Alterations of Gene Expression in Preterm Birth: A Screening Study

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Abstract

Preterm birth is classified by a baby being born 37 weeks or earlier. However, if a baby is born 32 weeks or earlier, they are at a higher risk for death and disabilities. Preterm babies cause more than half of long-term morbidity (suffering from a disease or medical condition) and 75% of prenatal death. Using the Preterm Birth Transcriptomic Database, these genes were screened using a variety of ways. To narrow down these genes, we initially used P-value, then later used FC Value, Val. Fold Change, and Log Fold Change to narrow down the genes. Screening second trimester maternal blood and third trimester maternal blood for significantly regulated genes revealed 42 potential gene candidates. Of these 42 genes, 22 (52.3%) were significantly altered in the third trimester based on p-value less than 0.05. In the end 12 genes were picked for further evaluation. We grouped the genes into 4 major categories: immune system related, metabolism related, clot related, and development related. In summary of the 12 genes screened, TLR4 is our top gene candidate for preterm birth prevention. TLR4 is a part of the TLR (toll-like receptor) family, which plays an important role in pathogen recognition and innate immune activation. This gene and its inhibitor, TAK-242 would serve as a potential causative gene in preclinical mice models and testing of this drug needs to be conducted in various preclinical platforms with the end target of a Phase 1/2 clinical trial.

Introduction

The number of preterm births per year is estimated to be around 15 million. Preterm birth is classified by a baby being born 37 weeks or earlier.[1] However, if a baby is born 32 weeks or earlier, they are at a higher risk for death and disabilities. In 2021, 1 in 10 infants born were affected by preterm birth in the United States. Although, this does differ from other countries as well. Preterm birth rates range from 5% to 18% of all births across 184 countries. To list a few, in the United States, preterm birth rates have risen from 10.1% in 2020 to 10.5% in 2021. (Figure 1)

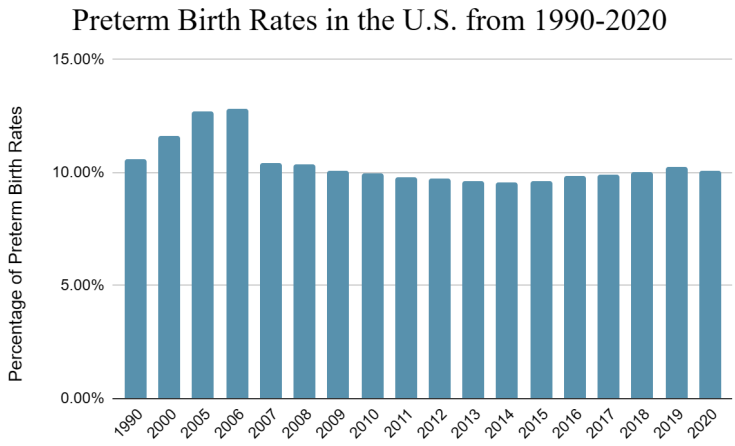

Figure 1 - Timeline of Preterm Birth Rates in the U.S. from 1990-2020

In Europe, preterm births range from 5-10% and in Africa, preterm birth rates were at a high of 14.8% in 2021. Preterm babies cause more than half of long-term morbidity (suffering from a disease or medical condition) and 75% of prenatal death.

Around 30-35% of preterm births are indicated, 40-45% are caused by spontaneous preterm labor, and 25-30% are caused by PPROM; births caused by both spontaneous labor and PPROM are referred to as spontaneous preterm births.[2] (Figure 2)

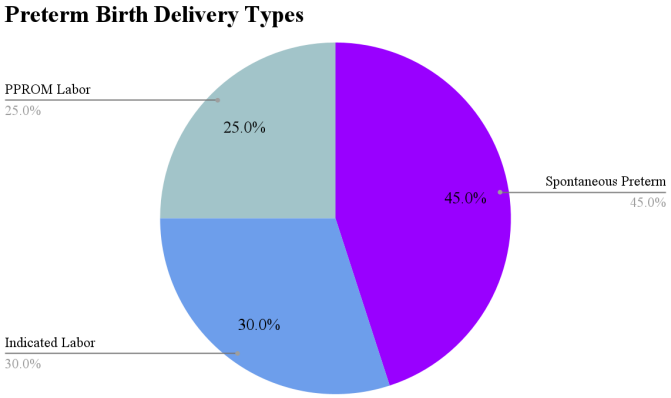

Figure 2 - 3 Different Types of Birth Delivery for Preterm Birth

Indicated births are when the delivery is medically induced. There could have been an issue with the baby or the mother, requiring an immediate delivery. Spontaneous preterm labor occurs when the mother's contractions have started but their water hasn't broken. Lastly, PPROM birth is when the mother's water breaks and goes into natural labor, however, before 37 weeks. This explains why the United States has a higher preterm birth rate, as induced labor is becoming increasingly common.

