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Transcriptional Profiling of Human Placentas from Pregnancies Complicated by Preeclampsia Reveals Disregulation of Sialic Acid Acetylesterase and Immune Signalling Pathways

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Abstract

The placenta plays an important role as a regulator of fetal nutrition and growth throughout development and placental factors contribute to gestational abnormalities such as preeclampsia. This study describes the genome-wide gene expression profiles of a large (n=60) set of human placentas in order to uncover gene expression patterns associated with preeclampsia. In addition to confirming changes in expression of soluble factors associated with preeclampsia such as sFLT1 (soluble fms-like tyrosine kinase-1), sENG (soluble endoglin), and INHA (inhibin alpha), we also find changes in immune-associated signaling pathways, offering a potential upstream explanation for the shallow trophoblast invasion and inadequate uterine remodeling typically observed in pathogenesis of preeclampsia. Notably, we also find evidence of preeclampsiaassociated placental upregulation of sialic acid acetylesterase (SIAE), a gene functionally associated with autoimmune diseases.

Keywords

placenta; pre-eclampsia; preeclampsia; maternal hypertension

†These authors contributed equally to this work.

Author's Contributions

ST performed all the experiments, and drafted the manuscript. NH and ST analyzed the data. BT, ST, and SB coordinated and collected patient samples. JP, AJ, and ST conceived of and designed the study; JP and AJ are the co-principal investigators. All authors read and approved of the final manuscript.

Introduction

Up to 8% of pregnancies are affected by hypertensive disorders [1]; most of these are due to preeclampsia, a multi-system pregnancy-associated disorder triggered by endothelial cell dysfunction and clinically characterized by the *de novo* onset of maternal hypertension and proteinuria [2]. About 15% of preterm births are due to preeclampsia; while early delivery can relieve maternal symptoms that can pose a risk to both mother and fetus, it also increases the risk of perinatal morbidity and mortality [3]. While infant mortality due to preeclampsia has been dramatically reduced in developed countries by clinical management, the risk for perinatal mortality is estimated to be 50% greater in pregnancies complicated by preeclampsia [4]. Preeclampsia and eclampsia are the third leading cause of maternal pregnancy-related deaths in the United States, behind embolism and hemorrhage [5]. Several risk factors have been identified including: first pregnancy (nulliparity), a family history of preeclampsia, diabetes, and multiple pregnancy [6,7].

Pregnancy is a fascinating and unique state where a semi-allogeneic fetus survives in spite of the usual rules of immunology [8]. A number of studies have focused on the connection between pregnancy and preeclampsia as it relates to immunological function. In women without preeclampsia in their first pregnancy, changing paternity in a subsequent pregnancy resulted in a 30% increase in risk compared to those without change, supporting the idea that tolerization to paternal antigens may have an effect [9]. Immune suppression has recently been shown to improve blood pressure and endothelial cell function in a rat model of preeclampsia, supporting the idea that maternal immune activation is involved in its etiology [10]. It has been proposed on the one hand that the innate, not adaptive immune system, controls immuno-regulation during pregnancy and preeclampsia and that a special population of uterine NK cells are key players in this process [8,11]. On the other hand, there is also evidence that regulatory T-cells are involved in mediating maternal tolerance during pregnancy [12].

Preeclampsia can arise from both feto-placental and maternal factors, as well as genetic factors [13]. The pathophysiology of preeclampsia is commonly divided into two stages: 1) abnormalities in placentation (from inadequate uterine remodeling and placental invasion, reduced or intermittent uteroplacental perfusion) and 2) the maternal syndrome associated with placental hypoxia and oxidative stress [14]. Clinical symptoms of preeclampsia generally improve and resolve after delivery suggesting the presence of a placental secreted factor that directly causes maternal endothelial dysfunction [2].

Known factors altered in preeclampsia include Activin-A and Inhibin A (INHA) [15,16], Leptin (LEP) [17], soluble Endoglin (sENG) [18], soluble fms-like tyrosine kinase-1 (sFlt-1) [19], and placental growth factor (PGF) [20] and have been recently reviewed by Carty *et al.* [21]. sFLT1 and sENG are both anti-angiogenic factors that oppose that action of vascular endothelial growth factor (VEGF). Interestingly, overexpression of sFLT1 has been shown to induce preeclampsia in rats [22]. sEng is a soluble TGF-beta co-receptor, which may cooperatively act with sFlt1 to induce severe preeclampsia [18]. Both levels of sEng and sFlt1 correlate with disease severity and decline after delivery [18,19].

While previous gene expression studies have been conducted on the preeclamptic human placenta, this is, to our knowledge, the largest of its kind. In this study, we place the detected gene expression changes in the context of an immune-mediated model of preeclamptic pathogenesis.

Materials and Methods

Study Design

23 preeclamptic, and 37 control placentas included in this study were collected during the years of 2004–2008 and hybridized in two batches to microarrays. Samples were randomized across arrays to control for array and batch variability.

Subjects

Subjects were women who received care at the Duke University Medical Center Obstetric Clinic. Enrollment began August 1, 2003. Women age 18 and older and their infants were eligible for inclusion. Women with multiple gestations, fetuses with documented congenital anomalies, fetuses with documented chromosomal abnormalities, and fetuses with prolonged premature rupture of the membranes (greater than 4 weeks) were excluded from enrollment. Inclusion was contingent upon live birth. Summary characteristics of the preeclamptic and control populations are presented in Table 1. The study was approved by the Duke University Medical Center Institutional Review Board (IRB 00016065).

