Published in final edited form as:

Placenta. 2011 November ; 32(11): 823–829. doi:10.1016/j.placenta.2011.08.005.

Increased endoplasmic reticulum stress in decidual tissue from pregnancies complicated by fetal growth restriction with and without pre-eclampsia

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Abstract

Objectives—Endoplasmic reticulum (ER) stress has been implicated in both pre-eclampsia (PE) and fetal growth restriction (FGR), and is characterised by activation of three signalling branches: 1) PERK-pEIF2 α , 2) ATF6 and 3) splicing of XBP1(U) into XBP1(S). To evaluate the contribution of ER stress in the pathogenesis of PE relative to FGR, we compared levels of ER stress markers in decidual tissue from pregnancies complicated by PE and/or FGR.

Study design—Whole-genome transcriptional profiling was performed on decidual tissue from women with PE ($n = 13$), FGR ($n = 9$), PE+FGR ($n = 24$) and controls ($n = 58$), and used for pathway- and targeted transcriptional analyses of ER stress markers. The expression and cellular localisation of ER stress markers was assessed by Western blot and immunofluorescence analyses.

Results—Increased ER stress was observed in FGR and PE+FGR, including both the PERK-pEIF2 α and ATF6 signalling branches, whereas ER stress was less evident in isolated PE. However, these cases demonstrated elevated levels of XBP1(U) protein. ATF6 and XBP1 immunoreactivity was detected in most (> 80%) extravillous trophoblasts, decidual cells and macrophages. No difference in the proportion of immunopositive cells or staining pattern was observed between study groups.

Conclusions—Increased PERK-pEIF2 α and ATF6 signalling have been associated with decreased cellular proliferation and may contribute to the impaired placental growth characterising pregnancies with FGR and PE+FGR. XBP1(U) has been proposed as a negative regulator of ER

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stress, and increased levels in PE may reflect a protective mechanism against the detrimental effects of ER stress.

Keywords

Pre-eclampsia; Fetal growth restriction; Endoplasmic reticulum stress; Gene expression; Decidua basalis

1. Introduction

Impaired spiral artery remodelling is a prominent feature of the decidua basalis in pregnancies complicated by pre-eclampsia (PE) and/or fetal growth restriction (FGR) [1]. The incompletely remodelled spiral arteries retain their musculo-elastic structure, which renders the vessels more responsive to vasoactive stimuli. Subsequent fluctuation in uteroplacental blood supply is proposed to cause ischemia-reperfusion insults and oxidative stress [2]. Several studies have shown that decidual tissue is an important source of oxidative stress [3,4], and increased levels of oxidative stress have been reported in pregnancies complicated by PE and FGR [3,5,6]. Furthermore, ischemia-reperfusion insults can induce endoplasmic reticulum (ER) stress in trophoblast-like cell lines [7].

The ER is an eukaryotic organelle involved in protein folding and maturation, lipid synthesis and calcium homeostasis [8], as well as sensing, coordinating and mediating stress responses [9,10]. Conditions that interfere with ER functions are collectively called ER stress. Stimuli such as accumulation of unfolded proteins, nutrient deprivation and oxidative stress can induce ER stress and activate the unfolded protein response (UPR) [8,9]. The UPR is a cellular self-defence mechanism, aiming to alleviate ER stress and re-establish homeostasis [8]. Three ER transmembrane sensors, PKR-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6), regulate the UPR through their respective signalling cascades [9]. Activated PERK phosphorylates the eukaryotic translation initiation factor 2 α (EIF2 α), which leads to attenuation of mRNA translation and a reduced ER workload [8]. IRE1 splices the constitutively expressed (unspliced) mRNA of the X-box binding protein 1, *XBPI(U)*, into a spliced isoform, *XBPI(S)* [11]. Both *XBPI(U)* and *XBPI(S)* mRNA are translated into transcription factors, but *XBPI(S)* has higher transcriptional activator activity [11]. The transcription factor ATF6 is activated by proteolytic cleavage in the Golgi compartment [12]. With partly overlapping functions, *XBPI* and ATF6 initiate transcription of genes that aim to increase protein folding capacity and degradation of misfolded proteins in ER [8]. These multiple signalling pathways allow for diversity in responses to ER stress, from minor homeostatic adjustments to oxidative stress [13] and activation of inflammatory pathways [10]. Additionally, if the cell fails to combat ER stress, the UPR can trigger apoptosis to eliminate damaged cells [9], but the manner in which the UPR switches from a protective to an apoptotic role is complex and not fully understood [14].

