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Identification of Fetal and Maternal Single Nucleotide Polymorphisms in Candidate Genes That Predispose to Spontaneous Preterm Labor with Intact Membranes

Roberto Romero, M.D.^{1,2,3}, Digna R. Velez, Ph.D.⁴, Juan Pedro Kusanovic, M.D.^{1,3}, Sonia S. Hassan, M.D.^{1,3}, Shali Mazaki-Tovi, M.D.^{1,3}, Edi Vaisbuch, M.D.^{1,3}, Chong Jai Kim, M.D.^{1,5}, Tinnakorn Chaiworapongsa, M.D.^{1,3}, Brad Pearce, Ph.D.⁶, Lara A. Friel, M.D., Ph.D.^{1,3}, Jacqueline Bartlett, M.S.⁷, Madan Kumar Anant, Ph.D.⁸, Benjamin A. Salisbury, Ph.D.^{*,8}, Gerald F. Vovis, Ph.D.^{*,9}, Min Seob Lee, Ph.D.¹⁰, Ricardo Gomez, M.D.^{11,12}, Ernesto Behnke, M.D.¹¹, Enrique Oyarzun, M.D.¹², Gerard Tromp, Ph.D.², Scott M. Williams, Ph.D.^{7,13}, and Ramkumar Menon, Ph.D.⁶

¹Perinatology Research Branch, NICHD/NIH/DHHS, Bethesda, Maryland, and Detroit, Michigan, USA

²Center for Molecular Medicine and Genetics, Wayne State University, Detroit, Michigan, USA

³Department of Obstetrics and Gynecology, Wayne State University, Detroit, Michigan, USA

⁴Department of Human Genetics, Dr. John T. Macdonald Foundation, and John P. Hussman Institute of Human Genomics and University of Miami, Miami, Florida, USA

⁵Department of Pathology, Wayne State University, Detroit, Michigan, USA

⁶Department of Epidemiology, Rollins School of Public Health, Emory University, Atlanta, Georgia, USA

⁷Center for Human Genetics Research, Vanderbilt University, Nashville, Tennessee, USA

⁸PGxHealth, LLC, New Haven, CT, USA

⁹Helix Therapeutics, LLC, New Haven, CT

¹⁰Genaissance Pharmaceuticals, Inc., New Haven, Connecticut, USA

¹¹CEDIP (Center for Perinatal Diagnosis and Research), Department of Obstetrics and Gynecology, Sotero del Rio Hospital, Santiago, Chile

¹²Department of Obstetrics and Gynecology, Pontificia Universidad Catolica de Chile, Santiago, Chile

¹³Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, Tennessee, USA

Abstract

Objective—To determine whether maternal/fetal SNPs in candidate genes are associated with spontaneous preterm labor/delivery.

Address correspondence to: Roberto Romero, M.D. and Scott M. Williams, Ph.D., Perinatology Research Branch, NICHD, NIH, DHHS, Wayne State University/Hutzel Women's Hospital, 3990 John R, Box 4 - Detroit, MI 48201, USA, Telephone (313) 993-2700; Fax: (313) 993-2694, prbchiefstaff@med.wayne.edu; smwilliams@chgr.mc.vanderbilt.edu.

*Formerly of Genaissance Pharmaceuticals, Inc., New Haven, CT, USA, where contributions to this work were initiated.

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Study Design—A genetic association study was conducted in 223 mothers and 179 fetuses [preterm labor with intact membranes who delivered <37 weeks (PTB)], and 599 mothers and 628 fetuses (normal pregnancy): 190 candidate genes and 775 SNPs were studied. Single locus/haplotype association analyses were performed; FDR was used to correct for multiple testing ($q^*=0.15$).

Results—1) The strongest single locus associations with PTB were IL6R (fetus: $p=0.000148$) and TIMP2 (mother: $p=0.000197$), remaining significant after correction for multiple comparisons; 2) Global haplotype analysis indicated an association between a fetal DNA variant in IGF2 and maternal COL4A3 (global $p=0.004$ and 0.007 , respectively).

Conclusion—A SNP involved in controlling fetal inflammation (IL6R) and DNA variants in maternal genes encoding for proteins involved in extracellular matrix biology approximately doubled the risk of PTB.

Keywords

Chorioamnionitis; DNA variants; extracellular matrix; genetic association study; genomics; genotype; haplotype; high dimensional biology; IL-6; parturition; prematurity; SNP

INTRODUCTION

Preterm birth (PTB) is the leading cause of perinatal morbidity and mortality worldwide.¹ The frequency of PTB varies from 5–12% depending on geographical region.^{2,3} PTB may be the result of spontaneous preterm labor, with intact or ruptured membranes, or indicated PTB (for fetal or maternal indications).

Genetic predisposition for PTB^{4–6} has been hypothesized based upon: 1) demonstration of familial aggregation;^{7–11} 2) measures of heritability;^{11–15} 3) identification of disease-susceptibility genes; and 4) racial disparity in preterm birth rate^{16–19} that may be related to frequency differences in risk predisposing alleles.^{20–24} Familial aggregation, defined as the co-occurrence of a trait in members of a family that can not be readily accounted for by chance, has been demonstrated for PTB.^{7–11} Studies in twins have demonstrated a heritability ranging from 17–40%.^{9,10} Polymorphisms in several genes have been studied and many have identified DNA variants associated with spontaneous preterm labor with and without intact membranes as well as in PTB.^{21;24–97}

The purpose of this genetic association study was to identify DNA variants in the maternal and fetal genomes that can alter the risk for spontaneous preterm labor and delivery with intact membranes. Seven hundred seventy five single nucleotide polymorphisms (SNPs) from 190 candidate genes, which have been implicated in the mechanisms of disease responsible for spontaneous preterm labor, preterm prelabor rupture of membranes (pPROM), small-for-gestational age (SGA), and preeclampsia, were analyzed. The study was conducted in a Hispanic population at a single site from Chile that has an estimated PTB rate of 6%^{98;99} and with extreme care to phenotypic characterization.

MATERIALS AND METHODS

Study Design

This was a case-control study that included patients with spontaneous preterm labor and intact membranes who delivered a preterm neonate (mothers: 223 and fetuses: 179) and controls (mothers: 599 and fetuses: 628). Spontaneous preterm labor was defined by the presence of regular uterine contractions occurring at a frequency of at least two every 10 minutes who delivered preterm (<37 weeks). The control group included women who

delivered at term (37–42 weeks of gestation) without complications of pregnancy including preterm labor with term delivery, preeclampsia, eclampsia, HELLP syndrome, pPROM, SGA, large-for-gestational age neonates, fetal demise, placental abruption, placenta previa, or chorioamnionitis.

Patients of Hispanic origin were recruited at the Sotero del Rio Hospital, in Puente Alto, Chile. All eligible mothers were enrolled in a research protocol, which requested permission to collect DNA from the mother and her neonate for research purposes. The exclusion criteria, beside those explained above for controls, included: 1) known major fetal chromosomal and/or structural anomalies; 2) multiple pregnancy; 3) serious medical illness (chronic renal failure, congestive heart failure, connective tissue disorders, etc.); 4) refusal to provide written informed consent; and 5) a clinical emergency, which prevented counseling of the patient about participation in the study, such as fetal distress or maternal hemorrhage. A blood sample was obtained from the mother at the time of enrollment in the protocol, and from the umbilical cord (blood of fetal origin) after delivery. Demographic and clinical characteristics of the mothers were obtained from a data collection form administered by trained medical and paramedical personnel. The collection of samples and their utilization for research purposes was approved by the Institutional Review Boards of the Sotero del Rio Hospital, Santiago, Chile (an affiliate of the Pontificia Catholic University of Santiago, Chile), and the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, NIH, DHHS.

Genotyping

Candidate genes were selected for analysis based on biological plausibility for a role in preterm labor and other pregnancy complications including SGA, pPROM and preeclampsia. Genes involved in processes such as the control of the immune response (pattern recognition receptors, cytokines, chemokines and their respective receptors), uteroplacental ischemia, or angiogenesis were considered appropriate candidates for this study. A complete list of the 190 genes and all SNPs genotyped are included in the supplemental materials (Supplemental Table 1).

