Differential gene expression analysis of placentas with increased vascular resistance and pre-eclampsia using whole-genome microarrays.

Centlow, Magnus; Wingren, Christer; Borrebaeck, Carl; Brownstein, M J; Hansson, Stefan

Published in:
Journal of Pregnancy

DOI:
10.1155/2011/472354

2011

Link to publication

Citation for published version (APA):

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain.
- You may freely distribute the URL identifying the publication in the public portal.

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.
Research Article

Differential Gene Expression Analysis of Placentas with Increased Vascular Resistance and Pre-Eclampsia Using Whole-Genome Microarrays

M. Centlow, C. Wingren, C. Borrebaeck, M. J. Brownstein, and S. R. Hansson

1 Departments of Obstetrics and Gynecology and Clinical Sciences, Lund University, BMC C14, Klinikgatan 28, 221 84 Lund, Sweden
2 Department of Immunotechnology and CREATE Health, Lund University, 22100 Lund, Sweden
3 National Institute of Mental Health, NIH, Bethesda, MD 20892-9663, USA

Correspondence should be addressed to S. R. Hansson, stefan.hansson@med.lu.se

Received 11 September 2010; Revised 30 November 2010; Accepted 30 December 2010

Pre-eclampsia is a pregnancy complication characterized by hypertension and proteinuria. There are several factors associated with an increased risk of developing pre-eclampsia, one of which is increased uterine artery resistance, referred to as “notching”. However, some women do not progress into pre-eclampsia whereas others may have a higher risk of doing so. The placenta, central in pre-eclampsia pathology, may express genes associated with either protection or progression into pre-eclampsia. In order to search for genes associated with protection or progression, whole-genome profiling was performed. Placental tissue from 15 controls, 10 pre-eclamptic, 5 pre-eclampsia with notching, and 5 with notching only were analyzed using microarray and antibody microarrays to study some of the same gene product and functionally related ones. The microarray showed 148 genes to be significantly altered between the four groups. In the preeclamptic group compared to notching only, there was increased expression of genes related to chemotaxis and the NF-kappa B pathway and decreased expression of genes related to antigen processing and presentation, such as human leukocyte antigen B. Our results indicate that progression of pre-eclampsia from notching may involve the development of inflammation. Increased expression of antigen-presenting genes, as seen in the notch-only placenta, may prevent this inflammatory response and, thereby, protect the patient from developing pre-eclampsia.

1. Introduction

In the western world, 3–7% of all pregnancies are affected by pre-eclampsia (PE). The etiology of PE is still poorly understood, and several theories have been put forward [1–4].

PE is considered a two-stage disease [5, 6]. The first stage is characterized by poor placentaation resulting from shallow invasion of the trophoblasts into the maternal spiral arteries. Instead of the low-resistance high-flow system seen in the normal placenta, the PE placenta has increased resistance, and a decreased blood flow and uneven perfusion leads to hypoxia, ischemia, and reperfusion injuries in the placenta [5, 6].

The second stage of PE is characterized by a general vascular endothelial damage, which eventually affects all maternal organs. Transition from stage one, to stage two of the disease is typically seen before 35 gestational weeks (GW) in early onset PE. Placentas from early onset PE more often show histological pathological findings associated with poor placental perfusion, such as infarcts and chronic inflammation [7]. In contrast, placentas from late-onset PE, manifest after 35 GW, rarely show these pathological findings. The placental factors linking the first and second stage are not fully known. However, some placental factors that may participate in the progression to the second stage have been identified [8].

Increased vascular resistance in the uterine arteries is associated with decreased placental perfusion. The former can be measured by Doppler ultrasound as early as 12 GW and quantified using pulsatility index (PI) which is calculated by subtracting the peak systolic velocity from the end
controls, 10 PE without notch, 5 PE with notches, and from 35 women admitted to Lund University Hospital (15
mum velocity averaged over one cardiac cycle. Increasing PI
(Philips Medical Systems, Voithell, USA). The uterine artery
formed at 18 and 24 GW, using a Philips HDI 5000 system

2.1. Sample Collection. Placental samples were obtained
2.2. RNA Extraction

2.2.1. Tissue. RNA was extracted with TRIzol according to
The pulsatility index (PI) was computed automatically by the
diastolic notch detected in early pregnancy
As many as 15–20% of all pregnancies show signs of
pattern described as “notching” detected in early pregnancy
uterine artery resistance is also correlated with a diastolic
increased vascular resistance, which has been shown to
correlate well with early onset and severe PE [9]. Increased
after delivery and immediately put on dry ice. Since the
placenta is a very heterogeneous tissue and the placenta gene
eexpression differs between different parts of the placenta,
sampling was always performed in a central portion of
the placenta in an attempt to keep the tissue samples as
homogenous as possible [18]. Maternal blood samples, 6 ml,
were collected before delivery into PAXgene blood RNA
tubes (Qiagen, Solna, Sweden) according to the manufac-
turer’s instructions. Samples were stored at −80 °C until
usage.

was visualized with color Doppler imaging on each side
at the level of crossing with the external iliac artery. The
pulsatility index (PI) was computed automatically by the
system in both the right and left uterine arteries. The
presence or absence of a diastolic notch in the waveform
was assessed visually. Only patients with bilateral notching
were included in the notch group. A 10 × 10 × 10 mm
cube of villous tissue from one cotyledon was removed
after delivery and immediately put on dry ice. Since the
placenta is a very heterogeneous tissue and the placenta gene
expression differs between different parts of the placenta,
sampling was always performed in a central portion of
the placenta in an attempt to keep the tissue samples as
homogenous as possible [18]. Maternal blood samples, 6 ml,
were collected before delivery into PAXgene blood RNA
tubes (Qiagen, Solna, Sweden) according to the manufac-
turer’s instructions. Samples were stored at −80 °C until
usage.