Increased levels of ER stress have been detected in FGR and PE+FGR placentas, where ER stress was associated with decreased cellular proliferation and apoptosis, and proposed as an important cause for the reduced placental growth characterising these phenotypes [15,16]. We recently performed whole-genome transcriptional profiling of decidual tissue from pre-eclamptic and normal pregnancies, identifying upregulation of several transcripts involved in ER stress in PE [17]. Thus, current data indicate that ER stress is involved in the pathogenesis of both PE and FGR, but whether the degree of ER stress differs between these pregnancy complications is unknown. Emerging observations indicate that PE and/or FGR may represent more or less severe stages on a continuous spectrum of responses to impaired placentation, where ischemia-reperfusion insults and oxidative stress following impaired

spiral artery remodelling appear to be common pathophysiological events [16,18]. However, as the clinical outcomes differ, disparate phenomena must take place at some point during the pathogenesis. Given the multiple responses ER stress may elicit, differential activation of UPR signalling branches might explain some of the differences in clinical outcome. The present study aimed to compare the degree of ER stress in pregnancies complicated by PE and/or FGR by analysing transcriptional- and protein expression of key mediators in each branch of the ER stress response.

2. Materials and methods

2.1 Study subjects

Women with pregnancies complicated by PE and/or FGR (cases) and women with uncomplicated pregnancies (controls) were recruited at Trondheim University Hospital (Norway) and Haukeland University Hospital (Bergen, Norway) from 2002 to 2006. PE was defined as persistent hypertension (blood pressure of ≥ 140 mmHg systolic or 90 mmHg diastolic), plus proteinuria (≥ 0.3 g in a 24 h urine collection or $\geq 1+$ according to a dipstick test), developing after 20 weeks of gestation [19]. PE was sub-classified as severe in accordance with criteria recommended by Sibai et al. [20]. FGR was defined as birthweight < 2.5 percentile adjusted for gestational age and sex according to a Scandinavian normogram [21], in addition to at least one of the following criteria: 1) reduced fundal height in serial measurements; 2) serial ultrasound biometry identifying failure to grow along a consistent percentile; or 3) abnormal umbilical artery waveform. Severe FGR was defined as birthweight < 1.7 percentile [22]. Cases diagnosed with PE or FGR before gestational week 34 were classified as early onset. Exclusively healthy women with no prior pregnancy complications were included as controls. Pregnancies with chromosomal aberrations, fetal and placental structural abnormalities or suspected perinatal infections were excluded from both study groups. Cases had caesarean section (CS) performed due to medical indications, whereas controls were undergoing CS for reasons considered irrelevant to the aim of this study (i.e. breech presentation, previous CS or maternal request). Only singleton pregnancies delivered by CS without labour activity were included. The study was approved by the Norwegian Regional Committee for Medical Research Ethics (REK no. 054-02) and informed consent was obtained from all participants.

2.2 Decidual tissue

Decidua basalis tissue was collected by vacuum suction immediately after separation of the placenta from the placental bed during CS [3,23]. The vacuum suction procedure was performed in less than one min. Collected tissue was flushed with saline to remove excessive blood. Decidual tissue was placed in RNAlater (for microarray analyses), 10% neutral-buffered formalin and paraffin embedded (for immunohistochemical analyses) and snap frozen in liquid nitrogen (for Western blot analyses) within 15 min of tissue collection.