SNP discovery within the candidate genes was performed by DNA sequencing at Genaissance Pharmaceuticals, Inc. (New Haven, CT, USA) using its Index Repository, which includes a total of 93 subjects with Native American, Hispanic/Latino, European, Asian, and African-American ancestry.¹⁰⁰ To determine which individuals in the Genaissance Index Repository were most representative of the genetic variation observed in the Chilean population, 96 unrelated Chilean individuals who are representative of the patient cohort were sequenced for 16 DNA fragments. A subset of 42 subjects from the Index Repository that is heavily weighted with the Native American and Hispanic/Latino subjects (although European, Asian, and African-American subjects contributed to the subset as well) was determined to be most representative of the variation for the Chilean population. This was based on the correlation in the minor allele frequencies for the SNPs in 16 DNA fragments that were sequenced in both the Index Repository and the sample of patients from Chile (mothers with the same ethnicity delivered at the same hospital). This subset of 42 individuals was used to select polymorphisms for the candidate genes. The selection was performed by applying the Shannon-Wiener diversity metric to this subset as previously described,¹⁰¹ and the SNPs selected for genotyping were intended to capture at least 90% of the haplotypic diversity of each gene, covering variation in the coding regions, 100 bases at each end of the introns, 1000 bases upstream of the start codon, and 100 bases downstream of the stop codon.¹⁰¹

Template DNA for genotyping was obtained by whole-genome amplification¹⁰² of genomic DNA isolated from blood using an automated DNA isolation protocol (BioRobot 9604,

Qiagen, Valencia, CA, USA). Genotyping was carried out using the MassARRAY™ System (Sequenom, Inc., San Diego, CA, USA) at the high-throughput genotyping facility at Genaissance. Each genotyping assay involved PCR amplification from template DNA in a target region defined by specific primers for the respective polymorphic sites, purification of the amplification product, annealing of the indicated extension primer to one strand of the amplification product adjacent to the polymorphic site, extending the primer by one nucleotide using the MassEXTEND™ reaction (Sequenom, Inc., San Diego, CA, USA), and detection of the allele-specific extension product by mass spectrometry.¹⁰³

Quality Control

Univariate and multivariate distributions were evaluated for each variable to identify significant outliers. The values of the outliers were removed only if found to be incorrect on reexamination. Each SNP was verified to ensure genetic consistency between the genotypes of mother and offspring. Numerous programs are available for detecting relationship errors,^{104–108} however, to produce accurate results, these programs require genotyping for a larger proportion of the genome than was available in this study. Therefore, we considered the number of Mendelian inconsistencies between mother and offspring to identify potential relationship errors (e.g. sample mix-ups or mislabeling). When an inconsistency for an individual marker was observed, those genotypes were removed at that marker. In the case of multiple inconsistencies in a given pair, the pair was excluded for further analysis (10 pairs in controls and 5 pairs in cases). Finally, we assessed the presence of genotyping errors. In some instances, genotyping errors will lead to Mendelian inconsistencies, which can be easily identified and removed from the analysis. However, most genotyping errors for SNPs will be Mendelian consistent.¹⁰⁹ For example, with mother-offspring pedigree structures, genotyping errors where a homozygous individual has been mistyped as a heterozygous individual will never lead to a Mendelian inconsistency for SNPs. Deviations from Hardy-Weinberg equilibrium (HWE) may indicate the presence of a genotyping error or hidden population stratification,¹¹⁰ or reflect a biological effect such as natural selection (or other evolutionary force) and/or the association of disease and genotype.^{111–115} Tests for deviations from HWE were performed for mothers and offspring separately and again separately for diagnostic subgroups. Because it is currently unclear how to unequivocally distinguish between deviations from HWE due to genotyping error, and deviations from HWE due to biological causes, such as location at or near a disease susceptibility locus, we noted SNPs that deviate from HWE, but we did not remove them from the analysis. If necessary, we could follow-up these observations with additional testing. In this study, no SNPs found to be associated with spontaneous preterm labor/delivery with intact membranes deviated from HWE in both cases and controls. We tested for population stratification in cases and controls using STRUCTURE.¹¹⁶ We used the CEU and Asian (CHB and JPT) populations as reference populations because Hispanic populations in the Americas, including those in Chile, are the products of admixture between European and Native American populations, and Native American populations are most recently derived from Asia. The analyses show that the Chilean samples, both cases and controls, cluster within the HapMap European (CEU) samples, indicating that our cases and controls do not represent stratified populations (Figure 1).

Statistical analysis

Continuous demographic and clinical characteristics of cases and controls (gestational age, birth weight, maternal age, and body mass index (BMI)) were tested for normality using Shapiro-Wilks test. All measurements deviated significantly from normality; therefore, Mann-Whitney two-sample rank sum tests were used for case-control comparisons. χ^2 tests were used to test for differences in parity, Apgar scores at 1 and 5 minutes, smoking, and

differences in fetal gender between cases and controls. Stata 10.0 statistical software (StataCorp, College Station, TX, USA) was used for all analyses.

Single locus tests of association—Statistical tests for single locus association and for deviations from HWE were calculated using PLINK software.¹¹⁷ Deviations from HWE in cases and controls were determined using χ^2 test. Single locus tests of association were performed with logistic regression using an additive genotypic model where the minor allele was coded as the risk allele. Standard summary statistics, odds ratios (OR) and confidence intervals (CI) were reported for these tests of association. Prior to performing single locus and haplotype analyses, linkage disequilibrium (LD) based SNP pruning was performed using PLINK software, with a cutoff of $r^2 = 0.8$. Of the 775 SNPs that passed quality control, we analyzed 697 maternal and 645 fetal SNPs. We excluded a small number of X chromosome SNPs for fetal data, as neonates included in the study were both male and female. This accounted for the most of the difference in the number of SNPs tested in mothers and fetuses.

Haplotype tests of association—Haplotype analyses were performed on genes with at least 1 significantly associated SNP ($p < 0.01$) and at least two SNPs in the same gene. Haplotype frequencies, as well as haplotype-based association analyses for the dichotomous PTB outcome with 2 and 3 marker sliding windows, were calculated using PLINK software. Only haplotypes that had a frequency of ≥ 0.05 were analyzed, and only SNPs that had less than 5% missing data were used. The strongest associated haplotype windows are reported ($p \leq 0.05$) and only these were analyzed for haplotype-specific effects. We present the calculation of OR for each haplotype (using the most common haplotype as referent), as well as determination of case and control haplotype frequencies. Standard summary statistics for pairwise LD, D' and r^2 , were calculated using Haploview.^{118;119} Haplotype blocks were assigned using the D' confidence interval algorithm created by Gabriel *et al.*¹²⁰ D is the standard population genetic measure of LD that calculates the deviation of haplotype frequencies from that expected if there were random combinations of alleles at two loci. Since D is allele frequency dependent, D' is often used because it normalizes the LD (to a range of 0 to 1) based on actual allele frequencies. In contrast, r^2 is the standard correlation statistic based on the population distribution of two SNPs. All three of these measures reflect similar patterns of LD.

Histologic chorioamnionitis analysis—All statistically significant single locus and haplotype associations were further analyzed for allele and haplotype differences between patients with spontaneous preterm labor and delivery (<34 weeks of gestation) with histological chorioamnionitis alone or with funisitis and term controls (delivered >37 weeks without histologic chorioamnionitis or funisitis). The purpose of these analyses was to further evaluate whether histologic chorioamnionitis was driving the observed associations.

Multiple testing corrections—A false discovery rate (FDR) correction was performed to adjust for multiple comparisons using a q^* of 0.15 in single locus tests of association.¹²¹ The q^* indicates the expected proportion of results that are identified as interesting that are actually false. This is in contrast to α (typically set to 0.05), which indicates the probability of obtaining a false positive result among all tests performed. FDR is used to measure global error, that is, the expected number of false rejections of the null hypothesis among the total number of rejections. The critical significance level was calculated by ranking the results by p values and then multiplying this rank by q^* divided by the total number of tests. Although the choice of q^* is somewhat arbitrary, a q^* of 0.15 was chosen to be permissive enough in our analyses not to run the risk of eliminating potentially interesting findings due to a Type II error.

Multi-locus analysis—Spontaneous preterm labor with intact membranes is a complex phenotype with no clear pattern of inheritance in pedigrees. Therefore, it is reasonable to assume that multiple variants, either alone or interacting, predispose to this phenotype. Because of this complexity it is important to explore genetic models that do not assume independent single locus effects as such analyses have the potential to reveal genetic models hidden by single locus analyses. Such analyses have previously revealed associations undetected by single locus analyses for hypertension,¹²² as well as associations with preterm birth.¹²³ One method that is designed to discover of complex models is Multifactor Dimensionality Reduction (MDR). We performed exploratory multi-locus analyses using MDR to identify interactions among maternal, fetal, and maternal/fetal SNPs. MDR has been previously described by Ritchie *et al.*¹²⁴ and is available as open source software at www.epistasis.org. Briefly, MDR is a non-parametric (does not assume any statistical model) and model free (no assumption mode of genetic inheritance) unique tool for identifying gene-gene interactions. MDR collapses all of the genetic data into two categories (high and low risk) by comparing all single locus and all multi-locus combinations, and then categorizing each genotype into either high-risk or low-risk on the basis of the ratio of cases to controls that have that genotype. MDR ultimately selects one genetic model, either single or multi-locus, that most successfully predicts phenotype or disease status. Analyses were performed: 1) separately for maternal and fetal data (tag SNPs only); and 2) combined for available maternal and fetal paired DNA samples [e.g. instead of an individual having 751 SNPs (the number of SNPs that completely overlapped for maternal and fetal DNAs), this increased the number of SNPs analyzed to 1502]. Data were analyzed for two- and three-way interactions with 10-fold cross-validation and average balanced accuracy as the metrics for evaluating a model.¹²⁵ Several filtering steps and parameters were explored and are described on Table 1. The MDR algorithm was implemented with the full array of tag SNPs as well as after filtering, using the Tuned ReliefF (TuRF) approach as described in detail by Moore and White.¹²⁶ TuRF is a modification of ReliefF. Briefly, ReliefF is a method that estimates the quality of attributes (e.g. SNPs) through a nearest neighbor algorithm that selects neighbors from the same and different classes based on the values of the attributes (in this case genotypes).¹²⁷ TuRF is a method that systematically removes attributes (e.g. SNPs) that poorly differentiate cases and controls.¹²⁶ ReliefF and similar computational algorithms have been developed, primarily in computer science and data mining fields, to provide means to screen attributes/variables on a rational basis that relate to quality in terms of ability to classify an outcome. These algorithms allow the reduction in importance of those attributes that are irrelevant to an outcome and will generate noise to any subsequent analyses. Many of the developed algorithms assume independent effects, and are therefore not appropriate when there are interactions among variables that affect outcome/disease. ReliefF does not make this assumption. It is an extension of Relief and we will briefly describe Relief in order to provide a better understanding of the attribute election process. In Relief, an instance (in this case an individual, either case or control) is randomly selected and then the two nearest neighbors (the nearest case and the nearest control, based on the multilocus genotype data). Attributes that are shared with those of the same status are increased in value and those that are shared among individual of opposite classes are devalued. Unshared values of an attribute in two cases are similarly decreased in value. This is repeated a preset number of times. ReliefF has the added feature of choosing just a single individual of the same and single individual of the opposite status an arbitrary number of individuals is selected to assess the attributes. In our case we use a sample of 10 nearest cases and 10 nearest controls on the genotype space. TuRF goes a step further and instead of just changing the attributes (e.g., SNP) values, it systematically removes attributes (e.g. SNPs) that poorly differentiate cases and controls. The motivation behind this algorithm is that the ReliefF estimates of the true associating SNPs will improve as the non-associating SNPs are removed from the dataset. In addition, SNPs were filtered based on results of the