Seeing that many babies are typically born at 40 weeks, developmental disabilities and undeveloped systems are more likely to affect prematurely born babies.[3] In a press statement,

Scott D. Berns, MD, Vice President for chapter programs of the March of Dimes, states that prematurity is a widespread, serious problem in America and that, regrettably, the number of preterm deliveries is increasing each year. “Too many babies are born extremely premature in this country, and the result is that many of them die in the hospital or suffer lifelong consequences, including cerebral palsy, mental retardation, chronic lung disease, blindness, and hearing loss.”

Premature delivery complications can range in severity, however, they are more likely to occur the sooner the baby is born. Extremely preterm is classified as a baby being born 25 weeks or earlier, very preterm is the baby being born less than 32 weeks, moderately preterm is the baby being born between 32-34 weeks, and late preterm is between 34-36 weeks of completed pregnancy. Although complications are not experienced by all premature babies, being born too early can have both short and long term complications. During the first few weeks of a premature infant, short term complications may include breathing problems, temperature control problems, and immune system problems. A premature baby may have trouble breathing due to their underdeveloped respiratory system. Their lungs aren’t able to expand and contract normally which can develop respiratory distress syndrome. Premature infants can lose their body heat quickly. They don’t have the stored body fat of a full term infant, meaning they are unable to produce enough heat to offset the heat that is lost through their skin. If their body temperature falls too low, hypothermia can occur. Premature babies frequently have weak immune systems, which increases their risk of infection. Sepsis, an infection that spreads to the bloodstream, can be quickly brought on by infection in a premature newborn. In the long term, premature birth can lead to cerebral palsy, dental problems, and vision problems. Cerebral palsy is a movement, muscle tone, or posture condition that can be brought up by an infection, poor blood supply, or trauma to the developing brain of a newborn, either early in pregnancy or while the child is still young. Dental problems can occur to infants that were critically ill. There can be a delay in tooth eruption, tooth discoloration, and improperly aligned teeth. Premature newborns are at risk of developing retinopathy of prematurity, a condition where blood vessels bulge and overgrow in the light-sensitive layer of neurons at the back of the eye (retina). Sometimes the retinal arteries damage the retina over time, forcing it out of position. When the retina pulls away from the back of the eye, the condition is known as retinal detachment, which can impair vision and ultimately blindness if left untreated.[4]

There are also some cognitive issues that come along with preterm birth. Many children that were born preterm suffer with mental processes such as attention, problem solving, and memory.[5] Studies have found that these cognitive issues start to become noticeable during elementary school. The study discovered that very preterm children (<32 weeks) had more difficulty remembering pairs of simple terms that appeared briefly on a screen, even if only a moment later. During the study, they also had unique patterns of brain activity on Magnetoencephalography (MEG) than full-term children of the same age. Very preterm children's brains, in particular, showed a decrease in coordinated activity in brain waves in the cortex, the outer layer of the brain. Full-term children, on the other hand, showed an increase in this type of connection when completing the same task.

As stated, the precise reason as to why preterm birth occurs is unknown, nevertheless, there are some known risk factors to preterm birth. These include smoking or drug and alcohol use,

being underweight or overweight before pregnancy, having a previous premature delivery, problems with the uterus, placenta, or cervix, and chronic conditions - such as high blood pressure or diabetes.

There are certain things that can be done to help women, especially those who are at a higher risk, these include progesterone supplements and cervical cerclage. Progesterone supplements, which have many uses, can specifically help women who have a history of preterm birth or a short cervix reduce their chances of preterm birth. Cervical Cerclage is a surgical procedure performed during pregnancy in women that have a short cervix. During this procedure, the cervix is stitched together with sutures, which may provide extra support to the uterus. When it is time to give birth, the sutures are removed. This method can be useful because it keeps the cervix closed during pregnancy and can prevent premature birth caused by a weaker cervix.

In this paper, we screened maternal and cord blood to find potential biomarkers related to the risk of preterm birth.

Methods

The genes were screened using a variety of ways. To begin, we used P-value to narrow down genes associated with premature birth.[6] In statistical analysis, the P-value (probability value) determines statistical significance. (figure) Generally, statistical significance is frequently stated in p-values ranging from 0 to 1. The lower the p-value, the stronger evidence there is, thus the result should be statistically significant. The null hypothesis is the statement you are trying to prove as false when looking for statistical significance.The next method used is the FC or F Value.[7] This was monitored when screening genes in the third trimester cord blood. The FC Value (Fold Change) is a type of ratio used in quantitative analysis. It displays the relationship between two variables by stating the ratio of their values. It also shows how many times something has changed in comparison to the original amount. Fold change is useful when searching for increases and decreases in data. Also used to screen genes was the Log Fold Change and the Val. Fold Change. These values sound very similar yet very different. The Log Fold Change is the log of the Fold Change, the Val. Fold Change is the raw value.