2.3 Illumina microarray analysis

Total RNA extracted from decidual tissue was used for synthesis of biotin labelled anti-sense RNA (aRNA) which was hybridised to Illumina HumanWG-6 v2 Expression BeadChips (Illumina Inc., San Diego, CA) as previously described [17]. Microarray expression data were preprocessed and analysed using Sequential Oligogenic Linkage Analysis Routines (SOLAR) [24] as previously described [25]. Microarray were performed in accordance with the Minimum Information About a Microarray Experiment (MIAME) guidelines [26]. Experimental data have been submitted to ArrayExpress (www.ebi.ac.uk/arrayexpress/) under accession no. E-TABM-682. The microarray dataset was validated by quantitative real-time polymerase chain reaction (qRT-PCR) analyses for six of the most differentially expressed transcripts, as previously described [17].

2.4 Pathway and targeted transcriptional analyses of ER stress markers

We recently performed whole-genome transcriptional profiling of decidual tissue from pre-eclamptic and normal pregnancies, identifying upregulation of several transcripts involved in ER stress in PE. In the previous study, we solely focused on *PE associated* transcriptional changes, i.e. using only a subset of the total microarray dataset used in this work [17]. To further explore the role of ER stress in the pathogenesis of *both* PE and FGR, we performed pathway analyses on a group of 20 ER stress related transcripts (as annotated by Ingenuity Pathway Analysis) (Supplementary Table 1) in cases and controls. Pathway analyses were performed using Rotation Gene Set Tests (ROAST) and Rotation Gene Set Enrichment Analysis (ROMER), implemented in the *limma* package [27] available via the Bioconductor Project (www.bioconductor.org). ROAST was used to test whether any of the transcripts in the pathway were differentially expressed [28] and ROMER was used to test whether the subset of transcripts in the pathway was more differentially expressed than any other subset of transcripts in the total dataset [29]. As a second step in our approach, three central ER stress marker from each branch of the UPR (ATF6, XBP1 and PERK), known to be upregulated by ER stress [30,31], were selected for a targeted transcriptional comparison to test if there was any differential UPR activation between cases with PE, FGR, PE+FGR and controls.

2.5 Western blotting

Decidual tissue was homogenised in lysis buffer (Active Motif, Rixensart, Belgium) using a rotor-stator homogeniser (Ultra-Turrax T25, Janke & Kunkel IKA Labortechnik, Staufen, Germany). Total protein extracts were prepared using a Nuclear extract kit (#40010, Active Motif), following manufacturer's instructions. Homogenised decidual tissue was lysed on ice for 30 min and cell debris pelleted at 14,000 g at 4°C for 20 min. The supernatant was recovered and equal amounts (100 µg) of protein were separated on precast 10% denaturing NuPAGE gels (Invitrogen Life Technologies, Carlsbad, CA) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). Membranes were blocked in Odyssey blocking buffer (Li-Cor Biosciences, Cambridge, UK) for 1 h at room temperature (RT) before incubating with primary antibodies against pEIF2 α , EIF2 α , XBP1 and ATF6. The antibody against XBP1 detected both spliced and unspliced variants of XBP1 [32,33], and the antibody against ATF6 detected both cleaved and uncleaved ATF6 [33,34]. Blots were reprobed with β -actin as a loading control. Primary antibodies were diluted in Odyssey blocking buffer (Li-Cor Biosciences) and hybridised to the membranes overnight at 4°C. Membranes were washed 3 $\times$ 10 min in tris-buffered saline (TBS) and incubated with fluorescently labelled secondary antibodies diluted in Odyssey blocking buffer (Li-Cor Biosciences) for 1 h at RT. Specifications for the primary and secondary antibodies are listed in Table 1. Membranes were scanned using the Odyssey Infrared Imaging System (Li-Cor Biosciences). Specific bands for pEIF2 α (37 kDa), EIF2 α (37 kDa), XBP1(U) (31 kDa), XBP1(S) (54 kDa), ATF6 (50 kDa) and β -actin (42 kDa) were detected in all samples. Band intensities were determined from two or three scans, normalised relative to loading control and quantified by densitometric analysis using Odyssey imaging software v3.0 (Li-Cor Biosciences).