2.2. RNA Extraction

2.2.1. Tissue. RNA was extracted with TRIzol according to
the manufacturer’s instructions (Invitrogen, Carlsbad, USA).
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 12000 g for 10 minutes after which
chloroform was added. The aqueous phase containing the
RNA was isolated. To precipitate the RNA, 2-propanol
with sodium citrate was used. The sodium citrate was
added in order to remove contaminating proteoglycans
and polysaccharides. The RNA pellet was washed once
with 75% (v/v) ethanol, and then allowed to air dry. The
pellet was dissolved in RNase-free water and assayed using
a NanoDrop spectrophotometer (Thermo Fisher Scientific,
Waltham, USA). To remove residual phenol, the RNA was
again precipitated with 2-propanol, and washed three times
with 75% (v/v) ethanol before it was used.

RNA integrity was assessed on a 1% (w/v) agarose gel
containing 6.7% (v/v) formaldehyde run with 1× MOPS
buffer. RNA samples were required to have clear bands at

**Table 1: Clinical characteristics of patients at delivery.**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PE</th>
<th>PE/N</th>
<th>Notch</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>15</td>
<td>10</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Age (y)</td>
<td>31 (24–37)</td>
<td>29 (22–34)</td>
<td>32 (27–39)</td>
<td>32 (23–39)</td>
</tr>
<tr>
<td>Gestational age (days)</td>
<td>280 (253–295)</td>
<td>264 (227–285)</td>
<td>241 (204–283)</td>
<td>250 (219–284)</td>
</tr>
<tr>
<td>Systolic pressure (mmHg)</td>
<td>120 (110–135)</td>
<td>159 (140–170)</td>
<td>151 (145–160)</td>
<td>110 (115–120)</td>
</tr>
<tr>
<td>Diastolic pressure (mmHg)</td>
<td>75 (60–80)</td>
<td>105 (100–120)</td>
<td>100 (98–105)</td>
<td>78 (60–80)</td>
</tr>
<tr>
<td>Proteinuria (g/l)</td>
<td>0 (0–0.3)</td>
<td>3 (0.3–5)</td>
<td>3 (0.3–5)</td>
<td>0 (0–1)</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>3780 (2900–4315)</td>
<td>3100 (2500–3860)</td>
<td>1590 (1100–3430)</td>
<td>2790 (1400–3850)</td>
</tr>
<tr>
<td>Gender M:F</td>
<td>7:8</td>
<td>5:5</td>
<td>2:3</td>
<td>3:2</td>
</tr>
<tr>
<td>Pulsatility index</td>
<td>NA</td>
<td>0.69 (0.59–0.73)</td>
<td>0.99 (0.67–1.43)</td>
<td>0.93 (0.83–1.76)</td>
</tr>
<tr>
<td>Mode of delivery (VD:CS)</td>
<td>15:0</td>
<td>7:3</td>
<td>3:2</td>
<td>4:1</td>
</tr>
</tbody>
</table>

All data is presented as median (range) except child gender (Male : Female) and mode of delivery (VD : CS). A Kruskal-Wallis post hoc Dunn test was used to calculate statistical differences between the groups. PE: pre-eclampsia, PE/N: pre-eclampsia with bilateral notching, NA: not available, VD: vaginal delivery, and CS: cesarean section. *A significant difference between controls versus PE/N (P < .05).
28S and 18S, respectively, with no other bands present on the gel. No samples with poor RNA quality were used for further analysis.

2.2.2. Blood. RNA was extracted using PAXgene Blood RNA Kit according to the manufacturer’s instructions (Qiagen). Briefly, samples were centrifuged and the supernatant discarded after which the pellet was resuspended in 5 ml RNase-free water. Following centrifugation, proteins were removed by the addition of proteinase K. Samples were added to PAXgene columns and washed with the corresponding buffers after which RNA was eluted in 80 μl elution buffer. RNA was quantified and assessed as above.

2.3. DNA Microarrays. Microarrays were produced at the Swegene DNA Microarray Resource Center, Department of Oncology, Lund University, Sweden (http://swegene.onk.lu.se). Human array-ready oligonucleotide libraries Version 2.1 and Version 2.1.1-upgrade, comprising approximately 27,000 unique probes, were obtained from Operon (Operon Biotechnologies, Germany). Probes were dissolved in Corning Universal Spotting solution (Corning, Acton, MA) and printed on aminosilane coated glass slides (Corning) using a MicroGrid2 robot (BioRobotics, Cambridgeshire, UK) equipped with MicroSpot 10K pins (BioRobotics, Cambridgeshire, UK). Following printing, arrays were left in a desiccator to dry for 48 hours, rehydrated for 1 second over steaming water, snap-dried on a hot plate (98°C), and UV cross-linked (800 mJ/cm²).

Complementary DNA for microarray was synthesized and labeled with fluorescence, Cy3 or Cy5 (Amersham Biosciences, Buckinghamshire, UK), using the Promega ChipShot labeling system according to the manufacturers’ instructions (Corning). Briefly, Cy3- and Cy5-labeled cDNA was prepared using 5 μg total RNA. Pretreatment of arrays, hybridization, and posthybridization wash carried out according to the manufacturers’ instructions (Promega ChipShot labeling system and Promega, Madison, USA). All experimental samples were hybridized individually against a common reference (a placental sample from a normotensive pregnancy without any complications). Samples were run in single experiments.