single SNP analyses and only SNPs that had a marginal p value of ≤ 0.1 were included, or only those with a p value <0.05 were analyzed separately. Permutation testing with 1,000 permutations was used to determine statistical significance of all MDR models.

MDR as described above is ideal for a balanced data set where the number of cases and controls are the same or close to the same. However, computational methods have been developed since the initial development of MDR to test for prediction accuracies in an imbalanced data set, such as ours.¹²⁵ The method, termed balanced accuracy, corrects for imbalanced data by taking an average of the sensitivity and specificity and is defined as the arithmetic mean of sensitivity and specificity. We tested for balanced accuracy in this manuscript.

Bioinformatics Tools

The SNPper (<http://snpper.chip.org>) database using dbSNP Build 125 was used to determine marker positions (bp), marker function, and identify amino acid changes.

Pathway analysis—As with the multilocus analyses, it is important to provide a more comprehensive assessment of our results in terms of pathway involvement in spontaneous preterm labor and delivery with intact membranes because it is likely that single genes are not by themselves adequate to affect disease risk, but multiple genes within a single pathway may affect function enough to increase risk. Therefore, as part of hypothesis generating exercise we perform pathway based analyses using Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, Inc., Redwood City, CA, USA).^{19;128–131} Specifically, IPA was used to examine whether the SNPs found to be putatively associated with spontaneous preterm labor and delivery with intact membranes mapped to different biological networks and disease functions. The genes with variants that were significantly associated with PTB ($p<0.05$) were entered into the IPA analysis tool. These genes were termed "focus genes." The IPA software was used to measure associations of these molecules with other molecules, their network interactions, and biological functions stored in its knowledge base. The knowledge base encompasses relationships between proteins, genes, cells, tissues, xenobiotics, and diseases. The information is scientist-curated, updated, and integrated from the published literature and other databases such as OMIM, GO, and KEGG. Our focus genes served as seeds for the IPA algorithm, which recognizes functional networks by identifying interconnected molecules, including molecules not among the focus genes from the IPA knowledge base. The software illustrates the networks graphically, and calculates a score for each network, which represents the approximate "fit" between the eligible focus molecules and each network. The network score is based on the hypergeometric distribution and is reported as the $-\log$ (Fisher's exact test result).

We also used IPA to identify functionally related genes that correspond to canonical pathways. We determined enrichment of our focus genes among the > 200 well-characterized metabolic and cell signaling "canonical" pathways curated by IPA scientists from journal articles, text books, and KEGG Ligand. The Fischer's exact test was used to calculate a p value representing the probability that the association between the genes in our dataset and the canonical pathway is explained by chance alone.

RESULTS

Significant differences between cases and controls were identified for gestational age at delivery ($p<0.0001$), birth weight ($p<0.0001$), Apgar scores at 1 ($p<0.0001$) and 5 minute ($p<0.0001$) (Table 2). However, we did not observe significant differences between cases and controls for BMI and smoking status, two variables that have been previously associated with risk for PTB.^{132–136}

Single locus association

SNPs associated with spontaneous preterm labor/delivery with intact membranes in maternal and fetal DNA ($p < 0.01$) are presented in Table 3. There were no statistically significant deviations from HWE in controls for any of these DNA variants. The most significant association in maternal DNA was observed at a synonymous coding SNP (S101S) in the gene for the tissue inhibitor of metalloproteinase 2 (*TIMP2*) rs2277698 (OR = 1.98 [95% CI 1.38–2.83], $p = 0.000197$) (Table 4). The minor allele frequency for this SNP (A) was 0.13 in cases and 0.07 in controls. This SNP was significant after FDR correction for multiple testing.

The most significant association observed in fetal DNA was also a synonymous SNP (A31A) in the interleukin 6 receptor 1 (*IL6R*) rs8192282 (OR = 2.07 [95% CI 1.42–3.02], $p = 0.000148$) (Table 4). The minor allele frequency for this SNP (A) was 0.13 for cases and 0.10 for controls. This SNP also remained statistically significant after FDR correction.

Additional SNPs that were associated with spontaneous preterm labor/delivery with intact membranes at a p value of < 0.05 are presented in Tables 5a and 5b. Although we have not emphasized these findings in the present report, in some instances these SNPs represent associations in genes previously reported that may lend support to previous findings, or may be additional SNPs in genes reported with a p value < 0.01 . Such findings strengthen the likelihood of an association because it will be based on multiple SNPs for the same gene (e.g. collagen type IV, LTF and TIMP2 in mothers and IGF2, IL2, TIMP2, collagen type IV, and TNF in fetuses).

Haplotype association

Haplotype analyses of genes with at least one significant SNP ($p < 0.05$) and two SNPs in the gene identified four genes in maternal samples [alpha 3 type IV collagen isoform 1 (*COL4A3*), interleukin 6 receptor (*IL6R*), lactotransferrin (*LTF*), and fibroblast growth factor 1 (acidic) isoform 1 (*FGF1*)] and three genes in fetal samples [insulin-like growth factor 2 (*IGF2*), interleukin 2 (*IL2*), and alpha 1 type IV collagen preprotein (*COL4A1*)] that were associated with the risk of spontaneous preterm labor/delivery with intact membranes (Table 6; Figure 2).

The most significant haplotype association for maternal DNA ($p < 0.01$) was for *COL4A3* at rs1882435-rs10178458-rs55997063 (global $p = 0.007$; Table 6). This haplotype included *COL4A3* SNP rs1882435, a SNP that independently associated with spontaneous preterm labor/delivery ($p = 0.007$; Table 6), where the A allele was the risk allele. Upon examination of the individual haplotypes, it was clear that all statistically significant haplotypes for rs1882435-rs10178458-rs55997063 contained the rs1882435 risk allele. Examination of the LD plot for *COL4A3* demonstrated that rs10178458 is in moderate LD with rs1882432, but the pattern of LD appears different between cases and controls (Figure 2a; 2b).

The most significant associations for fetal DNA ($p < 0.01$) were for *IGF2* and *IL2* (Table 6; Figure 3). The haplotypes for *IGF2* were at rs3213225-rs3213223 (global $p = 0.004$) and for *IL2* at rs2069772-rs2069771-rs2069762 (global $p = 0.005$) (Table 6). Both of these haplotypes contained a SNP that had a marginal effect; however, the *IGF2* rs3213225-rs3213223 had a global haplotype p value that was slightly more significant than the individual main effect (*IGF2* rs3213225, $p = 0.005$; Table 6), and the associations at individual haplotypes suggest that the risk allele for rs3213225 is not driving the association at this haplotype. Further examination of the LD plot suggests that there is strong LD (as measured by D') between rs3213225 and rs3213223 in controls but not cases (Figure 3a; 3b).

Histologic chorioamnionitis

Sub-analyses of all statistically significant single locus and haplotype associations for differences between cases with histologic chorioamnionitis and controls (Tables 7 and 8) demonstrated a decrease in the OR for the maternal sample *TIMP2* rs2277698 with the OR dropping from 1.98 (Table 4) to 1.49, as well as a loss of statistical significance (Table 7). However, there was an increase in the OR for fetal sample *IL6R* rs8192282 association (OR changing from 2.07 to 2.94) and this SNP remained statistically significant ($p = 0.045$). No significant global haplotype p values (Table 8) were observed for any of the maternal sample haplotypes that demonstrated significant association in Table 6. There were not enough fetal cases ($n=14$) with histologic chorioamnionitis to perform haplotype analyses of these samples. Overall, we caution that the interpretation of these findings should be tempered by the small samples sizes.