Preeclampsia Clinical Inclusion Criteria

Women were diagnosed with preeclampsia if their systolic blood pressure was at least 140 mm Hg, their diastolic blood pressure was at least 90 mm Hg and they had proteinuria with an estimated 300 mg of protein or greater excreted in 24 hour measured directly or indirectly by protein creatinine ratio.

Sample collection procedures

Eligible participants were approached by the study coordinator and after giving informed consent, participants completed a questionnaire administered by the study coordinator. The questionnaire included information on demographics (height, weight, race, ethnicity), lifestyle (smoking, substance abuse), and medical and reproductive history. An approximately 2 cm^3 placental sample, excluding fetal membranes, was obtained within 5 cm of the placental umbilical insertion site within an hour of delivery. Placental samples were flash frozen in liquid nitrogen and samples were homogenized under liquid nitrogen using a mortar and pestle. Maternal and infant medical records were reviewed for pertinent data including gestational age at delivery, fetal sex and weight, placental weight, ethnicity, maternal parity, and induction of labor.

RNA isolation and first-strand cDNA synthesis

Total RNA was isolated from term human placenta using the Totally RNA kit (Ambion) following the manufacturer's instructions with slight modifications. Briefly, homogenized tissue chilled in liquid nitrogen was lysed in 10 volumes of denaturation solution (200 mg tissue, 2 ml denaturation solution). The lysed sample was pre-cleared of bulk genomic DNA and cellular debris by spinning at 4,000×g for 5 minutes. The supernatant was sequentially extracted with equal volumes phenol:chloroform, and acid phenol:chloroform (pH 4.5) using phase divider gels (Sigma), then precipitated with an equal volume of 100% isopropanol. The pellet was washed twice with 70% ethanol and resuspended in elution buffer (1 mM Tris-Cl). Total RNA was treated with Turbo DNA-free to remove traces of genomic DNA contamination (Ambion). RNA integrity was assessed using a Bioanalyzer (Agilent Biotechnologies) and only samples with a RIN > 7 were used. Total RNA was converted into 1^{st} cDNA using the AffinityScript Multitemperature cDNA Synthesis Kit (Stratagene) according to manufacturer's instructions.

Microarray Hybridization

Labeling and hybridization for Illumina Human6-v2 BeadArrays was performed according to manufacturer's instructions. Briefly, the Illumina TotalPrep RNA Amplification Kit (Applied Biosystems, Foster City, CA) was used for a first and second strand reverse transcription step from 500 ng of total RNA. This was followed by an overnight *in vitro* transcription reaction that incorporates biotin-labeled nucleotides. 1.5 ug of biotin-labeled cRNA was mixed with an Illumina-supplied hybridization buffer containing several control oligonucleotides. Labeled cRNA was hybridized to BeadArrays at 58°C for 18 hr. Washing, blocking, and streptavadin-Cy3 staining was performed out following manufacturer's protocol. BeadChips were scanned on the BeadArray Reader and scanned images were analyzed using BeadStudio software.

qRT-PCR Validation

Quantitative RT-PCR was performed using a multiplex Taqman-format 5'-nuclease assays (Integrated DNA Technologies) where both gene of interest and reference gene are simultaneously queried. Expression of genes of interest were interrogated using FAM6-labeled double-quenched probes. POLR2A was chosen for a reference gene on the basis of low coefficient of variation in the microarray data and recommendations from the Microarray Quality Control Consortium (MAQC) [23] and interrogated with a HEX550-labeled dual-labeled-probe (Integrated DNA Technologies). A subset of 31 patients were chosen for this confirmatory q-RT-PCR study. Thermal cycling parameters were: [95 °C, 20 s, initial denaturation], [95 °C, 15 s, denaturation; 60 °C, 20 s, annealing and extension]$_{40\ \text{cycles}}$. qRT-PCRs were performed using Taqman Fast Advanced Master Mix (Applied Biosystems) on a StepOnePlus Real-Time PCR instrument (Applied Biosystems). Correlation between the qRT-PCR and microarray was evaluated to quantitatively confirm the microarray data.

Microarray Statistical Analysis

Transcript data was log2 transformed, and quantile normalized [24]. Following this, transcripts which did not attain a detection p-value of <= 0.05 (compared to synthetic sequences on the array not complementary to mammalian genomes, used as negative controls) in any sample were dropped from analysis, leaving 35580 transcripts out of the original 48701. This filtered expression data was scaled to values between 0 and 10, and mean-centered around 0.

JMP Genomics 4.0 was used to perform principle variants components analysis as previously described [25]. A series of models were fitted to calculate the contribution of each of the factors to the measured transcriptional variation: classification, gender, induction of labor, their pair-wise two-way interactions, and estimated gestational age. Gene-specific analysis of variance (ANOVA) was performed using the following model using PROC MIXED in SAS (SAS Institute, Cary, NC), treating classification, gender as fixed effects:

$$\text{expression}=\mu+\text{classification}+\text{gender}\times\text{classification}+\text{batch}+\varepsilon \quad \text{(model 1)}$$

$$\begin{aligned}\text{expression}=\mu&+\text{classification}+\text{induction}+\text{gender}+\text{classification}\\&\times\text{induction}\\&+\text{classification}\\&\times\text{gender}+\text{induction}\times\text{gender}+\text{batch}+\varepsilon\end{aligned} \quad \text{(model 2)}$$

Custom hypothesis tests were constructed to test for differential expression between pre-eclamptic and control groups, as well as male and female samples. Raw p-values were corrected for multiple comparisons via the conservative Benjamini-Hochberg FDR at $\alpha < 0.05$ (for pathway analysis) and Bonferonni at $\alpha < 0.05$ (heatmap visualization) methods [26] as implemented in PROC MULTTEST in SAS (SAS Institute, Cary NC).