Samples were run individually (i.e., no groups were pooled). The arrays were not run in replicates, each sample was only assessed once.

2.3.1. Imaging and Data Analysis. To image hybridized microarray slides, an Agilent G2565AA microarray scanner (Agilent Technologies, Palo Alto, USA) was used. Image analysis was done in GenePix Pro 4.0 (Axon Instruments Inc., Foster City, USA). Fluorescence intensities were extracted and spots with grains of dust and other contaminants were identified and eliminated. The retrieved intensity values were uploaded into Bio Array Software Environment (BASE) (http://base.thep.lu.se) for further analysis [19]. In BASE, Cy3 and Cy5 signals were corrected for background using the median spot pixel intensity and median local background pixel intensity. Fluorescence ratios were calculated as Cy3/Cy5 and log (base 2) transformed. Spots were filtered for intensity below 350 in any of the two channels and biased spots identified in GenePix. Following that, filtering spots were normalized using Lowess normalization and then corrected for common reference bias using gene median centering, after which the data was filtered for fold change equal to or above 1.5. [20]. Group comparison was conducted using a false discovery rate modified t-test [21]. A P-value < .005 was considered statistically significant. The data has been uploaded by MIAMExpress submission and annotation tool European Molecular Biology Laboratory European Bioinformatic Institute Microarray Informatics.

2.3.2. Bioinformatic Analysis. We used Gene Ontology (GO, http://www.geneontology.org/), the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/), and InterPro (http://www.ebi.ac.uk/interpro/) to classify significantly altered genes according to their molecular function, their participation in signaling pathways, and the presence of protein domains, respectively, as previously described [15]. The genes were also annotated and analyzed using the database for annotation, visualization, and integrated discovery (DAVID) with the whole human genome, approximately 27,000 genes, as background [22]. Statistical differences in GO or KEGG were determined by comparing the expected number of genes in each GO-category/KEGG-pathway (as determined by the background genome) with the actual number of altered genes as determined by the microarray results. In each category, both activating and suppressing genes may be present. Altered categories as well as the genes corresponding to them are listed in Table 3. A P-value below .05 with a fold change above 2 was used as cutoff.

2.4. Quantitative Real-Time PCR

2.4.1. cDNA Synthesis. cDNA was synthesized from total RNA using reverse transcriptase according to the manufacturer’s instructions (Applied Biosystems, Foster City, USA) as previously described [16]. cDNA samples were stored in −20°C until usage.

2.4.2. Real-Time Assays. Taqman Gene Expression Assays were ordered from Assays-on-Demand (Taqman Gene Expression: Hs00171257_m1, HP: Hs00978377_m1, TIP30: Hs00185131_m1 and INHA: Hs00171410_m1; Applied Biosystems, Foster City, USA). Primers were designed to target exon boundaries in order to avoid amplifying genomic DNA. Transcripts were assayed using an ABI Prism 7000 sequence detection system and quantified by using a standard curve by means of a 4-fold dilution series (80–0.08 ng). Each sample was run individually, and the reactions were run in duplicates as previously described [16]. Samples were removed from statistical analysis if only one of the two reactions gave a reliable result or if the standard deviation (SD) between the duplicates was above 2SD.

2.4.3. Analysis. β-actin was used to normalize the real-time PCR data. A Kruskal-Wallis test was used to determine the statistical distribution between the four groups (PE, PE with notching, PE without notching, notching without PE, and...
Table 2: The most significantly altered genes in the microarray experiments, with their respective gene ontology classification (where available).