MDR analysis

Exploratory MDR analyses were performed using different filtering approaches. The only statistically significant associations were in the analyses in which maternal and fetal genotypes were combined after filtering for SNPs with statistically significant main effects (both $p < 0.05$ and $p < 0.10$) (Table 9). The only model with a p value < 0.05 was found in analyses of SNPs filtered for main effects with a $p < 0.10$ (Table 9c, Figure 4). This model included rs3752896_maternal, rs34003_fetal, and rs598893_fetal, and had a testing balanced accuracy of 0.62 ($p = 0.043$), but a cross validation consistency of only 6/10, suggesting that this model is unlikely to generalize. The maternal SNP, rs3752896, is an intronic SNP in collagen type IV alpha 4 (*COL4A4*) and the two fetal SNPs are both intronic and include a SNP (rs34003) in fibroblast growth factor 1 (*FGF1*) and a SNP (rs598893) in *COL4A1*. Only fetal SNP, rs34003, was among the most associated SNPs in the single locus analysis (Table 4). Two other models are worth mentioning: 1) a maternal model using TuRF that included rs2069762-rs5369-GNSC_30713312, SNPs in *IL2*, Endothelin1 (*EDN1*) and *IL6*, respectively (Testing Balanced Accuracy = 0.61; $p = 0.057$, CVC = 9/10) (Table 9a); and 2) a fetal model following TuRF filtering that included rs2304573-rs2621208-rs2252070, SNPs in Fibronectin 1 (*FN1*), *COL1A2* and *MMP13*, respectively (Testing Balanced Accuracy = 0.62, $p = 0.063$, CVC = 8/10) (Table 9b).

Pathway analysis

In mothers, the IPA network algorithm discovered that the focus molecules were significantly interconnected in four networks, scoring 12 to 20 ($p = 10^{-12}$ to $p = 10^{-20}$) (Table 10). Using the “overlapping network” feature of IPA, we found these networks were joined together by a few molecules, namely *TIMP-2* as well as several network partners supplied by the IPA knowledge base (e.g. *MMPs* and *ACAN*, which is involved in cell adhesion and physiologic functions associated with extracellular matrix). An example of the top scoring network is shown in Figure 5. Using the fetal genes as input, the IPA network algorithm discovered that fetal focus genes are also highly interconnected. It modeled these into three networks, scoring 7 to 35 (Table 11). In contrast to the maternal model, these networks did not have overlapping molecules joining them, though *TIMP2* did play a role in the top-ranked network along with several inflammatory genes (including *TLR*) and coagulation factors (Figure 6).

Our focus genes were identified by IPA as part of several well-established canonical signaling pathways. In mothers, the IPA algorithm identified the “hepatic fibrosis/hepatic stellate cell activation” as the best scoring of the canonical pathways involving the “focus genes” ($p = 4.9 \times 10^{-12}$). This pathway was also the best scoring of the canonical pathways in the fetal genes ($p = 9.1 \times 10^{-14}$). The coagulation system was the second ranked canonical pathway for the fetal genes ($p = 1.0 \times 10^{-6}$).

COMMENT

Principal findings of the study

We report the results of a large, carefully phenotyped genetic association study of women with preterm labor and delivery with intact membranes and their offspring in a single Hispanic population. This study identified maternal and fetal DNA variants that predispose to preterm labor with intact membranes leading to preterm delivery. The main observations were: 1) a SNP in TIMP2 in mothers was significantly associated with the phenotype; 2) a SNP in IL6R in fetuses increased the risk for preterm labor/delivery; 3) haplotypes for COL4A3 in the mother and IGF2 and IL2 in the fetus were associated with preterm labor/delivery with intact membranes; 4) pathway analysis suggested that maternal and fetal genes involved in the control of inflammation and extracellular matrix metabolism are involved in the biological processes that predispose to this pregnancy outcome; and 5) importantly, TIMP2 was consistently found in the different networks (maternal and fetal) associated with preterm labor/delivery with intact membranes. Collectively, these findings support the concept that there is a genetic component in the risk of spontaneous preterm labor/delivery with intact membranes in this population.

Single locus analysis for mothers

The observed association between TIMP2 and spontaneous preterm labor with intact membranes is novel and implicates extracellular matrix metabolism as an important factor predisposing to preterm parturition. TIMP2 is expressed in the amnion¹³⁷ and plays an important role in the regulation of the activity of matrix degrading enzymes. At low concentrations, TIMP2 can enhance activation of MMP2, while at high concentrations it inhibits the activity of MMP2.^{138;139} Similarly, higher concentrations of TIMP2 inhibit MMP9 (gelatinase B) activity. MMP2 is a constitutive enzyme, while MMP9 is inducible in response to infection.¹⁴⁰ Both have been found in amniotic fluid^{141–145} and the activity and concentration of MMP9 is increased in the amniotic fluid of women with preterm labor and intact membranes, as well as in women with preterm PROM.^{141;143} We have previously reported that amniotic fluid TIMP2 concentrations are low in women with spontaneous labor (term and preterm), with intact or ruptured membranes, regardless of the microbial status of the amniotic cavity.¹⁴⁴ A decrease in TIMP2 is thought to favor the activity of MMPs, promoting extracellular matrix degradation associated with labor. In addition, TIMP2 has been implicated in the metabolism of collagen IV, a component of the basement membrane region of the amnion/chorion and uterine cervix.^{146–148} Moreover, TIMP2 has been found to inhibit angiogenesis through mechanisms that are independent of MMPs.¹⁴⁹ We have recently reported that a subset of women who develop preterm labor with intact membranes have an increased plasma concentration of anti-angiogenic factors prior to the development of this complication.¹⁵⁰ In a parallel study, we have found a significant association between the same TIMP2 SNP and pPROM (submitted for publication). Collectively, this evidence provides biological support for the association of a TIMP2 SNP with preterm labor/delivery with intact membranes.

Single locus analysis in fetuses demonstrates an association between preterm labor/delivery and IL6 receptor

This is the first demonstration of an association between IL6R and spontaneous preterm labor with intact membranes in Hispanics. This observation is consistent with previous reports of IL6R associations with spontaneous PTB in individuals of European and African descent.⁸⁹ The SNP found to be associated in our study was rs8192282. In contrast, the positive results of Velez *et al.*⁸⁹ were based on multiple haplotypes in the IL6R gene in both European-American and African-American populations. However, the SNPs found to be significant in the previous study⁸⁹ are in strong LD in Caucasian HapMap samples with the

SNP we found in the current study (Figure 7; $D' = 0.78\text{--}1.00$). The consistency of the association between IL6R and spontaneous preterm labor/delivery among different ethnic populations lends support to the importance of this finding.

Although IL-6 has been considered neither sufficient nor necessary for preterm delivery in murine models,¹⁵¹ the IL-6/IL-6R system has been previously implicated in preterm labor.^{152–154} The median amniotic fluid concentration of IL-6 in the midtrimester of pregnancy is higher in women destined to have a pregnancy loss or a preterm delivery than that of women who deliver at term.^{155–157} Moreover, among patients with preterm labor and intact membranes, amniotic fluid IL-6 concentrations are associated with preterm delivery, the amniocentesis-to-delivery interval, the likelihood of delivery within 48 hours and 7 days, as well as with neonatal morbidity and mortality.^{158;159} IL-6 is a major mediator of the acute phase response to infection and tissue injury and is differentially expressed by intrauterine tissues in the context of labor (term and preterm).^{22;160;161} Indeed, the fetal concentration of this cytokine has been used to define the fetal inflammatory response syndrome, which is associated with the onset of preterm labor, fetal multisystemic organ involvement, and neonatal morbidity.^{159;162–167} In addition, DNA variants in the IL-6 gene have been associated with sepsis, brain injury and cerebral palsy, specially in preterm neonates.^{168–171} IL-6 mediates its biological functions through the IL-6R. A cleaved form of the IL-6R can be found in biological fluids and tissues. The amniotic fluid concentrations of the soluble IL-6R are elevated in preterm labor with intra-amniotic infection.¹⁵⁴ It is of relevance to the findings of this study that Velez *et al.*^{24;89} reported that amniotic fluid IL-6 concentrations were strongly associated with IL6R haplotypes in mothers and fetuses in a Caucasian population in the US. The effects of IL-6R genetic variants on IL-6 concentrations has also been reported in non-pregnant subjects and in one case it was found to be more important than IL-6 DNA variants themselves in determining IL-6 concentrations. This finding is consistent with our observation of both IL-6 and IL-6R associations in previous studies.^{172;173} Pathway analyses indicated that IL6R operates in a network entailing other fetal genes associated with spontaneous preterm labor/delivery with intact membranes, including collagens and IL2. Therefore, the statistical findings in this study, the consistency of the findings among ethnic groups, and the biological role of IL-6 in preterm labor indicate the importance of the IL-6/IL-6R system in the predisposition to spontaneous preterm labor/delivery.