Unsupervised hierarchical clustering of the gene expression patterns of placental differentially expressed genes between control and preeclamptic pregnancies was performed using JMP Genomics 8 (SAS Institute, Cary, NC). Differences in demographics between the control and preeclamptic groups were tested using logistic regression using JMP 8 (SAS Institute, Cary NC).

Pathways Analysis

Molecules from the data set that met the *FDR<0.05,* that were annotated as high-quality ("perfect" or "good") in an updated Illumina probe set annotation [27], and were associated with a canonical pathway in Ingenuity's Knowledge Base were considered for the analysis. Canonical pathways analysis (Ingenuity Pathways Analysis library of canonical pathways) identified the pathways that were most significant to the data set. The significance of the association between the data set and the canonical pathway was measured by using Fisher's exact test to calculate a p-value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone.

Results

Power Analysis

To determine how likely the statistical tests are to detect differential expression with varying sample sizes, we conducted a retrospective mixed model power analysis. The median effect size detected by our full experimental design (n=60) at a Bonferonni-level of significance was 0.41. At this effect size, the power to detect differential expression is 0.985, 0.318, and 0.003, for experimental designs with 60, 30, and 15 patients, respectively (See Supplementary Figure 1). Thus, the power to detect realistic changes in differential expression is dramatically decreased in the reduced-sample designs, with neither the n=30 or n=15 design meeting the conventional threshold of 80% statistical power for an effect size of 0.41, equivalent to a 1.32X fold change (See Supplementary Figure 1). Power analysis of a design analyzing a subset of births between the gestational ages of 34–37 weeks (n=23) indicates that this design is insufficiently powered to detect preeclamptic differential expression (See Supplementary Figure 1).

Demographics of Study Population

No significant differences in sex, placental weight, corrected birth weight, or maternal weight were detected between uncomplicated pregnancies and pregnancies complicated by preeclampsia. A significant effect was detected, however, for uncorrected birth weight, maternal parity, fetal estimated gestational age, and induction of labor (See Table 1.).

Variance Components Analysis and Mixed Model Selection

The majority of preeclamptic pregnancies are induced as part of clinical management, while the converse is true for normal pregnancies (See Table 1.) To control for this effect, we fit model 2 including gender, classification, labor, their pair-wise interactions, and a batch effect. No significant differentially expressed genes were detected for the labor effect at either a conservative Bonferonni or relaxed FDR-corrected $p<0.05$ significance threshold. The differentially expressed genes detected for the classification (preeclampsia or normal)

effect in model 1 and model 2 were similar (See Figure 1a). As no significant effect of labor was detected, we chose to present the results of model 1 (which is higher-powered).

Additionally, to assess the independent contributions of other factors, such as estimated gestational age and induction of labor, to the observed transcriptional profiles, we conducted variance component analysis. This analysis indicates that classification (preeclampsia or normal) is the main contributing effect on expression variation, with minor contributing effects of sex and labor. Estimated gestational age has no significant contribution to the principle components of expression variation (See Figure 1b).

Quality Control: Differentially Expressed Genes Between Placentas from Male and Female Births

The estimate of the fixed sex effect between placentas of male and female infants from our gene-specific ANOVA found 36 genes to be differentially expressed at a conservative Bonferroni-corrected significance level of $p < 1.405E\text{-}6$ (See Figure 2a, Supplementary Table 1). As expected, nearly all of these differentially expressed genes (25/26, 96%) localize to the sex chromosomes, confirming the quality of the microarray data. These genes represent transcripts that are either specifically expressed from the Y chromosome or genes that escape X-inactivation, consistent with previous reports of human sex-specific expression variability [28]. The detection of these genes by using sex as a model phenotype confirms the sensitivity of our genome-wide gene expression profiling approach. The dataset is freely available at GEO repository under accession XXXX.

Differentially Expressed Genes and Pathways Between Normal and Preeclamptic Placentas

We found 128 genes to be differentially expressed between normal and preeclamptic placentas at a Bonferonni-corrected significance threshold. These genes include known preeclampsia-associated genes ENG, PAPPA2 (pappalysin-2), and RDH13 (retinol dehydrogenase 13) [29]. (See Figure 2b, Supplementary Table 2.) 2,109 genes were differentially expressed between normal and preeclamptic placentas at FDR<0.05 and include INHA, LEP, and FLT1. These FDR<0.05 genes were used to search for canonical pathways enriched in differentially expressed genes. Pathways significant above FDR<0.1 are listed in Table 2.

Inhibin A is upregulated in preeclamptic placentas. Interestingly, the magnitude of upregulation is greater in females than in male preeclamptic placentas pointing to a potential link to the increased number of preeclamptic pre-term deliveries of females [30]. *ENG* and *FLTI* are also significantly upregulated and confirm observations of others [18] [31]. We also detected the significant upregulation of three factors involved with sialic acid synthesis, transfer, and modification: N-acetylneuraminic acid synthase (NANS), and sialic acid acetylesterase (SIAE). Additionally, Sialic acid binding Ig-like lectin 6 (Siglec-6), a gene identified in previous gene expression profiling work on preeclampsia [32], was also found to be differentially expressed in our study (See Table 3).