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Genebank</th>
<th>P-value</th>
<th>Gene ontology</th>
<th>FC</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE compared to control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*C1orf90</td>
<td>NM_032648</td>
<td>7.0 × 10^{-5}</td>
<td>Cell death, inflammatory response, negative regulation of cell proliferation, and so forth</td>
<td>1.9</td>
</tr>
<tr>
<td>*TGFBI</td>
<td>NM_000660</td>
<td>.0011</td>
<td>Cytokine activity, hormone activity, hemoglobin biosynthetic process, cell-cell signaling, and so forth</td>
<td>3.5</td>
</tr>
<tr>
<td>*INHA</td>
<td>NM_002191</td>
<td>.0014</td>
<td>Extracellular matrix structural constituent, proteinaceous extracellular matrix</td>
<td>-2.2</td>
</tr>
<tr>
<td>FBN2</td>
<td>NM_001999</td>
<td>.0015</td>
<td>Cell adhesion, plasma membrane, synaptic transmission</td>
<td>2.4</td>
</tr>
<tr>
<td>HOXA13</td>
<td>NM_000522</td>
<td>.0027</td>
<td>Skeletal development hematopoiesis</td>
<td>2.1</td>
</tr>
<tr>
<td>FLT1</td>
<td>NM_002019</td>
<td>.03</td>
<td>Cell adhesion, plasma membrane, synaptic transmission</td>
<td>2.4</td>
</tr>
<tr>
<td>PE compared to notch</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*NCAM1</td>
<td>NM_00615</td>
<td>3.9 × 10^{-4}</td>
<td>Cell adhesion, plasma membrane, synaptic transmission, regulation of angiogenesis, regulation of apoptosis</td>
<td>1.7</td>
</tr>
<tr>
<td>*TIP30</td>
<td>NM_006410</td>
<td>.00048</td>
<td>RNA polymerase II transcription factor activity, and so forth</td>
<td>2.2</td>
</tr>
<tr>
<td>CCL8</td>
<td>NM_005623</td>
<td>.00060</td>
<td>Cell adhesion, calcium ion transport, chemotaxis, signal transduction, and so forth</td>
<td>3.4</td>
</tr>
<tr>
<td>LY6D</td>
<td>NM_003695</td>
<td>.00091</td>
<td>Calcium transporting ATPase activity, cell adhesion, integral to plasma membranes, and so forth</td>
<td>3.3</td>
</tr>
<tr>
<td>ATP2A2</td>
<td>NM_001681</td>
<td>.0013</td>
<td>RNA polymerase II transcription factor activity, DNA binding, transcription initiation, and so forth</td>
<td>-2.4</td>
</tr>
<tr>
<td>SMARCA5</td>
<td>NM_003601</td>
<td>.0015</td>
<td></td>
<td>2.4</td>
</tr>
<tr>
<td>PE with notch compared to notch</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KLHL31</td>
<td>NM_01003760</td>
<td>.0019</td>
<td>Cell adhesion, and so forth</td>
<td>1.9</td>
</tr>
<tr>
<td>SPP1</td>
<td>NM_000582</td>
<td>.0020</td>
<td></td>
<td>-2.8</td>
</tr>
<tr>
<td>TBCD</td>
<td>NM_005993</td>
<td>.0047</td>
<td>Chaperon binding</td>
<td>-1.9</td>
</tr>
<tr>
<td>CIDEA</td>
<td>NM_001279</td>
<td>.0055</td>
<td>Cell death, apoptotic, program, and so forth</td>
<td>-1.8</td>
</tr>
<tr>
<td>Notch compared to controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TUBG1</td>
<td>NM_001070</td>
<td>.0020</td>
<td>Structural constituent of cytoskeleton, protein binding, and so forth</td>
<td>-3.4</td>
</tr>
</tbody>
</table>


Table 2: Continued.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Genebank</th>
<th>P-value</th>
<th>Gene ontology</th>
<th>FC</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAL3ST4</td>
<td>NM_024637</td>
<td>.0022</td>
<td>Cell-cell signaling, proteoglycan biosynthetic process, and so forth</td>
<td>−2.7</td>
</tr>
<tr>
<td>CD320</td>
<td>NM_016579</td>
<td>.0027</td>
<td>Regulation of cell growth</td>
<td>−3.7</td>
</tr>
<tr>
<td>*HP</td>
<td>NM_005143</td>
<td>.0061</td>
<td>Cellular iron ion homeostasis and defense response</td>
<td>−3.8</td>
</tr>
</tbody>
</table>

FC: fold change. All FC values are relative to the first group in the comparison. PE: pre-eclampsia
*Gene validated with quantitative real-time PCR.

Table 3: GO categories. All genes on the array were assigned to their respective GO categories and used as a background for the analysis.

<table>
<thead>
<tr>
<th>GO Term</th>
<th>GO ID</th>
<th>P-value</th>
<th>FC</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE compared to controls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transcription factor binding</td>
<td>GO:0008134</td>
<td>.0019</td>
<td>3.6</td>
</tr>
<tr>
<td>JUNB, CALCOCO1, ATF5, HOXC6, PARD6A, NR2F2, FAF1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative regulation of signal transduction</td>
<td>GO:0009968</td>
<td>.0027</td>
<td>8.5</td>
</tr>
<tr>
<td>FRZB, ZA20D1, and RHOH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transcription corepressor activity</td>
<td>GO:0003714</td>
<td>.0086</td>
<td>6.2</td>
</tr>
<tr>
<td>JUNB, ATF5, HOXC6, and NR2F2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steroid hormone receptor activity</td>
<td>GO:0003707</td>
<td>.014</td>
<td>7.8</td>
</tr>
<tr>
<td>PGRMC2, NR2F2, NR1D2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PE compared to notch</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transcriptional activator activity</td>
<td>GO:0016563</td>
<td>.0005</td>
<td>3.2</td>
</tr>
<tr>
<td>CALCOCO1, SMARCC1, Pias1, SRCAP, RUNX1, MAML3, and Dyrk1B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscle fiber development</td>
<td>GO:0048747</td>
<td>.0058</td>
<td>10.6</td>
</tr>
<tr>
<td>IGFBP3, and CACNA1H</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell motility</td>
<td>GO:0006928</td>
<td>.012</td>
<td>2.4</td>
</tr>
<tr>
<td>FLNA, FEZ2, CRK, MAPK14, PXN, and TLN1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive regulation of transcription, DNA-dependent</td>
<td>GO:0045893</td>
<td>.012</td>
<td>3.6</td>
</tr>
<tr>
<td>PIAS1, SMARCC1, RUNX1, MAML3 and Dyrk1B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucosamine metabolism,</td>
<td>GO:0006041</td>
<td>.018</td>
<td>7.1</td>
</tr>
<tr>
<td>CTBS and NAGK</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PE with notch compared to notch</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemotaxis / Locomotory behavior</td>
<td>GO:0006935</td>
<td>.021</td>
<td>6.7</td>
</tr>
<tr>
<td>CCBP2, SPP1, and CMKLRI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive regulation of cell proliferation</td>
<td>GO:0008284</td>
<td>.026</td>
<td>6.1</td>
</tr>
<tr>
<td>SPP1 and EBI3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vesicle-mediated transport</td>
<td>GO:0016192</td>
<td>.029</td>
<td>6.7</td>
</tr>
<tr>
<td>ERGIC1, ZFYVE1, and RABGEF1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytokine binding</td>
<td>GO:0019955</td>
<td>.048</td>
<td>8.4</td>
</tr>
<tr>
<td>CCBP2, EBI3, and CMKLRI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Notch compared to control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Regulation of apoptosis</td>
<td>GO:0042981</td>
<td>.017</td>
<td>3.9</td>
</tr>
<tr>
<td>DIABLO, IGFBP3, STAT1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sterol transport</td>
<td>GO:0015918</td>
<td>.021</td>
<td>9.3</td>
</tr>
<tr>
<td>NPC1 and CAV1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PE: pre-eclampsia, FC: fold change, GO ID: gene ontology identification.
controls). If the distribution was significant, a post hoc
Dunn's multiple comparison test was used to determine
significance between individual groups. A P-value < .05 was
considered statistically significant.