2.6 Immunohistochemical analyses

Cellular localisation and expression of ATF6 and XBP1 in decidual tissue was assessed using the same antibodies against XBP1 and ATF6 as used in Western blot experiments, in combination with antibodies against cytokeratin 7 (CK7) to detect extravillous trophoblasts (EVTs), vimentin and prolactin to detect decidual cells (DeCs), and CD68 to detect macrophages (M ϕ s). Double immunofluorescence staining was performed manually after deparaffination in xylene, rehydration and heat-induced antigen retrieval in TBS. Protein Block (X0909, Dako, Glostrup, Denmark) was added for 10 min to inhibit non-specific

binding, and slides were incubated in a primary antibody mixture overnight at 4°C. The slides were incubated with appropriate species-specific secondary antibodies for 30 min in a dark chamber. Slides were examined using a fluorescent microscope (Nikon Eclipse 90i with CytoVision 3.7 software, Applied Imaging, New Milton Hampshire, UK) at magnification $\times 600$. Decidual glands, tonsil tissue and pituitary glands were used as positive controls for CK7, vimentin/CD68 and prolactin, respectively. For negative controls, primary antibodies were substituted with isotype-matched rabbit- (#011-000-120, Jackson ImmunoResearch, PA) and mouse (#400102, BioLegend, CA) immunoglobulins. Specifications for the primary and secondary antibodies are listed in Table 1. The proportion of XBP1 and ATF6 immunopositive EVTs, DeCs and M ϕ s was counted in five randomly selected fields on each slide, and calculated as the number of positive cells among the total number of CK7, vimentin/prolactin or CD68 positive cells, respectively.

2.7 Statistical analyses

Nonparametric data were analysed using Mann-Whitney U test, whereas parametric data were analysed using the Students *t*-test. Fisher's exact test and χ^2 test were used for categorical data, and Spearman's rank correlation was used for correlation analyses. The significance threshold was set to 0.05. All analyses were performed using SPSS v.15 (SPSS, Chicago, IL).

3. Results

3.1 Clinical characteristics of the study subjects

Clinical characteristics of the study subjects included in the microarray analysis are presented in Table 2. A total of 104 samples were included (PE, *n* = 13; FGR, *n* = 9; PE +FGR, *n* = 24 and controls, *n* = 58). As expected, elevated blood pressure was observed in pregnancies with PE and PE+FGR, and lower gestational age and birthweight were observed in all case groups (Table 2).

A total of 30 samples (PE, *n* = 7; FGR, *n* = 7; PE+FGR, *n* = 8 and controls, *n* = 8) were included in Western blot analysis (Table 3). Twenty-four of these were also included in the microarray study population, whereas the remaining six were selected from our total study population, but not included in microarray analyses due to low RNA quality. As ER stress probably arises secondary to ischemia-reperfusion insults, we selected cases with clinical characteristics suggesting vascular malperfusion, which is closely associated with abnormal uterine artery Doppler findings and more common in early onset, severe PE [35] and severe FGR [36]. Thus, only cases with severe clinical characteristics were included, i.e. cases with uterine artery score ≥ 1 and/or early onset PE or FGR (Table 3). The mean uterine artery score was higher in all case groups compared to controls, whereas the placental weight ratio was lower in cases with FGR and PE+FGR as compared to controls (Table 3). Sixteen decidual samples selected from the Western blot study population were included in immunohistochemical analyses (PE, *n* = 4; FGR, *n* = 4; PE+FGR, *n* = 4 and controls, *n* = 4). The clinical characteristics of these samples did not differ from those included in Western blot analyses.