From the pathway analysis, three pathways that are dysregulated in preeclampsia are associated with the activation of an immune response: Fcγ receptor-mediated phagocytosis in macrophages and monocytes, leukocyte extravasation signaling, and CXCR4 signaling (See Supplementary Figures 2, 3).

Finally, the heterogeneity in gene expression within each placental class is shown in the unsupervised two-way hierarchical-clustering heatmap in Figure 3, in which all preeclamptic samples cluster together in the first major split, as do the majority of normal samples. However, there are some normal samples that do cluster with the preeclamptic samples,

indicating an interesting biological heterogeneity to the patterns of gene expression and suggesting the presence of molecular subclassifications of preeclampsia worthy of further investigation.

Quality Control: Taqman qPCR Validation

To validate the microarray data, we chose 6 genes exhibiting a range of differential expression between control and preeclamptic groups: SIAE, ENG, PIK3R1, RHOG, CD4, and CXCR4. ENG was selected as a positive control gene previously associated with preeclampsia [18]. Of these SIAE, ENG, PIK3R1, and CD4 were significantly correlated with the microarray data, while RHOG was not ($p<0.089$). There is conflicting microarray data with respect to CXCR4. There are two expressed CXCR4 probes in the array, one targeted to the 5' UTR (ILMN_1801584) and the other targeted to the 3' UTR (ILMN_1728927). The 5' UTR probe reported significantly upregulated expression, while the 3' UTR did not. Two independent qPCR assays to CXCR4 were designed and both correlated with the 3' UTR microarray results (See Table 4) suggesting CXCR4 is not upregulated or that there are different CXCR4 isoforms, only some of which are affected. Overall, these results quantitatively confirm the gene expression patterns observed in the microarray data, with the exception of RHOG (See Table 4).

Discussion

One of the difficulties in interpreting expression profiling studies involved preeclamptic placentas is controlling for differences in gestational age and induction of labor. In this study, while most demographic factors were not significantly different, preeclamptic and normal groups differed in estimated gestational age and induction of labor. This is unsurprising, as early induction of labor is part of the routine clinical management of preeclampsia. Nonetheless, these clinical differences can potentially confound transcriptional profiling for preeclampsia-associated genes, as the gene expression differences detected may actually be associated with gestational age or treatments used to induce labor. To determine the effects of such demographic factors we used variance component analysis and two different linear mixed models, one including labor induction and one without it. We did not detect significant differences in gene expression associated with induction of labor. This should not be interpreted as induction of labor having no effect on placental gene expression, but rather that the study was not designed to detect these differences. Our variance component analysis found that estimated gestational age has minimal independent contribution to this study's gene expression variation. Taken together, while our results do not exclude the possibility of an effect of gestational age or labor on placental gene expression, they support that most of the differences we detect are associated with preeclampsia and not these two comfounding factors (See Supplementary Figure 1).

Additionally, since tissue sampling for the study was from placentas obtained from live births, gene expression profiles obtained potentially include both causative factors as well as downstream symptomatic responses. One important consideration is that if preeclampsia is caused by either: 1) placental factors, 2) maternal disposition and response, or 3) an interaction of the two, gene expression profiling of the preeclamptic placenta is limited by the possibility that it can arise from the combination of a normal placenta with a hypertensive maternal disposition. A comparison with gene expression profiles obtained from chorionic villi sampling earlier in gestation may help distinguish etiological factors from late acute response. In spite of these limitations, however, our data supports the use of microarray studies to understand the basis of preeclampsia.

Preeclampsia Associated Factors and Pathways

One current of model of the development of preeclampsia is that it is an exaggerated systemic maternal inflammatory response to oxidative stress from a placenta that has failed to sufficiently invade and remodel the maternal vasculature releasing placental factors into maternal circulation [14]. Through our transcriptional profiling we identified a number of transcripts encoding factors previously associated with preeclampsia such as ENG, FLT1, INHA [15,18,19] as well as novel factors such as SIAE.

In the analysis of the set of differentially expressed genes between placentas of uncomplicated control and pre-eclamptic pregnancies, we identified several pathways that were enriched in differentially expressed genes. Of major interest are pathways involved in an immune response: Fcy Receptor Mediated Phagocytosis in Macrophages and Monocytes, CXCR4 signaling, and leukocyte extravasation signaling (the process by which white blood cells migrate out of capillary circulation). It has been previously reported that the placenta co-opts adhesion molecules involved in the leukocyte extravasation pathway for uterine remodeling [33], and our study suggests that downregulation of these leukocyte extravasation signals are associated with the clinical manifestation of preeclampsia. The CXCR4 pathway is composed of 169 genes, of which 31 are differentially expressed at FDR < 0.05. Our qPCR data support a model where expression levels of the CXCR4 receptor itself is unchanged, but where binding of the receptor activates downstream G-protein mediated signaling including upregulation of PIK3R1. PIK3R1 is the top differentally expressed gene in each of the three immune-associated pathways.