2.5. Antibody Microarrays. An in-house designed human
recombinant antibody microarray technology platform was
applied for targeted protein expression profiling [23, 24]. The
applicability of the technology platform has been extensively
validated [23–26] and demonstrated in various clinical
applications targeting, for example, pre-eclampsia [27] and
pancreatic cancer [28].

2.5.1. Extraction and Labeling of Placenta Tissue Proteins.
Predominantly water-soluble proteins were extracted from
tissue biopsies as recently described [27]. Briefly, 50 mg
tissue was resuspended in 375 μl extraction buffer—2% (w/v)
saponin (Sigma-Aldrich, St Louis, MO, USA), 100 μg/ml
soybean trypsin inhibitor (Sigma-Aldrich), 350 μg/ml phe-
nylmethylsulfonyl-fluoride (Sigma-Aldrich), and 0.1% (w/v)
bovine serum albumin in PBS—and incubated at 4°C
over night. After centrifugation at 13000 g for 5 min, the
supernatants containing the extracted proteins were collected
and dialyzed against PBS for 72 h at 4°C. The protein con-
centrations were determined using Micro BCA Protein Assay
kit (Thermo Fisher Scientific Pierce, Rockford, IL, USA)
according to the instructions provided by the manufacturer.

The samples were biotinylated, using previously opti-
mized labeling conditions [23, 24, 29]. Briefly, the samples
were diluted with PBS to a final protein concentration of
about 2 mg/ml before EZ-Link Sulfo-NHS-LC-Biotin
(Thermo Fisher Scientific Pierce) was added to a final
concentration of 0.6 mM. The reaction mixture was then
incubated for 2 h on ice, with vortexing every 20 min.
Unreacted biotin was removed by dialysis against PBS for
72 h at 4°C. Finally, the samples were aliquoted and stored
at −20°C prior to use.

2.5.2. Production and Purification of scFvs. Eleven human
recombinant scFv antibodies against 4 soluble proteins,
including, TGF-β1 (clones 1 to 3), VEGF (clones 1 to 4),
C5 (clones 1 and 2), and RANTES (clones 1 and 2), were
included in the study. Further, 22 additional antibodies
directed against a range of analytes were included for array-
array normalization (see below). The antibodies were
selected from the n-CoDeR library [30] and kindly provided
by BioInvent International AB (Lund, Sweden). Thus, some
of the antigens were recognized by up to four different scFv
clones, which was part of the quality control of the arrays.

All scFvs were produced in E. coli. Briefly, soluble scFvs,
all carrying a C-terminal his6-tag, were purified from expres-
sion supernatants or periplasmic space by affinity chroma-
tography on Ni²⁺-NTA (Qiagen, Hilden, Germany). Bound
molecules were eluted with 250 mM imidazole, dialyzed
against PBS, and stored at 4°C until further use. The integrity
and degree of purity of the produced scFvs were evaluated
by 10% SDS-PAGE (Invitrogen, Carlsbad, CA, USA). The
protein concentrations were determined by measuring the
absorbance at 280 nm.

2.5.3. Fabrication and Processing of scFv Antibody Microarrays.
The fabrication and processing of scFv microarrays were
performed according to previously optimized techniques
[27]. Briefly, scFv antibody microarrays were fabricated by
dispensing Ni²⁺-NTA purified scFvs (200–500 μg/ml) onto
black polymer MaxiSorp slides (NUNC A/S) using the
noncontact sciFLEXARRAYER S11 (Scienion AG, Berlin,
Germany). The scFvs were arrayed, in eight replicates each,
by spotting 2 drops (300 pL/drop) on top of each other (the
spots were allowed to dry out in between), and individual
subarrays were created using a hydrophobic marker pen
(DakoCytomation).

A 36 × 8 array, composed of 33 antibodies, 1 negative
control (PBS), and 2 position markers (10 μg/ml Alexa-
647 labeled streptavidin in PBS) was printed per slide. The
arrays were blocked with 5% (w/v) fat-free milk powder
and 0.05% (v/v) Tween-20 in PBS for 1 h on shaking table
and then washed two times with 0.05% (v/v) Tween-20
in PBS (PBS-T). All incubation steps were performed in
humidity chamber unless otherwise stated. Labeled tissue
extracts diluted 10 times in 1% (w/v) fat-free milk pow-
der and 1% Tween-20 in PBS (PBS-MT) were added to
arrays (100 μl/slide) and incubated on a shaking table
for 1 h. Subsequently, the arrays were washed two times
with PBS-T. Bound proteins were detected using 1 μg/ml Alexa-
647 labeled streptavidin in PBS-MT, and incubated on
a shaking table for 1 h. The slides were washed two times
with PBS-T. Finally, the arrays were dried under a stream
of nitrogen and immediately scanned at 5 μm resolution
using a confocal fluorescence scanner (ScanArray Express
microarray scanner; Perkin Elmer Life Analytical Sciences).
The intensity of each spot was quantified by the fixed
circle method using the ScanArray Express software V4.0
(Perkin Elmer Life Analytical Sciences). Each data point
represents the mean value of four replicates (the two highest
and two lowest replicates were automatically excluded) after
subtracting local background. For protein analytes displaying
saturated signals, values from lower scanning settings were
scaled and used instead.