3.2 Pathway and targeted transcriptional analyses of ER stress markers

Pathway analyses (ROAST and ROMER) showed that the ER stress pathway was upregulated in cases with FGR and PE+FGR, whereas in PE, a trend towards upregulation was observed (Table 4). The targeted comparison of transcript data for *XBPI*, *ATF6* and *PERK* demonstrated that cases with FGR had increased expression of *ATF6*, cases with PE +FGR had increased expression of *XBPI*, *ATF6* and *PERK*, whereas *XBPI* was increased in PE (Table 4). The Illumina probe interrogating *XBPI* (ILMN_1710675) detected both the

spliced and unspliced *XBP1* variants. No differences between the case groups were observed for any of these three transcripts (*data not shown*).

3.3 Western blot analyses

Cases with FGR and PE+FGR showed increased phosphorylation of EIF2 α ($p < 0.05$), increased ratio of pEIF2 α /EIF2 α ($p < 0.05$) and increased levels of ATF6 ($p < 0.01$) (Fig. 1A–C). Although cases with PE had high mean levels of pEIF2 α /EIF2 α and ATF6, large variations within this group rendered the results non-significant in comparison to controls ($p = 0.09$ and 0.43 , respectively), as indicated by the larger standard deviation bars for the PE group in Fig 1B–C. No differences in protein levels of the spliced form of XBP1, XBP1(S), were observed between any of the case groups compared to controls. However, increased levels of the unspliced form of XBP1, XBP1(U), and a decreased ratio of XBP1(S)/XBP1(U) were observed in isolated PE ($p < 0.05$ and 0.01 , respectively) (Fig. 1A and C). The levels of XBP1(U) was higher in PE compared to that of FGR, and the ratio of XBP1(U)/XBP1(S) was lower in PE compared to FGR (all p 's < 0.05). No differences in protein levels of pEIF2 α /EIF2 α , ATF6 or XBP1(S) were observed between case groups.

3.4 Immunohistochemical analyses

Both cytoplasmic and nuclear immunoreactivity for XBP1 and ATF6 was observed in most ($> 80\%$) EVT, DeCs and M ϕ s (Fig. 2A, A'–R, R'). No differences in the proportion of XBP1 or ATF6 immunopositive cells or staining pattern were observed between the study groups. In general, XBP1 and ATF6 immunoreactivity was present in most cells in the decidual tissue, in contrast to tonsil tissue and pituitary glands, in which only a few cells were positive (*not shown*). Decidual glands displayed intense nuclear immunoreactivity for XBP1 in both cases and controls (Fig. 2S). No differences in staining intensity of XBP1 and ATF6 were observed between the study groups in any of the evaluated cell types.

3.5 Correlation analyses

Correlation analyses demonstrated a negative correlation between the pEIF2 α /EIF2 α ratio and the placental weight ratio ($r_s = -0.561$, $p < 0.05$). A similar tendency was observed for ATF6 ($r_s = -0.352$, $p = 0.072$). None of the ER stress markers were correlated with gestational age.

4. Discussion

In this work, we have shown that decidual ER stress is increased in pregnancies complicated by FGR and PE+FGR. Initially, this was demonstrated by pathway analyses, showing upregulation of the ER stress pathway in FGR and PE+FGR. In addition, targeted transcriptional and protein analyses showed upregulation of the PERK-pEIF2 α and ATF6 signalling branches of the UPR. In PE, none of these branches were significantly altered. However, a marked increase in XBP1(U) was observed. These results indicate divergent activation of the UPR in FGR and PE+FGR compared to isolated PE.

Consistent with our finding of increased pEIF2 α in deciduas from FGR and PE+FGR, increased placental levels of pEIF2 α have previously been reported in these cases [15]. In trophoblast-like cell lines, increased levels of pEIF2 α were associated with reduced proliferation through suppression of protein synthesis and decreased survival [15]. The net effect of reduced proliferation and cell survival was proposed as a cause for reduced placental growth in pregnancies with FGR and PE+FGR [15,16], which are characterised by decreased placental villous tissue volume and surface area [37]. Of relevance, we observed that the pEIF2 α /EIF2 α ratio was negatively correlated with placental weight ratio, with a

similar tendency for ATF6, suggesting an association between ATF6 and PERK-pEIF2 α signalling and reduced placental weight.