Haplotype analyses suggest novel genes predisposing to preterm labor/delivery

Maternal haplotype analysis found that haplotypes in four genes were associated with preterm labor/delivery: COL4A3, IL6R, LTF, and FGF1 (Table 6). The most significant global association was in the COL4A3 gene. One haplotype (ACT) was associated with a 48% increased risk of preterm labor/delivery. Collagen IV is a major component of the basement membrane of several organs, including the uterine cervix.^{146–148;174} Treatment of cervical smooth muscle cells with TNF-alpha results in increased MMP-9 mRNA expression, induction of MMP-9 production, and decreased TIMP-2 secretion.¹⁷⁵ Therefore, our findings with haplotype analysis for collagen IV and the single locus association in the mother supports the relationship between structural proteins of the extracellular matrix (collagen IV) and a regulator of its degradation (TIMP2) lending substantial biological plausibility to both associations.

The significant association between a haplotype in IL6R in the mothers and spontaneous preterm labor/delivery further supports the role of the IL6/IL6R system in the predisposition for this phenotype. In addition, the IL6R SNP rs8192282 in mothers was of marginal significance, even though it did not remain after multiple testing corrections (Table 5). A maternal haplotype in lactoferrin (LTF) was significantly associated with predisposition to preterm labor/delivery. This protein is a major component of the innate immune system and

has antimicrobial properties, including the ability to protect against biofilm infections. Lactoferrin has been reported to prevent the formation of biofilms by *Pseudomonas*.¹⁷⁶ Biofilms have been recently reported in the amniotic cavity¹⁷⁷ and it is possible that a deficiency in the concentration of this protein may predispose to biofilm formation in this unique niche and its consequences, such as the difficulty in obtaining a positive culture and refractoriness to treatment.^{178–180} A proposed mechanism of action for lactoferrin to inhibit biofilm formation is via iron chelation and stimulation of a specialized form of bacterial-surface motility (twitching), thereby, preventing the formation of cell clusters and biofilms.¹⁷⁶ This protein is normally present in the amniotic fluid and its concentration increases with gestational age and decreases at the time of the onset of spontaneous labor.¹⁸¹ Another important aspect of lactoferrin is that it downregulates pro-inflammatory cytokines and the production of reactive oxygen radicals. We have previously reported that the amniotic fluid concentrations of lactoferrin are increased in women with preterm labor and intra-amniotic infection.¹⁸¹

A maternal haplotype for fibroblast growth factor 1 (FGF1) was also associated with the risk of spontaneous preterm labor/delivery. This protein is very pleiotropic, and has been implicated in vascularization.¹⁸² A subgroup of patients with spontaneous preterm labor/delivery are thought to have vascular lesions as a primary mechanism of disease.¹⁸³ It is possible that defective angiogenesis plays a role in some cases of preterm labor.¹⁵⁰ Further studies are required to explore the role of FGF1 in this condition.

Three genes in fetal DNA had significant haplotype associations: IGF2, IL2, and COL4A1. The association with COL4A1 in the fetus is consistent with the finding of a haplotype in COL4A3 in the mother in this study, and TIMP2, as discussed above. The role of IL2 in preterm parturition is less clear. This cytokine is a T cell growth factor that has been reported to stimulate prostaglandin production by chorion.^{184;185} However, a role for IL-2 in parturition is not well established. It is possible that this cytokine may play a role in tolerance regulation of the fetal allograft. Finally, fetal haplotypes in IGF2 were found to be a large modifier of the risk of preterm delivery; one haplotype increased the risk, and another one decreased the risk. This gene was studied because we were interested in examining the relationship between DNA variants and SGA. Spontaneous preterm labor has been associated with SGA,¹⁸⁶ and also with accelerated fetal growth.^{187;188} The finding reported herein calls for further examination of the role of insulin-like growth factor 2 and pathway in spontaneous preterm parturition.

Exploratory Analysis

Spontaneous preterm parturition is syndromic in nature, and multiple mechanisms of disease are likely to be involved.^{189–191} Discovery tools employing unbiased methods have been used to study complex problems in Obstetrics.^{192–195} To address the complexity of the genetic predisposition to spontaneous preterm labor and delivery, we performed two exploratory analyses. MDR explicitly addressed the potential role of interactions among genes (maternal, fetal, and maternal-fetal). The results of these analyses were not compelling in identifying complex genetic models (e.g. epistasis). Although such gene-gene interactions have been previously reported for spontaneous preterm birth involving IL6, IL6R, and TNF-alpha, these were in the mother in Caucasian individuals.¹²³ Therefore, our negative results may reflect the different ethnic group under study, different DNA variants between studies, and the sensitivity of interaction tests to changes in allele frequencies.¹⁹⁶ However, it is of note that the models we detected, although not significant, did include SNPs in genes that are in extracellular matrix metabolism and inflammatory pathways, especially IL6, MMP13, and several collagen genes.

Additionally, there is an increasing realization of the importance of pathways in predisposition to complex diseases. We utilized IPA to examine the contribution of genetic variants in determining networks and disease functions. The prominence of hepatic fibrosis among established signaling pathways in both maternal and fetal analysis presumably reflects involvement of inflammatory cascades and extracellular matrix remodeling. The findings suggest that maternal and fetal gene variants involving inflammation and extracellular matrix metabolism pathways predispose to spontaneous preterm labor/delivery. These findings are consistent with a large body of literature supporting this view.

Strengths and limitations of the study

The strengths of our study include a well-defined phenotype (spontaneous preterm labor and delivery with intact membranes) and a relatively homogeneous population. This is the largest study to examine the genetic predisposition to spontaneous preterm birth in Hispanics. Moreover, the study includes both maternal and fetal DNA and a relatively large number of genes and DNA variants. The number of DNA variants selected was estimated to cover 90% of the coding DNA variation in the candidate genes. Importantly, we identified that both maternal and fetal DNA variants contributed to modify the risk. Limitations of these types of studies are that confirmation of the findings is required and that we have not examined the effect of environmental factors,^{50;197;198} which are known to play a role in the risk of preterm delivery. In addition, functional studies are needed to assess the precise physiologic implications of the DNA variants and their iterations identified in this study.

Conclusion

This large genetic association study of candidate genes involved in adverse pregnancy outcome revealed that both maternal and fetal DNA variants are associated with spontaneous preterm delivery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. STRUcTURE¹¹⁶ analysis of spontaneous preterm labor/delivery with intact membranes samples to detect population stratification. A) Each mother is denoted as a character with cases in RED and controls in GREEN. Also included are samples from HapMap (Phase 3). These samples are the European (CEU) (BLUE) and Asian samples (Chinese and Japanese, YELLOW) to test the hypothesis of population stratification. Clustering at the vertices indicate genetic similarity and little stratification. The samples from Chile cluster within the space defined by the European samples, supporting the hypotheses of little or no genetic stratification. B) The same analyses conducted with fetal samples included in the current study using the criteria and color coding as in (A). As with the maternal there is no evidence of substantial population stratification.

Figure 2.

Patterns of linkage disequilibrium for genes associated in haplotype analyses of maternal samples in this study. Case and control patterns of LD are shown separately. Haploview¹¹⁹ plots are presented with the SNPs and relative locations within the gene across the top of each panel. The bottom of each figure shows the pair-wise linkage disequilibrium values of all SNPs as measured by D' . D' is a standardized measure of linkage disequilibrium D' values are given in each diamond. D' between each pairwise combination of SNPs is given in each diamond on a scale of 0–100. A value of 100 represents maximum possible linkage disequilibrium. Specifically, in panel (a) the D' between rs1882435 (687) and rs35467545 (693) can be seen by tracing down from each SNP to the diamond where the lines intersect.

In this case, D' is 52. Solid red diamonds represent a D' of 100 and blue diamonds are values with low logarithm of the odds (LOD) scores (<2) and hence unreliable values. White diamonds represent evidence of strong recombination and hence little LD. **a.** *COL4A3* cases; **b.** *COL4A3* controls; **c.** *IL6R* cases; **d.** *IL6R* controls; **e.** *FGF1* cases; **f.** *FGF1* controls; **g.** *LTF* cases; **h.** *LTF* controls.

Figure 3.

Patterns of linkage disequilibrium for genes associated in haplotype analyses of fetal samples in this study. Case and control patterns of LD are shown separately. Haploview¹¹⁹ plots are presented with the SNPs and relative locations within the gene across the top of each panel. The bottom of each figure shows the pair-wise linkage disequilibrium values of all SNPs as measured by D' . D' is a standardized measure of linkage disequilibrium on a scale of 0–100. D' values are given in each diamond. A value of 100 represents maximum possible linkage disequilibrium. Solid red diamonds represent a D' of 100 and blue diamonds are values with low logarithm of the odds (LOD) scores (<2) and hence unreliable values. White diamonds represent evidence of strong recombination and hence little LD. **a.** *IGF2* cases; **b.** *IGF2* controls; **c.** *IL2* cases; **d.** *IL2* controls; **e.** *COL4A1* cases; **f.** *COL4A1* controls.

Figure 4.