Novel Sialic Acid Associated Factors

Recently, work from Surolia et al. [34] has described the association of functional rare genetic variation in sialic acid esterase (SIAE) with the regulation of human immune tolerance. SIAE catalyzes the removal of O-acetyl moities from the 9-OH position of sialic acids that decorate the surface of cells [35,36]. ST6 beta-galactosamide alpha-2,6-sialyltranferase 1 (ST6GAL1) transfers sialic acid to galactose in an α2–6 linkage [37]. ST6GAL1 ablation in mice leads to a hyper-immune phenotype [38] although the mechanisms behind this is not well understood [39]. Together, ST6GAL1 and SIAE produce the natural ligands for CD22 (an inhibitor of B-cell receptor signaling and immune activation): 9-O-deacetylated, α2–6-linked sialic acid. Defects in the ST6GL1-SIAE-Siglec pathway could lead to the “unmasking” of CD22 due to the unavailability of its natural cis-ligands as proposed in a model by Pillai et al [39]. Interestingly, our microarray data indicates that SIAE is upregulated, and ST6GAL1 downregulated in the preeclamptic placenta (See Table 4.) We also corroborate the placental upregulation of SIGLEC6 in preeclampsia previously reported by others [40] (See Table 4). The functional consequence of SIAE, ST6GAL1, and SIGLEC6 transcriptional disregulation in preeclamptic placentas remains to be understood, particularly in the context of genetic variation of these transcripts.

Immune-mediated Model of Preeclamptic Pathogenesis

Our data is consistent with a model where trophoblast invasion is defective as a consequence of an immune-associated mechanism affecting cell migration, invasion, and survival. We propose that Sialic acid modifications play a role in either initiating or maintaining maternal immune tolerance, eventually leading to the clinical onset of preeclampsia. Failure of extravillous cytotrophoblasts to invade and remodel the maternal vasculature and replace the endothelial cells lining the spiral arteries are then predicted to be sufficient to decrease the amount of low-pressure blood flow, induce placental hypoxia and oxidative stress, and increase the expression of factors such as sFLT1, sENG, and INHA that lead to the clinical manifestation of preeclampsia in maternal hypertension and proteinuria. (See Figure 4.)

Future

This work presents the molecular characteristics of the preeclamptic placenta, by identifying a number of both known and novel factors and pathways. Molecular classification of these expression profiles might help to elucidate fetal versus maternal causes of the pregnancy-associated syndrome. While we have identified a number of genes and pathways we believe to be implicated in the etiology of preeclampsia, it is our hope that researchers in the field will use this comprehensive gene expression dataset to help guide their own efforts towards understanding the pathogenesis of this disorder. Finally, while many preeclampsia studies have focused on maternal genetic factors for association analysis, genetic analysis of the interactions between fetal and maternal genetics for disregulated genes such as SIAE could prove to be insightful.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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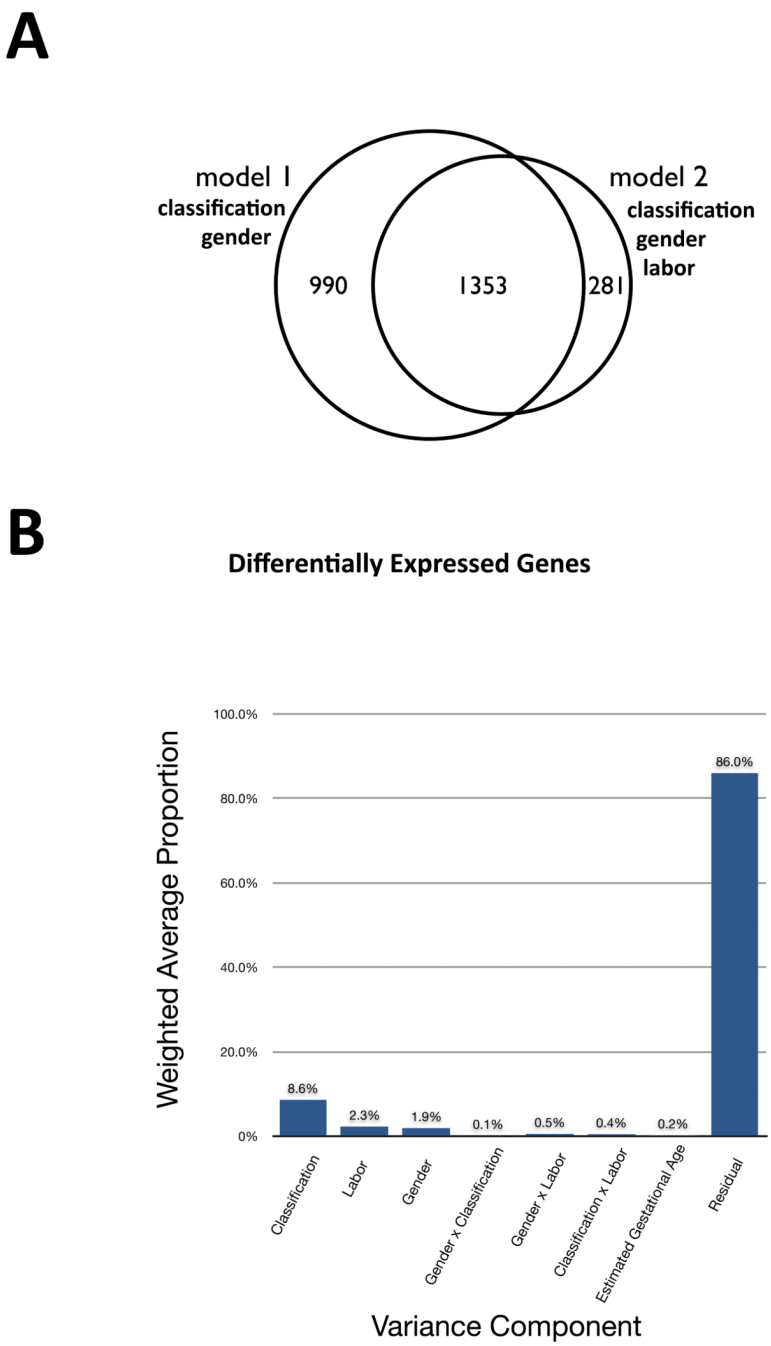