Cases with isolated PE only showed a trend towards upregulation of the ER stress response. However, we observed increased levels of *XBP1* mRNA and XBP1(U) protein in isolated PE. It was recently shown that XBP1(U), the constitutively expressed form of XBP1, accumulates in the recovery phase of ER stress [38] and is able to inhibit XBP1(S) and ATF6, implying that XBP1(U) functions as a negative regulator during ER stress [39–40]. This correlates well with our finding of the highest level of XBP1(U) and the lowest level of XBP1(S) in PE, and that ATF6 was not significantly increased in these cases. Based on this, it is tempting to speculate that IRE1-XBP1 signalling via upregulation of XBP1(U) may protect against or decrease the effects of ER stress in PE, which could be reflected by the normal placental weight ratio observed in this group. Correspondingly, reduced placental weight in FGR and PE+FGR, but not in isolated PE, was recently reported in a Norwegian cohort of 317,688 pregnancies [41].

Both nuclear and cytoplasmic immunoreactivity for XBP1 and ATF6 was observed in EVT, Decs and M ϕ s, but no differences in the proportion of immunoreactive cells or staining pattern was observed between the study groups. No differences in cytoplasmic staining intensity for XBP1, representing XBP1(U), or nuclear staining intensity of ATF6, representing cleaved ATF6, were detected in any of these cell types between the study groups. In general, nuclear staining intensity was difficult to assess due to photobleaching. Thus, no determination of the cellular source of increased levels of XBP1(U) or cleaved ATF6 in decidual tissue, as detected by Western blot, could be made by immunofluorescence analysis. However, we cannot exclude that other cell types in the decidua, that were not evaluated in the present work may have contributed to the increased ATF6 and XBP1(U) levels.

In summary, we found that decidua basalis is a source of ER stress, and that ER stress is increased in pregnancies complicated by FGR and PE+FGR. In PE, we found increased levels of XBP1(U), which may be a protective mechanism against the detrimental effects of ER stress. This could explain some of the observed clinical differences in between PE and FGR. However, future studies are warranted to test this hypothesis and elucidate the implications of our findings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Mette Langaas for her contribution to the pathway analyses and Linda T. Roten for her valuable comments made during manuscript preparation. We would like to thank Kristine Pettersen, Anne Gøril Lundemo, Caroline H.H. Pettersen and Svanhild A. Schønberg at the Department of Cancer Research and Molecular Medicine, Faculty of Medicine, Norwegian University of Science and Technology (NTNU), for their technical support and help in optimising the Western blot protocols. We are deeply grateful to Toril Rolfseng at Department of Laboratory Medicine, Children's and Women's Health, NTNU, for performing the immunofluorescence staining. This study was supported by grants from NTNU (IAL, ML, SBM, IPE), functional genomics (FUGE) mid-Norway (RA), Haukeland University Hospital, Bergen, Norway (LB), and in part by grants from Southwest Foundation Forum (MPJ), National Institutes of Health (NIH) Grant R01 HD049847 (EKM) and Research Facilities Improvement Program Grant C06 RR017515 from the National Center for Resources, NIH. The funding sources had no involvement in study design, data collection and analyses, or in the preparation and submission of this article.

Abbreviations

ER	endoplasmic reticulum
UPR	unfolded protein response
PERK	PKR-like ER kinase
IRE1	inositol-requiring enzyme 1
ATF6	activating transcription factor 6
EIF2α	eukaryotic translation initiation factor 2 α
XBP1	X-box binding protein 1

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Fig 1.