MDR model for a three-way interaction involving maternal SNP rs3752896 (*COL4A4*) and fetal SNPs rs34003 (*FGFI*) and rs598893 (*COL4A1*). Each panel represents a three locus genotype; the genotype for each SNP is labeled on the figure. Each large square (3 × 3 box) represents a different genotype for rs598893_2 (GG on left, AG in the middle, and AA on the right). Within each square, each row of cells delineates rs34003_2 genotypes (top TT, middle GT and bottom GG) and each column the rs3752896 genotypes. Therefore, each small cell describes a single and unique three locus genotype. Within each cell are two bars that represent the number of cases with this genotype (left hand bar) and number of controls (right hand bar). Each multilocus cell is denoted as “high risk” (dark gray) or “low risk” (light gray) for spontaneous preterm labor/delivery with intact membranes. Risk status is determined by the ratio of cases to controls adjusted by the number of cases and controls studied. The testing average balanced accuracy is 62% (p-value = 0.043) with a cross-validation consistency of 6 out of 10.

Figure 5.

Connection map for the first ranked network generated by IPA from maternal focus gene input. The biomarkers passing the $p < 0.05$ significance threshold (focus molecules, depicted in pink) were entered into the IPA software for an unsupervised functional analysis to discern regulatory networks involving these molecules. The asterisk indicates that there was more than one SNP probe for the gene tested and the most significant value was placed into the analysis. Solid lines show direct interaction (binding/physical contact); dashed line, indirect interaction supported by the literature but possibly involving one or more intermediate molecules that have not been investigated definitively. Molecular interactions involving only binding are connected with a line without an arrowhead since directionality cannot be inferred.

Figure 6.

Connection map for the first ranked network generated by IPA from fetal focus gene input. The biomarkers passing the $p < 0.05$ significance threshold (focus molecules, depicted in pink) were entered in to the IPA software for an unsupervised functional analysis to discern regulatory networks involving these molecules. The asterisk indicates that there was more than one SNP probe for the gene tested and the most significant value was placed into the analysis. Solid lines show direct interaction (binding/physical contact); dashed line, indirect interaction supported by the literature but possibly involving one or more intermediate molecules that have not been investigated definitively. Molecular interactions involving only binding are connected with a line without an arrowhead since directionality cannot be inferred. The symbol, "SAA@" indicates serum amyloid, which includes SAA1, SAA2, SAA3P, and SAA4.

Figure 7.

Linkage disequilibrium pattern of IL6R region in the HapMap CEU samples of IL6R SNPs from the current study and that of Velez *et al.*⁸⁹ The Haploview figure is as described in Supplemental Figure 2. The SNP that associated with spontaneous preterm labor/delivery with intact membranes in the current study (rs 8192282) is denoted by a rectangle surrounding it in the top of the panel. All other SNPs are from associating haplotypes in Velez *et al.*⁸⁹

Table 1

Parameters explored in MDR analyses

Algorithm	SNPs included	Population
Balanced Accuracy	all tag SNPs	Maternal
	tag SNPs with $p < 0.05$	
	tag SNPs with $p < 0.10$	
	all tag SNPs	Fetal
	tag SNPs with $p < 0.05$	
	tag SNPs with $p < 0.10$	
	all tag SNPs	Maternal-Fetal Combined
	tag SNPs with $p < 0.05$	
	tag SNPs with $p < 0.10$	
Balanced Accuracy with TuRF with 10 SNP filter		Maternal
	all tag SNPs	Fetal
		Maternal-Fetal Combined

Table 2

Demographic and clinical characteristics of the study population

Variable	Cases (n = 223)	Controls (n = 599)	p-value
Parity (number of previous pregnancies)	1 [0–2]	1 [0–1]	0.193
Maternal age (years)	24 [19–32]	24 [20–30]	0.984
BMI	23 [21–26]	24 [22–26]	0.455
Smoking	13%	14%	0.506
Gestational age at delivery (weeks)	34 [30–35]	40 [39–41]	<0.0001
Birth weight (grams)	2200 [1680–2600]	3440 [3230–3650]	<0.0001
1 st minute Apgar score	8 [5–9]	9 [9–9]	<0.0001
5 th minute Apgar score	9 [18–9]	9 [9–9]	<0.0001

Results are expressed as median (25th to 75th percentile)

BMI: body mass index

Table 3

Gene summary information strongest associations ($p < 0.01$)

Population	Gene Name	Gene Code	rs #	Chromosome	Position (bp)	Function
Maternal	Interleukin 6 receptor isoform 1	<i>IL6R</i>	rs8192282	1	152668303	Coding Exon (A/A 31)
	Alpha 3 type IV collagen isoform 1	<i>COL4A3</i>	rs1882435	2	227810996	Intron
	Lactotransferrin	<i>LTF</i>	rs2269435	3	46462007	Intron
	Fibroblast growth factor 1 (acidic) isoform 1	<i>FGF1</i>	rs34003	5	141955251	Intron
	Guanine nucleotide-binding protein, beta-3	<i>GNB3</i>	rs5440	12	6819160	Promoter
	Insulin-like growth factor 1 (somatomedin C)	<i>IGF1</i>	rs5742612	12	101398994	Promoter
	Tissue inhibitor of metalloproteinase 2	<i>TIMP2</i>	rs2277698	17	74378612	Coding Exon (S/S 101)
	Coagulation factor III precursor	<i>F3</i>	rs610277	1	94771147	Intron
	Natriuretic peptide receptor A/guanylate cyclase	<i>NPRI</i>	rs3891075	1	151924811	Intron
	Interleukin 6 receptor isoform 1 precursor	<i>IL6R</i>	rs8192282	1	152668303	Coding Exon (A/A 31)
Fetal	Tenascin R (restriclin, janusin)	<i>TNR</i>	rs2228359	1	173591274	Coding Exon (D/D 1079)
	Interleukin 1, beta protein	<i>IL1B</i>	rs1143643	2	113304773	Intron
	Fibronectin 1 isoform 3 preproprotein	<i>FNI</i>	rs2304573	2	215951895	Intron
	Interleukin 2 precursor	<i>IL2</i>	rs2069772	4	123592583	Intron
	Fibroblast growth factor 1 (acidic) isoform 1	<i>FGF1</i>	rs34003	5	141955251	Intron
	Interferon gamma receptor 1	<i>IFNGRI</i>	rs1887415	6	137560931	Coding Exon (L/P 467)
	Insulin-like growth factor 2	<i>IGF2</i>	rs3213225	11	2113112	Intron
	Alpha 1 type IV collagen preproprotein	<i>COL4A1</i>	rs16975492	13	109631703	Coding Exon (P/P 710)
	Tissue inhibitor of metalloproteinase 2	<i>TIMP2</i>	rs2277698	17	74378612	Coding Exon (S/S 101)

SNPs found to be significantly associated with spontaneous preterm labor/delivery in single locus tests of association ($p < 0.01$)

Table 4

Population	Gene Code	rs#	Minor Allele	Minor Allele Frequency		OR ¹	95% CI ²		p-value
				Cases	Controls		Lower	Upper	
Maternal (Cases n = 223; Controls n = 99)	<i>IL6R</i>	rs8192282	A	0.13	0.08	1.70	1.19	2.41	0.003
	<i>COL4A3</i>	rs1882435	A	0.33	0.26	1.40	1.10	1.79	0.007
	<i>LTF</i>	rs2269435	C	0.38	0.3	1.39	1.10	1.76	0.007
	<i>FGF1</i>	rs34003	G	0.32	0.41	0.69	0.54	0.87	0.002
	<i>GNB3</i>	rs5440	G	0.36	0.43	0.73	0.58	0.92	0.007
	<i>IGF1</i>	rs5742612	C	0.06	0.10	0.56	0.36	0.87	0.009
	<i>TIMP2</i>	rs2277698	A	0.13	0.07	1.98	1.38	2.83	0.000197*
	<i>F3</i>	rs610277	C	0.10	0.01	2.47	1.31	4.67	0.005
	<i>NPR1</i>	rs3891075	T	0.09	0.07	2.04	1.27	3.30	0.003
	<i>IL6R</i>	rs8192282	A	0.13	0.10	2.07	1.42	3.02	0.000148*
Fetal (Cases n = 179; Controls n = 628)	<i>TNR</i>	rs2228359	T	0.41	0.3	1.47	1.14	1.91	0.004
	<i>IL1B</i>	rs1143643	A	0.2	0.23	1.55	1.18	2.03	0.002
	<i>FN1</i>	rs2304573	C	0.62	0.49	0.70	0.54	0.91	0.008
	<i>IL2</i>	rs2069772	G	0.00	0.13	0.53	0.35	0.80	0.003
	<i>FGF1</i>	rs34003	G	0.30	0.42	0.67	0.51	0.87	0.003
	<i>IFNGR1</i>	rs1887415	C	0.04	0.07	0.50	0.32	0.79	0.003
	<i>IGF2</i>	rs3213225	C	0.09	0.36	0.67	0.51	0.89	0.005
	<i>COL4A1</i>	rs16975492	A	0.41	0.27	0.67	0.51	0.89	0.006
	<i>TIMP2</i>	rs2277698	A	0.13	0.14	1.82	1.27	2.62	0.001

None of these markers had statistically significant deviations from HWE in cases or controls.

¹OR is the Odds Ratio for the additive genotypic model

²95% CI is the 95% confidence interval of the Odds Ratio.

