plasma (µg/ml)
D
r=0.30
p-value 0.03
[α1m] Plasma (µg/ml)
30
15
2.5
5.0
7.5
[Hb] Plasma (µg/ml)

Figure 7

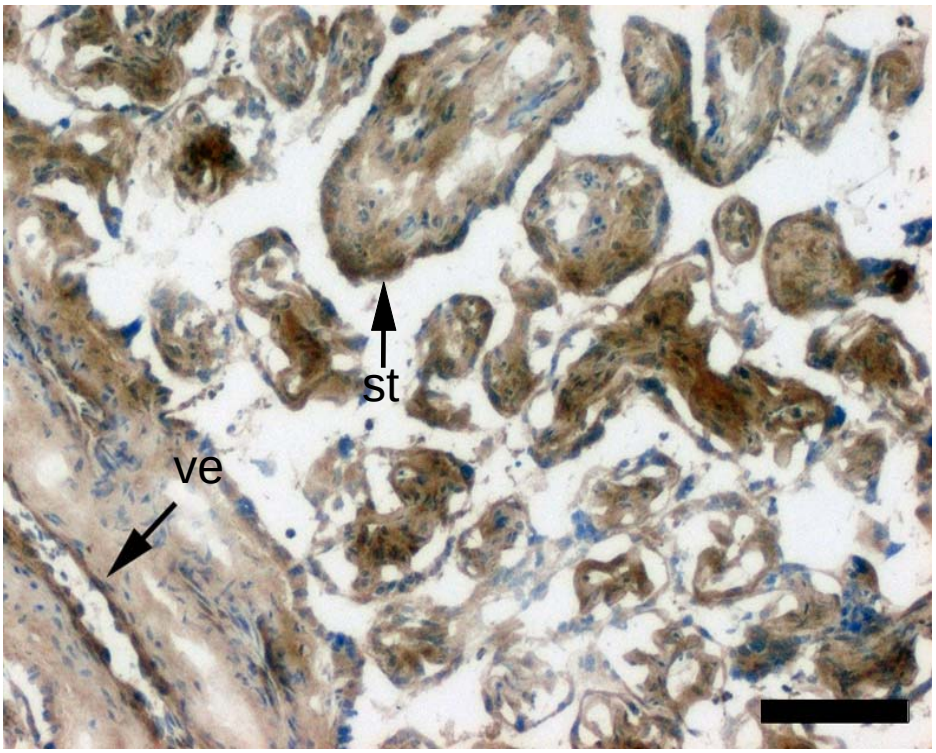

Figure 8

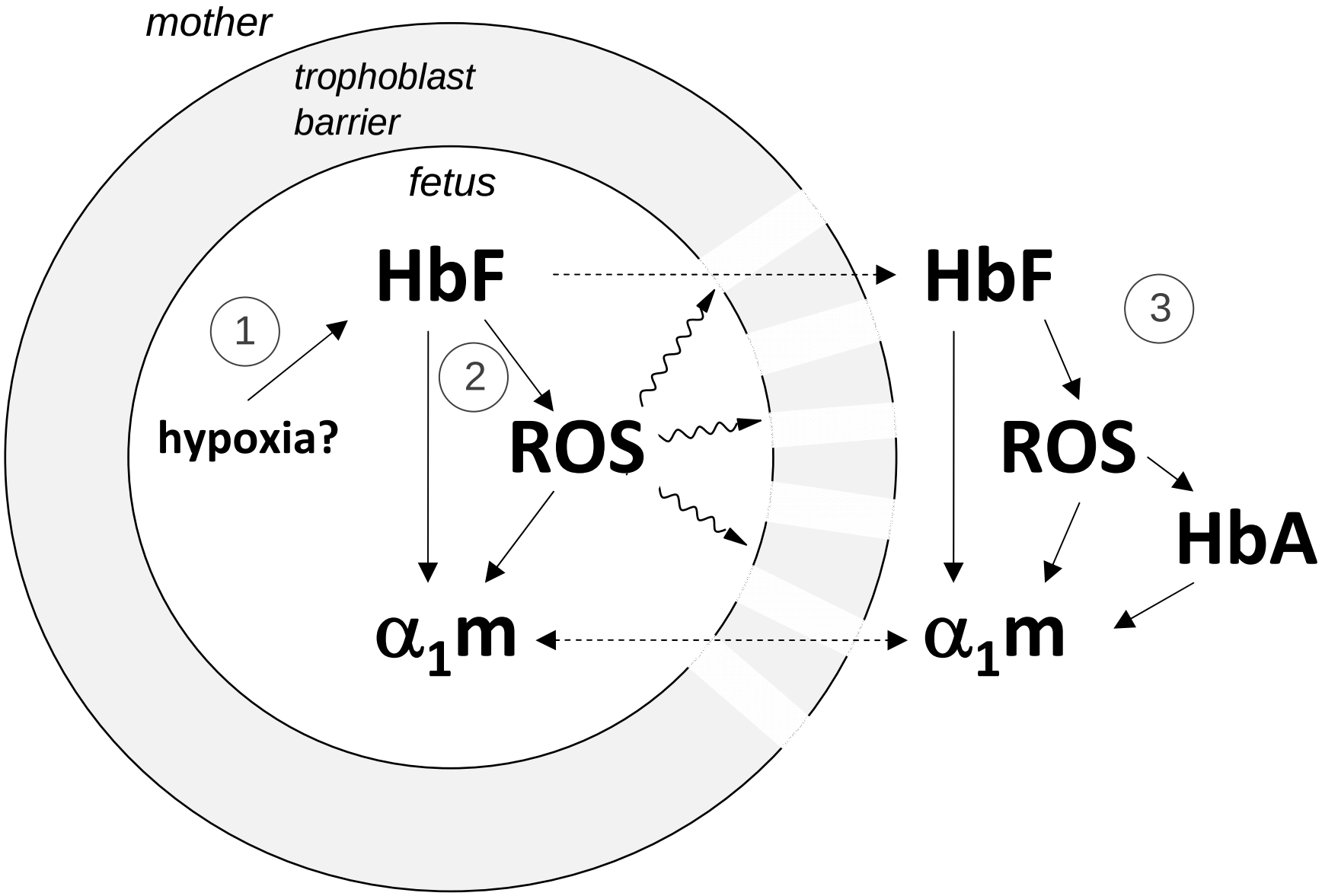
mother
trophoblast barrier
fetus
HbF
HbF
1
2
3
hypoxia?
ROS
ROS
HbA
α1m
α1m

Table 1. Demographics of patients and control group.

Data for maternal age, gestational age and parity are shown as median (range). Systolic pressure, diastolic pressure and albuminuria are shown as mean ± SEM. Child gender is shown as female:male.

	Normal pregnancy C (n=27)	Preeclampsia PE (n=28[a])
Maternal age (years)	32 (23-44)	30 (22-45)
Gestational age (days)	270 (247-295)	266 (253-287)
Parity [b]	1 (0-5)	0 (0-4)
Systolic pressure (mm Hg)	117 ± 11	149 ± 12
Diastolic pressure (mm Hg)	67 ± 4	103 ± 8
Albuminuria (mg/ml)	0.04 ± 0.06	2.78 ± 4.02
Child gender (F:M)	11:16	14:14

[a] One complicated by fetal growth restriction.

[b] Eleven women in the control group were primigravidae whereas 19 women were primigravidae in the PE group.