Western blot analyses of endoplasmic reticulum (ER) stress markers in decidual tissue from pregnancies complicated by preeclampsia (PE, $n = 7$), fetal growth restriction (FGR, $n = 7$), PE+FGR ($n = 8$) and controls ($n = 8$). (A) A representative Western blot of phosphorylated and total EIF2α, ATF6, spliced (S) and unspliced (U) forms of XBP1 and loading control (β-actin). (B and C) Densitometry of bands expressed relative to controls (100%), showing increased levels of pEIF2α and ATF6 in pregnancies complicated by FGR and PE+FGR. Increased levels of XBP1(U) and a decreased XBP1(S)/XBP1(U) ratio were observed in pregnancies complicated by PE. Phosphorylation status of EIF2α is presented as the ratio between phosphorylated and total level of EIF2α. No significant differences in EIF2α were observed between any of the study groups. Data are presented as means $\pm$ SE, * $p < 0.05$ as compared to controls.

Fig 2.

Immunofluorescence analyses of XBP1 and ATF6 in extravillous trophoblasts (EVTs), decidual cells (DeCs) and macrophages (Mφs), identified using antibodies against cytokeratin 7, vimentin/prolactin and CD68, respectively. Nuclei were counterstained with 4'-6-diamidino-2-phenylindole (DAPI) (blue signal). Cytoplasmic and nuclear immunoreactivity of XBP1 (red signal) was observed in EVT, DeCs and Mφs in both cases with PE and/or FGR (A–I) and controls (A'–I'). A similar staining pattern was observed for ATF6 (green signal) for the same cell types in cases with PE and/or FGR (J–R) and controls (J'–R'). In decidual glands, strong nuclear immunoreactivity for XBP1 (red signal) was observed (S). No positive staining was found in negative controls where primary antibodies were substituted with isotype-matched immunoglobulins of the appropriate species (T). Magnification ×600 in D'–F', S and T, otherwise ×1000. Scale bar 2μm.

Table 1
Primary and secondary antibodies used for Western blot and immunohistochemical analyses.

Species	Manufacturer	Catalogue no.	Dilution Western Blot	Dilution immunofluorescence
<i>Primary antibodies</i>				
XBP1	Rabbit anti-human	Santa Cruz Biotechnology, CA	sc-7160	1:500
ATF6	Mouse anti-human	Imgenex, San Diego, CA	IMG-273	1:200
pEIF2 α	Rabbit anti-human	Cell Signaling Technology, Danvers, MA	#9721	1:1500
EIF2 α	Rabbit anti-human	Cell Signaling Technology, Danvers, MA	#9722	1:3000
β -actin	Mouse anti-human	Abcam, Cambridge, UK	ab6276-100	1:30,000
CK7	Mouse anti-human	Dako, Glostrup, Denmark	M7018	1:750
CK7	Rabbit anti-human	Abcam, Cambridge, UK	ab52870	1:50
Vimentin	Mouse anti-human	Dako, Glostrup, Denmark	M0725	1:200
Prolactin	Rabbit anti-human	Dako, Glostrup, Denmark	A0569	1:60
CD68	Mouse anti-human	Dako, Glostrup, Denmark	M0814	1:1000
CD68	Rabbit anti-human	Santa Cruz Biotechnology, CA	sc-9139	1:5
<i>Secondary antibodies</i>				
IRDye 680	Goat anti-rabbit	Li-Cor Biosciences, Cambridge, UK	#926-32221	1:5000
IRDye 800CW	Donkey anti-mouse	Li-Cor Biosciences, Cambridge, UK	#926-32212	1:5000
<i>a</i> FITC	Goat anti-mouse	Dako, Glostrup, Denmark	F0479	1:10
<i>b</i> TRITC	Swine anti-rabbit	Dako, Glostrup, Denmark	R0156	1:10

^aFluorescein iso-thiocyanate

^bTetramethyl rhodamine iso-thiocyanate.

Table 2Clinical characteristics of the study subjects included in the Microarray analysis ($n = 104$).