* Significant after FDR correction.

Single SNP associations ($0.01 < p < 0.05$)

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a. Maternal DNA

Gene	SNP	A1	OR	L95	U95	P
LTF	GNSC_617330042	A	1.39	1.003	1.927	0.04825
OXTR	GNSC_631469608	A	1.575	1.003	2.473	0.04862

b. Fetal DNA

Gene	SNP	A1	OR	L95	U95	P
IL10RA	rs17121493	G	1.815	1.155	2.854	0.009803
TIMP2	rs55743137	C	1.494	1.097	2.033	0.01073
TLR1	rs5743553	A	2.728	1.252	5.946	0.01159
COL4A3	rs1882435	A	1.392	1.074	1.805	0.01253
COL4A4	rs3752896	A	0.7424	0.5823	0.9464	0.0162
CETP	rs891144	T	1.603	1.083	2.371	0.01821
COL4A2	rs7338575	C	1.369	1.055	1.778	0.01837
SERPINE1	rs6092	A	1.629	1.081	2.455	0.0198
IL4R	rs1029489	A	0.7479	0.5852	0.9559	0.02034
AGTR1	rs5183	G	2.555	1.151	5.673	0.02113
NPPA	rs5068	C	0.256	0.07936	0.8259	0.0226
HSPG2	GNSC_634098268	T	1.406	1.049	1.885	0.02264
ELN	rs2301995	A	1.44	1.05	1.974	0.02347
COL4A2	rs12873113	T	1.45	1.045	2.011	0.02617
IGF2R	rs894817	A	1.316	1.032	1.679	0.02698
IRS1	rs1801278	A	1.762	1.055	2.945	0.03053
COL4A3	rs56326869	C	0.1134	0.01558	0.8255	0.03161
COL4A4	rs3752895	C	0.7748	0.6118	0.9813	0.03433
IL9R	GNSC_14430798	C	0.4446	0.2085	0.9483	0.03596
LPA	rs4708871	G	2.195	1.05	4.587	0.03655
REN	rs3730103	G	1.753	1.033	2.975	0.03763
ELN	rs17855988	C	1.631	1.028	2.589	0.03784
IL2	rs2069771	T	2.655	1.05	6.71	0.03908
TIMP1	rs11551797	T	0.4538	0.2141	0.9618	0.03924
COL4A5	rs28465565	C	0.4558	0.2153	0.965	0.04006
IL3RA	GNSC_649033887	A	0.3728	0.1447	0.9606	0.04104

b. Fetal DNA

Gene	SNP	A1	OR	L95	U95	P
SERPINC1	rs677	C	1.601	1.017	2.521	0.04197
TNR	rs1385540	T	1.37	1.01	1.858	0.04276
IL1R1	rs3917318	G	1.288	1.008	1.647	0.04315
FIGF	GNSC_634828146	C	0.4742	0.2289	0.9824	0.04468
IGF2	rs2230949	T	1.568	1.01	2.434	0.04508
F7	rs6046	A	1.382	1.004	1.901	0.04705
COL4A2	rs391859	A	0.7216	0.522	0.9975	0.04827
COL4A6	GNSC_634841755	A	0.4671	0.2187	0.9975	0.04925
COL4A4	rs1800516	C	0.3023	0.09162	0.9977	0.04956

Table 6

Most Significantly Associating Haplotypes (Global $p < 0.05$)

Population	Gene	Markers	Haplotype	Haplotype Frequency		OR	95%CI		p-value
				Cases	Controls		Lower	Upper	
Maternal	COL4A3	rs1882435-rs10178458-rs55997063	Global <i>p</i> -value	-	-				0.007
			CTT	0.08	0.11	0.8	0.53	1.21	0.279
			ACT	0.31	0.23	1.48	1.14	1.90	0.002
	IL6R	rs8192282-rs7521458	CCT (referent)	0.60	0.66	-	-	-	-
			Global <i>p</i> -value	-	-				0.043
			AT	0.12	0.08	1.51	1.04	2.20	0.023
	LTF	rs2269435-rs2239692	GT	0.22	0.25	0.88	0.67	1.15	0.339
			GC (referent)	0.66	0.66	-	-	-	-
			Global <i>p</i> -value	-	-				0.013
	FGF1	rs34003-rs17217240-rs9324894	CG	0.18	0.13	1.53	1.12	2.09	0.005
CA			0.19	0.16	1.32	0.98	1.78	0.058	
TA (referent)			0.63	0.70	-	-	-	-	
Fetal	IGF2	rs3213225-rs3213223	Global <i>p</i> -value	-	-				0.004
			TT	0.23	0.07	2.78	1.96	3.94	<0.0001
			CC	0.10	0.36	0.24	0.16	0.35	<0.0001
	IL2	rs2069772-rs2069771-rs2069762	TC (referent)	0.67	0.57	-	-	-	-
			Global <i>p</i> -value	-	-				0.005
			ACG	0.19	0.41	0.26	0.19	0.35	<0.0001
	COL4A1	rs685884-GNSC_633869360	GCT	0.00	0.14	-	-	-	-
			ACT (referent)	0.81	0.45	-	-	-	-
			Global <i>p</i> -value	-	-				0.012
				TA	0.09	0.05	1.12	0.69	1.8
CG				0.19	0.49	0.24	0.15	0.33	<0.0001

Population	Gene	Markers	Haplotype	Haplotype Frequency		OR	95%CI		p-value
				Cases	Controls		Lower	Upper	
				TG (referent)					
				0.73	0.46	-	-	-	-

Table 7

Analysis of histologic chorioamnionitis for significantly associated SNPs

Population	Gene Code	rs#	OR ¹	95% CI ²		p-value
				Lower	Upper	
Maternal (Cases n=30 Controls n=464)	<i>IL6R</i>	rs8192282	1.82	0.87	3.80	0.110
	<i>COL4A3</i>	rs1882435	1.03	0.57	1.89	0.912
	<i>LTF</i>	rs2269435	0.95	0.54	1.67	0.845
	<i>FGF1</i>	rs34003	0.59	0.33	1.05	0.074
	<i>GNB3</i>	rs5440	0.43	0.24	0.79	0.006
	<i>IGF1</i>	rs5742612	0.50	0.15	1.65	0.256
	<i>TIMP2</i>	rs2277698	1.49	0.62	3.61	0.377
Fetal (Cases n=14 Controls n=465)	<i>F3</i>	rs610277	3.78	0.79	18.01	0.096
	<i>NPR1</i>	rs3891075	1.68	0.36	7.76	0.505
	<i>IL6R</i>	rs8192282	2.94	1.02	8.43	0.045
	<i>TNR</i>	rs2228359	1.01	0.42	2.42	0.979
	<i>IL1B</i>	rs1143643	1.70	0.74	3.92	0.212
	<i>FN1</i>	rs2304573	1.23	0.54	2.82	0.626
	<i>IL2</i>	rs2069772	0.22	0.03	1.59	0.133
	<i>FGF1</i>	rs34003	0.43	0.16	1.18	0.101
	<i>IFNGR1</i>	rs1887415	0.60	0.14	2.58	0.495
	<i>IGF2</i>	rs3213225	0.73	0.30	1.79	0.496
	<i>COL4A1</i>	rs16975492	0.62	0.24	1.56	0.307
	<i>TIMP2</i>	rs2277698	2.14	0.72	6.34	0.169

¹OR is the Odds Ratio for the additive genotypic model;

²95% CI is the 95% confidence interval of the Odds Ratio

Table 8

Analysis of histologic chorioamnionitis for significantly associated haplotypes

Population	Gene	Markers	Haplotype	Haplotype Frequency	OR	95%CI		p-value
				Cases	Controls	Lower	Upper	
	<i>COL4A3</i>	rs1882435-rs10178458-rs55997063	Global p-value					0.914
			CTT	0.09	0.11	0.77	0.23	2.00
			ACT	0.23	0.23	0.98	0.48	1.88
			CCT (referent)	0.69	0.66			0.953
	<i>IL6R</i>	rs8192282-rs7521458	Global p-value					0.229
			AT	0.15	0.09	1.97	0.80	4.37
			GT	0.27	0.25	1.19	0.60	2.25
			GC (referent)	0.58	0.66			0.584
Maternal	<i>LTF</i>	rs2269435-rs2239692	Global p-value					0.403
			CG	0.17	0.12	1.35	0.59	2.84
			CA	0.12	0.17	0.68	0.25	1.57
			TA (referent)	0.72	0.71			0.357
	<i>FGFI</i>	rs34003-rs17217240-rs9324894	Global p-value					0.103
			GGT	0.01	0.06	0.21	0.01	1.32
			TGT	0.04	0.06	0.62	0.12	2.05
			GGC	0.26	0.35	0.56	0.28	1.06
			TGC (referent)	0.69	0.53			0.060

* There were only 14 cases available to do haplotype analyses for the sub-analysis of histologic chorioamnionitis. These were not enough cases to do haplotype analyses under the same criteria used for haplotype analyses on Table 4.