2.5.4. Data Normalization. The chip-to-chip normalization
was performed using a semiglobal normalization approach,
as previously described [30, 31]. To this end, 22 antibodies
were used to calculate a chip-to-chip normalization factor.
The normalization factor Ni was calculated by the formula
Ni = Si/u, where Si is the sum of the signal intensities of the
22 analytes for each sample, and u is the average Si from
each sample. The normalization factor Ni was divided by the
clean signal intensity of the 22 analytes for each sample, and u is the average Si from
each sample. Each dataset generated from one sample was

2.5.5. Data Analysis. Significantly upregulated or downregu-
lated analytes (P < .05) were identified using Wilcoxon test
using R [32].

3. Results
We compared the gene profiles of PE placentas to those of
normal placentas using oligonucleotide microarrays. A total
of 35 samples were included in the experiments, 10 PE
placenta samples, 15 control placenta samples, 5 placenta samples from patients with bilateral notching with PE, and 5 placenta samples from patients with bilateral notching without PE (Table 1). In order to identify genes differentially expressed between the examined groups, we performed a false discovery rate modified $t$-test. To limit the number of significant genes a cutoff of value was set, $P \leq 10^{-3}$. At this $P$-value, 148 genes were significantly altered in at least one group comparison. The differentially expressed genes are listed in the supplementary table (see supplementary Material available online at doi: 10.1555/2011/472354). Among the most significantly altered genes were neural cell adhesion molecule 1 (NCAM1), haptoglobin (HP), transforming growth factor beta 1 (TGFBI), and inhibin alpha (INHA) (Table 2).

In order to assess the possibility that the results obtained derived from maternal blood in the placentas, 15 maternal blood samples (5 PE and 5 controls) were analyzed.

Using the same $P$-value as above, resulted in 10 genes with significantly altered expression between PE and controls; actin-related protein 2 (ACTR2), calponin 2 (CALP2), and superoxide dismutase 2 (SOD2). None of these genes matched the altered placenta genes indicating that maternal blood trapped in the placenta did not affect the results observed there.

Quantitative real-time PCR was used to verify the gene microarray results. Real-time mRNA data are presented as box-plots in Figure 1.

**PE Compared to Controls.** TGFBI was higher in the PE with and without bilateral notching than in the controls ($P = .02$ and $P = .01$, resp.). INHA showed increased expression in the PE groups (with and without notching) versus controls ($P = .005$ and .01, resp.).

**PE Compared to Notch without PE.** the transcription factor Tat-interacting protein, 30KD, (TIP30) was underepressed in both the notch groups compared to the PE group ($P = .02$ and $P = .04$, resp.). Neural cell adhesion molecule 1 (NCAM1) was not significantly altered in the real-timePCR analysis. A larger sample size will be needed to validate or invalidate the NCAM result definitively.

**Notch Compared to Controls.** haptoglobin (HP), a plasma heme scavenger responsible for transporting heme to the liver, showed lower expression in the notch group compared to controls ($P = .04$).

To couple changes in gene expression to biological processes, we performed three different bioinformatic analyses: gene ontology (GO), KEGG signaling pathway analysis, and InterPro protein domain analysis (IRP). Results from the GO and KEGG/IRP analyses are listed in Tables 3 and 4, respectively.

When comparing genes differentially expressed in the PE group against controls, 18 GO categories were significantly altered $P < .05$; the most significant changes were in genes associated with transcription factor binding (GO:0008134, $P = .0019$), negative regulation of signal transduction (GO:0009968, $P = .0027$), and transcription corepressor activity (GO:0003714, $P = .0086$). When performing a signal pathway analysis, of the same genes, the neurodegenerative disorders pathway (KEGG pathway ID HSA01510, $P = .04$) was the only one significantly altered. This pathway includes genes such as heat shock protein 5 and superoxide dismutase 2. In the InterPro analysis, the category Ras small GTPase, Rab (IPR003579) type was the only category altered ($P = .05$).

When comparing PE to the notch group, 36 GO categories were found to be altered, although several of these were "hierarchical duplicates," meaning they were subgroups within the same categorical branch. Again, the top categories were transcription related, transcriptional activator activity (GO:0016563, $P = .0005$), and transcription coactivator activity (GO:0003713, $P = .0009$). Muscle fiber development was also significantly altered (GO:0048747, $P = .0058$). The signaling pathway antigen processing and presenting (KEGG pathway ID HSA04612, $P = .045$) showed a significant alteration. In the InterPro analysis, six protein domain functions were altered. Among them, were the HEAT repeat element (IPR000357, $P = .0028$) and H+ transporting ATPase, proton pump (IPR000695, $P = .0078$).