Figure 1. Model selection and variance component analysis

(a) Venn diagram of overlap between placental differentially expressed genes between preeclamptic and normal pregnancies for model 1 and model 2. Model 2 controls for the effect of induction of labor. No differentially genes were detected for the effect of induction of labor. The genes detected for the classification effect (preeclampsia or normal) overlapped substantially for model 1 and model 2. 82% of the genes detected by model 2 at FDR < 0.05 are also detected by model 1. Model 1 detects a larger number of differentially expressed genes due to having a higher statistical power. The results of model 1 were used in pathway analysis. **(b) Variance components analysis of the first five principle components** of variation indicates that classification is the dominant contributor to

transcriptional variation in this study. There are minor contributions of labor and gender, and minimal contributions of the pairwise interaction effects and estimated gestational age. This analysis suggest that most of the differentially expressed genes we detect are associated with classification and is corroborated by the results of model 2 in which we control for the effect of labor.

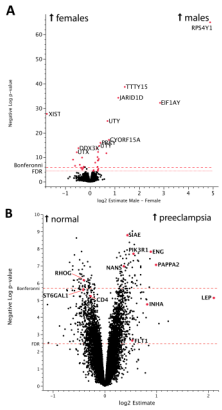

Figure 2.

(a) Volcano plot of differences in gene expression between placentas from male and female neonates. The x-axis represents the estimate of the difference in expression between males – females on a log2 scale. The y-axis represents the negative log of the uncorrected p-value on a log10 scale. Genes upregulated in placentas from male neonates are found in the upper right while those upregulated in placentas from female neonates are in the upper left. The points highlighted in red are differentially expressed genes that exceed a significance threshold of FDR<0.05 and map to the X or Y chromosome. **(b) Volcano plot of expression differences between preeclamptic and control placentas.** The x-axis represents the estimate of the difference in expression between preeclampsia – control on a log2 scale. The y-axis represents the negative log of the uncorrected p-value on a log10 scale. Genes upregulated in preeclamptic placentas are found in the right, while those upregulated in controls are in the left. A number of known preeclampsia associated factors such as ENG, FLT1, INHA, PAPPA were also found to be differentially expressed in our study. The differentially expressed genes at FDR<0.05 were used for pathway analysis. Only genes that were annotated as high-quality in an updated Illumina re-annotation were included in this figure and in pathway analysis.