Table 9

Summary of MDR analyses

a. PTB Maternal				
Algorithm	Model	Testing Balanced Accuracy	Cross Validation Consistency	<i>p</i> -value
Balanced Accuracy with all tag SNPS	rs2069762	0.55	6/10	0.377
	rs2069762 rs17689966	0.59	6/10	0.132
	rs3917318rs2069762 rs7338575	0.55	2/10	0.397
Balanced Accuracy with TuRF – 10 SNPS	rs2069762	0.55	10/10	0.321
	GNSC_635148713 rs2069762	0.55	5/10	0.330
	rs2069762rs5369 GNSC_30713312	0.61	9/10	0.057
Balanced Accuracy with Tag SNPs with $p < 0.05$	rs1882435	0.56	9/10	0.331
	rs2269435 rs5440	0.56	3/10	0.306
	rs1882435rs34003 rs10459953	0.59	4/10	0.139
Balanced Accuracy with Tag SNPs with $p < 0.10$	rs1882435	0.56	8/10	0.356
	rs2269435 rs5440	0.53	2/10	0.579
	rs1882435rs34003 Rs10459953	0.55	2/10	0.406
b. PTB Fetal				
Algorithm	Model	Testing Balanced Accuracy	Cross Validation Consistency	<i>p</i> -value
Balanced Accuracy with all tag SNPS	rs2069762	0.54	4/10	0.581
	rs3752896 rs2069762	0.59	5/10	0.204
	rs1250215rs3752896 rs2069762	0.56	2/10	0.371
Balanced Accuracy with TuRF – 10 SNPS	rs2069762	0.55	8/10	0.432
	rs2304573 rs2621208	0.61	10/10	0.085
	rs2304573 rs2621208 rs2252070	0.62	8/10	0.063
Balanced Accuracy with Tag SNPs with $p < 0.05$	rs2069772	0.56	8/10	0.292
	rs2069772 rs16975492	0.59	5/10	0.174
	rs34003 rs321225 rs16975492	0.53	3/10	0.634
Balanced Accuracy with Tag SNPs with $p < 0.10$	rs2069772	0.56	8/10	0.292
	rs2069772 rs1800692	0.54	4/10	0.554
	rs2069772 rs2146323 rs3213225	0.55	4/10	0.414
c. PTB Maternal-Fetal Combined				
Algorithm	Model	Testing Balanced Accuracy	Cross Validation Consistency	<i>p</i> -value
Balanced Accuracy with all tag SNPS	rs2069762	0.55	6/10	0.461
	rs2069762_2 rs2274849	0.58	5/10	0.254
	rs2069762_2	0.53	8/10	0.614
Balanced Accuracy with TuRF – 10 SNPS	rs2069762_2 rs16145	0.62	9/10	0.084
	rs2069762_2 rs16145 rs2243290	0.51	4/10	0.943

c. PTB Maternal-Fetal Combined

Algorithm	Model	Testing Balanced Accuracy	Cross Validation Consistency	<i>p</i> -value
Balanced Accuracy with Tag SNPs with <i>p</i> < 0.05	rs8192282_2	0.61	7/10	0.050
	rs5356 rs1882435_2	0.58	2/10	0.160
	rs1882435_2 rs16975492_2 rs2277698	0.54	1/10	0.456
Balanced Accuracy with Tag SNPs with <i>p</i> < 0.10	rs8192282_2	0.61	7/10	0.050
	rs1143643_2 rs7338575_2	0.54	2/10	0.522
	rs3752896 rs34003_2 rs598893_2	0.62	6/10	0.043

* Bold indicates a statistically significant interaction (permutation $p < 0.05$).

Combined analysis consisted of matching fetal to maternal individuals and adding the SNPs to the analysis (e.g., instead of an individual having 751 SNPs this would increase to 1449). Any results with a “_2” indicates that the genotypes were fetal. Any maternal or fetal genotypes without matches were removed from the combined analysis.

Table 10

IPA networks of maternal genes

Network	Molecules*	Calculated Score	Focus molecules	Top Functions of Network
1	AGT, AGTR1, Akt, <u>COL4A2</u> , Collagen(s), Creb, Cyclin A, Cyclin E, ERK, ERK1/2, FAK, <u>FGF1</u> , G-protein beta, <u>GNB3</u> , <u>IGF1</u> , <u>IL6R</u> , Insulin, Laminin, Mapk, Mek, Mlc, <u>OXTR</u> , p70 S6k, PDGF BB, PI3K, Pkc(s), PLC, Ras, Ras homolog, Rsk, Shc, STAT, <u>THPO</u> , <u>TIMP2</u> , Vegf	20	10	Cardiovascular System Development and Function, Tissue Morphology, Cellular Growth and Proliferation
2	Angiotensin II receptor type 1, C8, Calcineurin protein(s), Cyclooxygenase, <u>F3</u> , Fibrinogen, Ifn gamma, Ige, IgG, Ikb, IKK (complex), IL1, <u>IL10</u> , <u>IL18</u> , IL12 (complex), IL12 receptor, <u>IL12B</u> , Interferon alpha, LDL, MHC Class II, Mmp, <u>MMP16</u> , Nfat (family), NFkB (complex), Nfkb-RelA, Nos, P38 MAPK, <u>SELE</u> , <u>SERPINE1</u> , Sod, STAT5a/b, Tgf beta, Tlr, <u>TLR2</u> , <u>VEGFC</u>	18	9	Cell-mediated Immune Response, Cellular Movement, Hematological System Development and Function
3	ACAN, acetaldehyde, ADAMTS1, Ap1, Calmodulin, Caspase, <u>COL4A3</u> , <u>COL4A4</u> (includes EG:1286), FGFR1, FSH, Histone h3, Hsp70, <u>HTR2A</u> , IL17A (includes EG:3605), <u>IL1B</u> , ITGA3, Jnk, LRPAP1, <u>LTF</u> , MIF, MMP3, MMP7, MMP13, platelet activating factor, PTH, RAPGEF1, <u>REN</u> , SNCG, <u>THBS4</u> , Timp, <u>TIMP2</u> , Tnf receptor, Trypsin, xanthine, XDH	16	8	Connective Tissue Disorders, Genetic Disorder, Cellular Movement
4	ACAN, ADAM17, AFP, alcohol, CCL7, <u>COL5A2</u> , <u>CRHR2</u> , CRK, CXCL12, EGFR, FCER2, Fgfr, FGFR1, HMGA2, <u>HSPG2</u> (includes EG:3339), IL6, IL17A (includes EG:3605), ITGA5, Mmp, MMP3, MMP7, <u>MMP10</u> , MMP13, MMP17, NQO1, platelet activating factor, PLC gamma, PTPN6, SAA2, <u>TBXAS1</u> , testosterone, Timp, <u>TIMP2</u> , TIMP4, TNF	12	6	Cellular Movement, Connective Tissue Disorders, Genetic Disorder

* Focus molecules are underlined.

Table 11

IPA networks of fetal genes

Network	Molecules*	Calculated Score	Focus molecules	Top Functions of Network
1	Angiotensin II receptor type 1, <u>CETP</u> , Collagen(s), <u>F3</u> , <u>F7</u> , Fibrin, Fibrinogen, <u>FN1</u> , HDL, <u>HSPG2</u> (includes EG:3339), Ifn gamma, <u>IFNGR1</u> , Ige, <u>IGF2</u> , IgG, IL1, <u>IL10RA</u> , IL12 (complex), <u>IL1R1</u> , <u>IL4R</u> , Interferon alpha, Laminin, LDL, <u>LPA</u> , Mmp, NFkB (complex), P38 MAPK, p70 S6k, SAA@, <u>SERPINC1</u> , <u>SERPINE1</u> , Tgf beta, <u>TIMP2</u> , Tlr, <u>TLR1</u>	35	15	Cardiovascular Disease, Hematological Disease, Infectious Disease
2	<u>AGTR1</u> , Akt, Ap1, <u>COL4A1</u> , <u>COL4A2</u> , <u>COL4A3</u> , Creb, Cyclin A, Cyclin E, ERK, ERK1/2, FAK, <u>FGF1</u> , <u>IGF2R</u> , <u>IL2</u> , <u>IL6R</u> , Insulin, <u>IRS1</u> , MAP2K1/2, Mapk, Mek, MHC Class II, <u>NPPA</u> , <u>NPR1</u> , PDGF BB, PI3K, Pkc(s), PLC, Ras, <u>REN</u> , Shc, Sos, STAT, TCR, Vegf	27	12	Cardiovascular Disease, Cardiovascular System Development and Function, Embryonic Development
3	ADAM8, BDNF, C11ORF82, CARD18, CHAT, Ck2, <u>COL4A4</u> (includes EG:1286), CXCL6, <u>ELN</u> , FSH, GBP6, GNL1, H2-Q4, Histone h3, IL1/IL6/TNF, IL17B, IL17C, <u>IL1B</u> , Jnk, MHC CLASS I D2D ANTIGEN, MIF, PENK, PLD3, Rac, SAA2, SCUBE1, SCUBE2, SLC14A1, SLC5A2, SLC5A4B, SLFN12L, TMEM176B, TNF, <u>TNR</u> , TREM3	7	4	Cancer, Cell Cycle, Cell-To-Cell Signaling and Interaction

* Focus molecules are underlined.