In the comparison between PE with notching and notch without PE, 19 GO categories were altered. Among the most significantly altered categories were chemotaxis (GO:0006935, $P = .021$), positive regulation of cell proliferation (GO:0008284, $P = .026$), and cytokine binding (GO:0042102, $P = .048$). The ubiquitin mediated proteolysis signaling pathway (KEGG pathway ID HSA04120, $P = .02$) was altered. The InterPro analysis showed changes in ATPase protein domains (IPR002379, $P = .02$).

To validate a selected differentially expressed gene, TGFB1, on the protein level, a recombinant antibody microarray technology platform, optimized for sensitive serum protein expression profiling, was applied. For this purpose, we selected 6 PE and 5 control samples to assay. Three additional proteins commonly involved in inflammatory responses—C5, VEGF, and RANTES—were also analyzed. It is worth noting that the mRNA encoding the VEGF receptor, FLT1, was found to be increased in PE samples in our DNA array study.

**TGF-β1, VEGF, C5, and RANTES were significantly upregulated in PE compared to controls, ($P < .05$) (Figure 2). Taken together, the data show that TGF-β1 was differentially expressed on both gene and protein level. Like its receptor, VEGF protein was upregulated leveling PE samples (FLT1, Table 2).**

### 4. Discussion

To find genes or mechanisms that might promote or mitigate the development of PE, we used gene profiling to study women with bilateral notching who developed or failed to develop PE. Some of the altered genes (INHA, FLT1, HO-I, and NCAM-1) have been reported in other studies [8, 33, 34]. The V-rel reticuloendotheliosis viral oncogene homolog A (RELA) has previously been implicated in PE and, we now show that RELB is increased in PE as well [15]. A few HLA
isoforms, HLA-G3, and HLA-DRA have previously been shown to be either increased or decreased in PE [34, 35].

Notching in early pregnancy is considered a risk factor for PE [9, 14]. We have previously suggested notch to be an early form of PE [15]. Thus, one would expect the notch group to have a different placental gene profile compared to the control and PE groups. Indeed, our bioinformatic analyses, as well as our previous study, show alterations in several functional categories for the notch group. Based on the gene expression results, we suggest that there may be a placental mechanism that determines how PE progresses (Figure 3). Depending on which inflammatory genes are being expressed, the placenta may either progress from a state of notch to early onset PE or remain clinically asymptomatic only showing signs of bilateral notch. One mechanism that provides protection may be the increased capacity for antigen presentation. Overexpression of HLA genes such as HLA-B, CD74, PSME1, and PSME2, may inhibit the inflammatory processes and thereby prevent progression of PE. Recently, we have shown that HLA-DPA1 is increased in the notch placenta [16].

Increased gene expression and accumulation of free fetal hemoglobin have also been shown in the PE placentas [16]. Hemoglobin, in its free form, disrupts cell membranes causing cytolysis and formation of reactive oxidative species (ROS) [36, 37]. Moreover, the hemoglobin metabolites, heme and iron, are both potent redox agents disrupting cell membranes and membrane proteins by cytolysis. Due to its
Table 4: Results from the KEGG signaling pathway analyses and the InterPro protein domain analyses.

<table>
<thead>
<tr>
<th>KEGG pathway analyses</th>
<th>Pathway</th>
<th>KEGG ID</th>
<th>P-value</th>
<th>FC</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE compared to controls</td>
<td>Neurodegenerative disorders</td>
<td>HSA01510</td>
<td>.04</td>
<td>9.1</td>
</tr>
<tr>
<td>PE without notch compared to notch</td>
<td>Antigen processing and presentation</td>
<td>HSA04612</td>
<td>.04</td>
<td>3.1</td>
</tr>
<tr>
<td>PE with notch compared to notch</td>
<td>Ubiquitin mediated proteolysis</td>
<td>HSA04120</td>
<td>.02</td>
<td>12.0</td>
</tr>
<tr>
<td>Notch compared to controls</td>
<td>Leukocyte transendothelial migration</td>
<td>HSA04670</td>
<td>.03</td>
<td>5.7</td>
</tr>
<tr>
<td>Notch compared to controls</td>
<td>Focal adhesion</td>
<td>HSA04510</td>
<td>.03</td>
<td>4.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>InterPro protein domain analyses</th>
<th>InterPro term</th>
<th>InterPro ID</th>
<th>P-value</th>
<th>FC</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE compared to controls</td>
<td>HEAT</td>
<td>IPR000357</td>
<td>.05</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>TNPO1 and PPP2R1A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ras small GTPase, Rab type</td>
<td>IPR003579</td>
<td>.05</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>RAB9B, RAB17, and RHOH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PE compared to notch</td>
<td>HEAT</td>
<td>IPR000357</td>
<td>.003</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>ARMG8, IPO13, PPP1R12B, and GCN1L1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>H+ transporting ATPase, proton pump</td>
<td>IPR000695</td>
<td>.008</td>
<td>21.4</td>
</tr>
<tr>
<td></td>
<td>ATP13A1 and ATP2A2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Haloacid dehalogenase-like hydrolase</td>
<td>IPR005834</td>
<td>.01</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td>ATP13A2, ATP13A1, and ATP2A2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PE with notch compared to notch</td>
<td>ATPase, F0/V0 complex</td>
<td>IPR002379</td>
<td>.02</td>
<td>7.4</td>
</tr>
<tr>
<td></td>
<td>ATP6V0C and ATP5G1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Notch compared to controls</td>
<td>Actin</td>
<td>IPR004001</td>
<td>.04</td>
<td>44.1</td>
</tr>
<tr>
<td></td>
<td>ACTG1 and FKSG30</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PE: pre-eclampsia, FC: fold change.