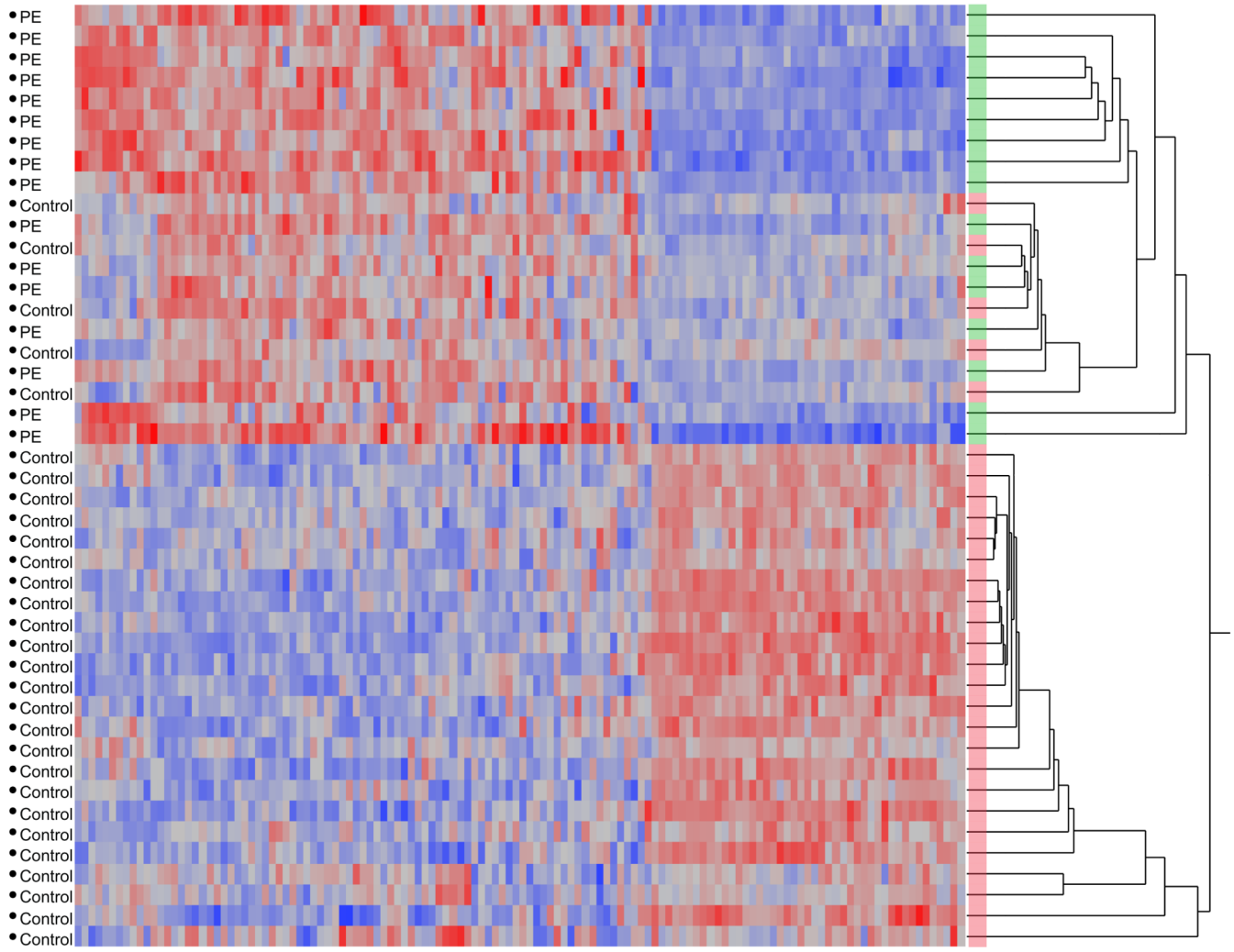

Figure 3. Heatmap of Bonferonni-significant differentially expressed between control and preeclamptic placentas

While there is significant heterogeneity in human gene expression, the relatively large sample size of our microarray study allowed us to sensitively detect differential expression using a linear mixed model analysis. Reassuringly, unsupervised hierarchical clustering of the expression patterns of differentially expressed genes groups all preeclamptic and the majority of control gene expression profiles together. Some normal samples do cluster with preeclamptic samples, indicating that there may be different molecular subclassifications of preeclampsia worthy of further investigation. Preeclamptic/control status is indicated in the text to the left of the heatmap and by color (preeclampsia = green, control = red) to the right of the heatmap at each branch of the dendrogram. Patients are grouped along the Y-axis, while genes are clustered along the X-axis.

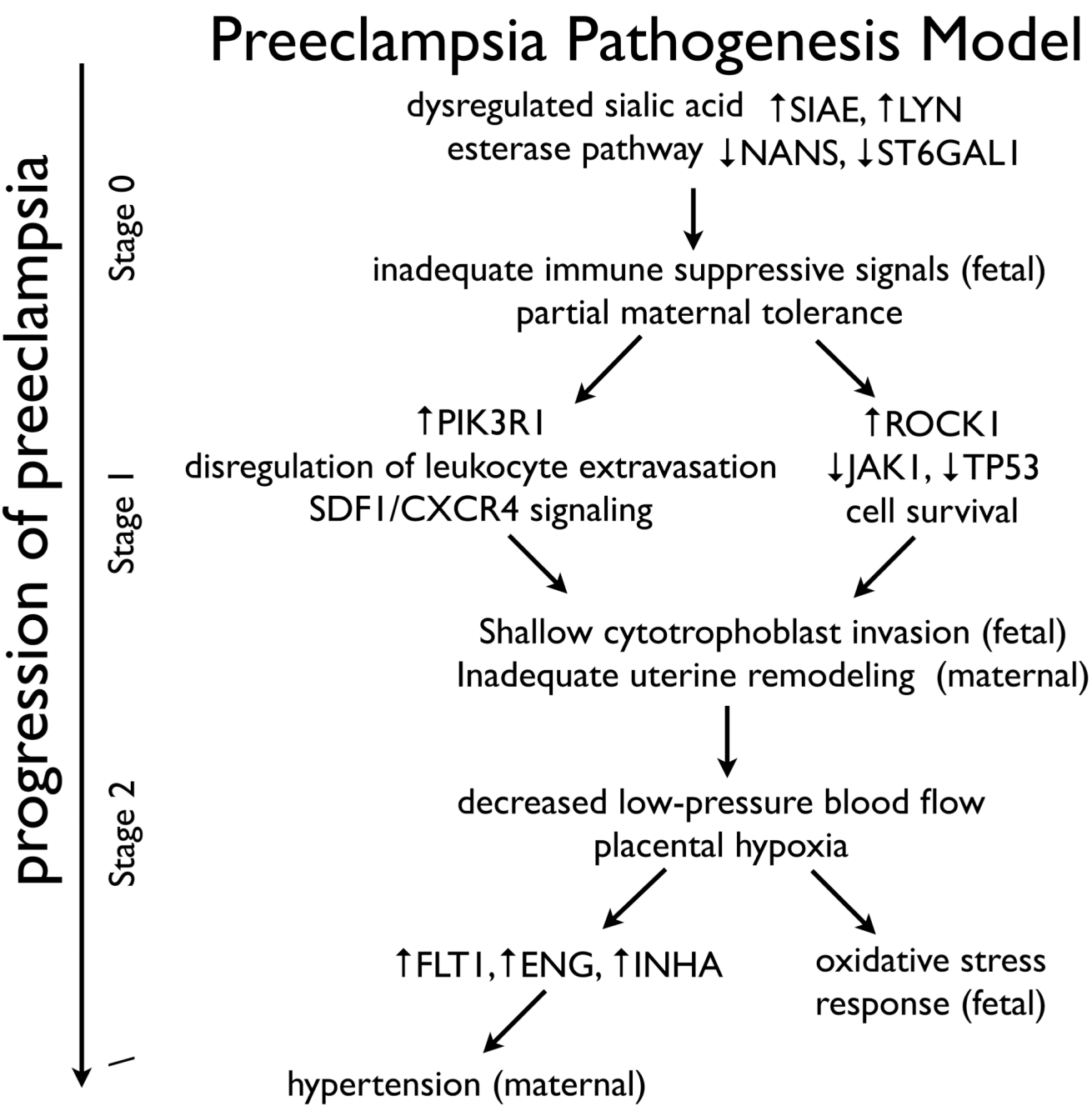

Figure 4. Preeclampsia pathogenesis model

Our data supports a model where dysregulation of immune-associated signaling pathways, associated with atypical sialic acid modification, leads to shallow trophoblast invasion and inadequate uterine vascular remodeling. These changes are predicted to be sufficient to decrease the amount of low-pressure blood flow, induce placental hypoxia and oxidative stress, and increase the expression of factors such as sFlt1, sEng, and INHA that lead to the clinical manifestation of preeclampsia in maternal hypertension and proteinuria.