	PE ($n = 13$)	FGR ($n = 9$)	PE+FGR ($n = 24$)	Controls ($n = 58$)
Maternal age (y)	30.1 $\pm$ 5.4	32.7 $\pm$ 6.4	29.7 $\pm$ 5.5	32.4 $\pm$ 5.1
Systolic BP (mmHg)	153.1 $\pm$ 17.5**	128.3 $\pm$ 14.7*	151.0 $\pm$ 14.9**	116.2 $\pm$ 10.4
Diastolic BP (mmHg)	94.7 $\pm$ 8.6**	74.4 $\pm$ 8.3	96.2 $\pm$ 12.1**	69.9 $\pm$ 8.8
^a BMI (kg/m ²)	29.2 $\pm$ 6.3	28.5 $\pm$ 7.5	26.4 $\pm$ 6.1	26.0 $\pm$ 4.5
Primipara	8/13	4/9	16/24	^b 29/55
GA (weeks)	34.8 $\pm$ 2.7**	31.9 $\pm$ 5.0**	30.3 $\pm$ 3.5**	38.7 $\pm$ 0.8
Birthweight (g)	2363.9 $\pm$ 509.7**	1224.7 $\pm$ 672.4**	1118.2 $\pm$ 470.1**	3608.3 $\pm$ 491.9

Data are presented as means $\pm$ SD*
 $p < 0.05$ **
 $p < 0.001$ comparing pre-eclampsia (PE), fetal growth restriction (FGR) and PE+FGR to controls; BP, blood pressure^a BMI, Body Mass Index measured at first antenatal care visit^b No info available for three of the controls GA, gestational age.

Table 3

Clinical characteristics of the study subjects included in the Western blot analysis ($n = 30$).

	PE ($n = 7$)	FGR ($n = 7$)	PE+FGR ($n = 8$)	Controls ($n = 8$)
Birthweight (g)	2137.1 $\pm$ 259.6**	1363.1 $\pm$ 753.8**	1105.3 $\pm$ 329.1**	3576.3 $\pm$ 507.1
Placenta weight (g)	447.8 $\pm$ 30.3*	269.2 $\pm$ 115.0*	261.3 $\pm$ 55.9**	657.9 $\pm$ 163.9
^a Placenta Weight Ratio	1.0 $\pm$ 0.3	0.5 $\pm$ 0.2*	0.7 $\pm$ 0.1*	0.9 $\pm$ 0.1
Severe PE or FGR	5/7	6/7	8/8	
Early onset < 34 weeks	7/7	5/7	8/8	
^b Mean uterine artery score	2.4**	2.2*	3.5**	0

Data are presented as means $\pm$ SD

* $p < 0.05$

** $p < 0.001$ comparing pre-eclampsia (PE), fetal growth restriction (FGR) and PE+FGR to controls

^a Placenta Weight Ratio was calculated as observed/expected placenta weight according to gestational age and sex according to a Norwegian normogram [42]

^b Uterine artery score is based on pulsatility index and notching in Aa.uterinae [43]; Blood pressure, maternal age, gestational age, Body Mass Index and parity did not differ statistically from that of women included in the microarray analysis (Table 2).

Table 4

Pathway and targeted transcriptional analyses of ER stress related genes.

	PE vs. controls	FGR vs. controls	PE+FGR vs. controls
^a Pathway analysis			
ROAST	$p = 0.06$ (NS)	$\uparrow, p < 0.05$	$\uparrow, p < 0.001$
ROMER	$p = 0.09$ (NS)	$\uparrow, p < 0.05$	$\uparrow, p < 0.05$
^b Microarray analysis			
<i>XBPI</i>	$\uparrow, p < 0.01$	$p = 0.07$ (NS)	$\uparrow, p < 0.05$
<i>ATF6</i>	$p = 0.22$ (NS)	$\uparrow, p < 0.05$	$\uparrow, p < 0.001$
<i>PERK</i>	$p = 0.09$ (NS)	$p = 0.55$ (NS)	$\uparrow, p < 0.05$

^aRotation Gene Set Tests (ROAST) and Rotation Gene Set Enrichment Analysis (ROMER) pathway analyses of 20 transcripts involved in the endoplasmic reticulum (ER) stress response (Supplementary Table 1)

^bA targeted comparison of transcription data for ER stress markers from each branch of ER stress response; NS, not significant; $\uparrow$ upregulation.