Lipophillic nature, heme crosses cell membranes and damages cytosolic proteins, organelles, and DNA [38]. Furthermore, heme is a proinflammatory substance that stimulates both neutrophil activation and migration [39, 40]. There is an increased number of fetal cells, cell debris, and free fetal hemoglobin crossing over the damaged blood-placenta barrier into the maternal system [41, 42]. The notch placenta shows similar vascular damage as in PE. Fetal cells and cell debris may, therefore, also leak into the maternal circulation in the notch placenta. Increased expression of HLA genes and other genes responsible for antigen processing may result in an increased immune defense in the notch placenta. Hence, notch placentas may not develop the same inflammatory response as seen in notch with PE and PE.

In fact, genes related to inflammation were unaltered in the notch placenta.

When comparing PE with and without notch to notch without PE, we found pro-inflammatory genes with increased expression, TGFB1, INHA, and chemokine ligand 8 (CCL8). Among the most significantly altered GO functional categories are chemotaxis and locomotory behavior. Thus, PE with notching appears to be associated with increased cellular movement compared to notching only. More specifically, the mode of cellular movement appears to be induced by genes related to chemotaxis that were overexpressed. The GO functional category cytokine binding (GO:0019955) was also upregulated, suggesting that a cytokine gradient may be responsible for the chemotaxis in PE with notching.
Figure 2: Protein expression validation with antibody microarray. Protein expression profiling of PE (grey bars) versus N (open bars) using recombinant antibody microarray analysis. A focused microarray composed of 11 scFv antibodies directed against 4 proteins, including TGF-β1, VEGF, C5, and RANTES was applied. Six PE samples and five control samples were analysed. The index (1), and so forth, indicate the clone number of the antibody used, meaning that several clones targeting different epitopes on the same analyte was used to further strengthen the data. The expression levels between PE and N were found to be significant (P < .05) for all four analytes.

Figure 3: Pathophysiology. Based on the gene expression results, we suggest that there may be a placental mechanism that determines how PE progresses from stage one, characterized by inadequate perfusion of the placenta, to the clinical symptoms in stage two. Depending on which inflammatory genes that are expressed, the placenta may either progress from a state of notch to early onset PE or remain clinically asymptomatic only showing signs of bilateral notch.

Immunologically derived cells proliferate in response to cytokine gradients. In fact, T-cell proliferation was yet another category altered in the comparison between PE with notch and notch without PE. The progression from notch to PE may, therefore, be driven by induction of inflammation within the placenta. PE is known to be associated with increased levels of several pro-inflammatory cytokines, including interleukins –6, –8, and the tumor necrosis factor α (TNFα) [43, 44]. Moreover, genes related to the central inflammatory NF-kappa B pathway are altered in the PE placentas, which may explain the increase of pro-inflammatory factors in PE [15, 45]. Our results suggest that the notch state may be more than a risk factor, it may be a stepping stone towards PE, bridging an early decrease in placental perfusion with the final inflammatory steps needed to progress into the second stage of PE. By using the antibody array methodology, we have been able to display different inflammatory signatures between early and late-onset PE, further underlining the importance of inflammation in the PE pathogenesis [46].

Several confounding factors may contribute to the gene expression revealed by microarrays. Gestational age and mode of delivery are such factors. A placenta delivered at 35 gw differs from a term placenta. Due to the fact that there is no treatment for PE, the gestational ages of the included PE cases were generally lower than those of the controls. Although we aimed to match the groups based on gestational age, one early onset PE case was included in the PE group. Placentas from vaginal deliveries have been shown to have increased activity in the pro-inflammatory pathway NF-kappa B as well as increased levels of pro-inflammatory cytokines compared to placentas from Caesarian sections [47]. Hence, mode of delivery may well affect the gene expression and contribute to some of the changes seen in the study.

In this discovery study, we have performed gene expression analysis on both placenta and maternal blood, and validated targeted analytes on the protein level as well, to further enhance our fundamental knowledge of PE. Although these findings need to be validated and extended analysing novel, independent cohorts containing larger numbers of samples, the work has provided a clue about the pathophysiology of PE. Pro-inflammatory genes may drive the placenta towards PE, and protective genes may have the opposite effect. More specifically, the first stage, poor placentation, induces ROS formation and damage to the blood-placenta barrier as previously suggested [6]. A possible intermediate step may be brought on by increased apoptosis, activation of inflammatory pathways, imbalance in antioxidation, and increased vascular resistance as seen in bilateral notch. As the placenta attracts and/or activates immune cells, inflammation and irreversible damage to the blood-placenta barrier may occur. Increased, expression of HLA and antigen-presenting genes may ameliorate the inflammatory response.

Conflict of Interests

None of the authors have any conflicts of interest regarding this work.

Acknowledgments

This work was funded by grants from the Swedish Research Council: 5775, the Anna Lisa and Sven Erik Lundgrens
foundation for Medical Research, the Crafoord foundation, the Magnus Bergvalls foundation, the Swedish Society for Medical Research, the Swedish National Science Council (VR-NT), and the SSF Strategic Center for Translational Cancer Research (CREATE Health). Microarrays and protocols were obtained from the Swegene DNA Microarray Resource Center in Lund, supported by Knut and Alice Wallenberg foundation through the Swegene consortium. For technical support regarding the antibody microarray analysis, Dr. Linda Dexlin-Mellby is greatly acknowledged.

References