Table 1

Table of demographic information on study population.

	Control (n=37)		Pre-Eclamptic (n=23)		
	Mean (s.d.)	**Range**	**Mean (s.d.)**	**Range**	**p-value**
Fetal estimated Gestational Age (weeks)	37.6 (1.93)	29–40	33.6 (3.7)	27–38	P < .0001*
% Female	43.2		56.5		P < .4267
Placental weight (g)	491.7 (140.3)	240–990	448.2 (237.8)	130–1090	P < .4322
Birth weight (g)	3258.4 (630.9)	1190–4685	2278.4 (998.9)	765–3860	P < .0001*
Corrected percentile birth weight	50.6 (31.0)	4–99	38.4 (26.4)	2–85	P < .1153
Maternal parity	2.35 (1.7)	0–7	.42 (.69)	0–2	P < .0001*
Weight (lb)	213.9 (51.8)	125–358	209.2 (45.5)	140–322	P < .1474
% Induced	69.6		21.6		P < 0.0002*

*Significant at P<0.05

Table 2

Table of pathways significantly enriched for placental differentially expressed genes between control and preeclamptic pregnancies.

Canonical Pathways	FDR (B-H p-value)
Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes	0.0068
CXCR4 Signaling	0.0068
Leukocyte Extravasation Signaling	0.0083
Semaphorin Signaling in Neurons	0.0219
Regulation of Actin-based Motility by Rho	0.0617
N-Glycan Biosynthesis	0.0891
NRF2-mediated Oxidative Stress Response	0.0891
Germ Cell-Sertoli Cell Junction Signaling	0.0891

Table 3

Table of sialic-acid associated placental differentially expressed genes between preeclampsia and normal pregnancies with FDR<0.05.

Rank[a]	Illumina Probe ID	Gene Symbol	Gene Description	GO Molecular Function	log2 Estimate	FDR	p-value
4	ILMN_1669146	SIAE	Sialic acid acetylesterase	hydrolase activity; serine esterase activity; sialate O-acetylesterase activity	0.44	1.4E-05	1.6E-09
44	ILMN_1815874	NANS	N-acetylneuraminic acid synthase	transferase activity; N-acetylneuraminate synthase activity; N-acylneuraminate-9-phosphate synthase activity; N-acylneuraminate cytidylyltransferase activity	0.37	8.3E-05	1.1E-07
166	ILMN_1756501	ST6GAL1	ST6 beta-galactosamide alpha-2,6-sialyltranferase 1	beta-galactoside alpha-2,6-sialyltransferase activity	−0.42	5.2E-04	2.5E-06
1535	ILMN_1685630	SIGLEC6	sialic acid binding Ig-like lectin 6	protein binding; sugar binding	0.21	2.2E-02	1.0E-03
1632	ILMN_1756937	ST8SIA4	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 4	sialyltransferase activity	0.21	2.4E-02	1.2E-03

[a] Genes ranked by p-value of preeclamptic differential expression from low to high.

[b] Benjamini-Hochberg FDR

Table 4

qPCR validation of microarray data for selected genes

Rank[a]	Illumina Probe ID	Gene	Gene Description	GO Molecular Function	log2 Estimate	Array p-value	qPCR correlation p-value
4	ILMN_1669146	SIAE	Sialic acid acetylesterase	hydrolase activity; serine esterase activity; sialate O-acetylesterase activity	0.44	1.6E-09	7.1E-03*
13	ILMN_1760778	ENG	Endoglin	protein binding	0.89	1.5E-08	1.7E-17*
16	ILMN_1760303	PIK3R 1	Phosphoinositid e-3-kinase, regulatory subunit 1 (alpha)	kinase activity; protein phosphatase binding; insulin-like growth factor receptor binding; insulin binding; insulin receptor binding; phosphatidylinositol binding; ErbB-3 class receptor binding; insulin receptor substrate binding; phosphoinositide 3-kinase regulator activity	0.55	2.0E-08	8.6E-13*
91	ILMN_1739792	RHOG	Ras homolog gene family, member G	GTPase activity; nucleotide binding; GTP binding; protein binding	−0.41	6.8E-07	8.9E-02
236	ILMN_1727284	CD4	CD4 molecule	protein homodimerization activity; protein kinase binding; transmembrane receptor activity; MHC class II protein binding; extracellular matrix structural constituent; zinc ion binding; coreceptor activity; glycoprotein binding	−0.27	5.9E-06	1.8E-03*
539	ILMN_1801584	CXCR 4	Chemokine (C-X-C motif) receptor 4	C-C chemokine receptor activity; receptor activity; rhodopsin-like receptor activity; C-X-C chemokine receptor activity	−0.54	5.9E-05	1.3E-01
7138	ILMN_1728937	CXCR 4*	Chemokine (C-X-C motif) receptor 4	C-C chemokine receptor activity; receptor activity; rhodopsin-like receptor activity; C-X-C chemokine receptor activity	−0.20	6.5E-02	3.2E-03*

[a] Genes ranked by p-value of preeclamptic differential expression from low to high.

* qPCR significant Pearson correlation p<0.05.