Results

Screening second trimester maternal blood and third trimester maternal blood for significantly regulated genes revealed 42 potential gene candidates. Of these 42 genes, 22 (52.3%) were significantly altered in the third trimester based on p-value less than 0.05. Secondly, third trimester cord blood was examined using an absolute val. fold change threshold of 2.0. This led to 111 genes being included in the screening. Finally, overlap between third trimester maternal blood and third trimester cord blood was investigated. In the end 12 genes were picked for further evaluation. (Figure 3)

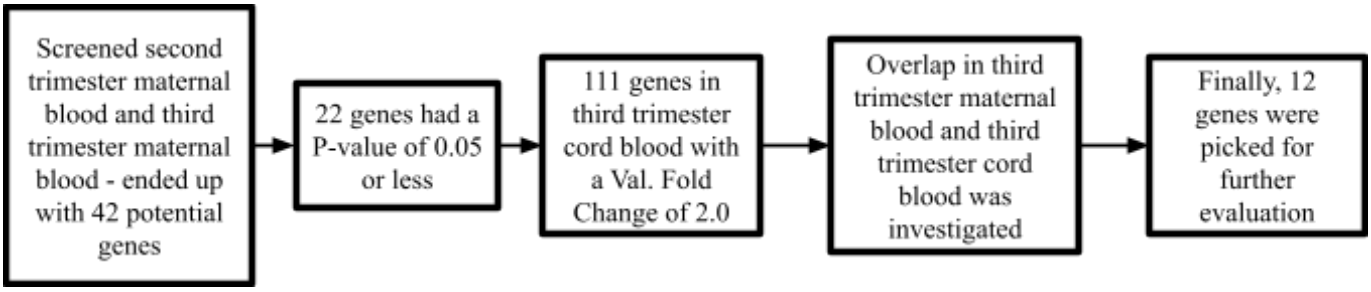

Figure 3 - Flow Chart on How Genes Were Screened and Picked for Further Evaluation

We grouped the genes into 4 major categories: immune system related, metabolism related, clot related, and development related.

Development Related Genes

The H19 gene is a long noncoding RNA gene found in humans and other animals. H19 is involved in the regulation of body weight and cell proliferation. This gene is also involved in the development of various cancers and the regulation of gene expression.[8] This gene can also act as tumor suppressor, keeping cells from dividing too fast or in an uncontrolled way. The H19 gene is highly active in numerous tissues before birth and appears to play an important role in early development.[9] In our Preterm Birth Transcriptomic Database we used to compare genes, H19 is shown as an upregulated gene.[10] A search of PubMed showed no results for a correlation between H19 and preterm birth, however, our research narrows down and concludes that H19 could be a potential candidate for being related to preterm birth.

Clot Related Genes

There were two narrowed down genes in the clot related category, THBS1 and SERPINI1. THBS1 is a glycoprotein that acts as an adhesive and enables cell-to-cell and cell-to-matrix connections.[11] This protein can bind fibrinogen, fibronectin, laminin, collagen types V and VII, and alpha-V/beta-1 integrins. Platelet aggregation, angiogenesis, and cancer have all been linked to this protein. THBS1 was found to be upregulated in preterm births. THBS1 has been studied in the context of preterm births prior and found to be a potential reactor in preterm birth, not just only in humans but in animals as well.[12] Additionally, researchers have inhibited THBS1 through use of CD47 antisense antibodies increasing the feasibility of targeting THBS1 to prevent preterm births.

The SERPINI1 gene encodes a protein called neuroserpin, which is a type of serine protease inhibitor.[13] Serpins contribute to the regulation of many chemical reactions by inhibiting the activity of specific proteins. Neuroserpin suppresses the action of a tissue plasminogen activator enzyme, which is associated with cell movement, blood clotting, and inflammation. It has been found that a functional SNP in the promoter of the SERPINI1 gene drastically lowered promoter activity in amnion fibroblast cells, increasing the risk of preterm premature rupture of membranes (PPROM), the major cause of preterm delivery.[14] This correlated with our results showing SERPINI1 downregulated in maternal and fetal cord blood. However, targeting of

SERPINI has not been pursued in the literature currently and potential off target effects need to be considered.

Metabolism Related Genes

There were three genes narrowed down to being metabolism related, NET1, EPM2A, and ALPL. The NET1 gene is part of the guanine nucleotide exchange factor family.[15] This gene is involved in various cancers such as gastric cancer, breast cancer, and glioma. Normally, this gene interacts with RhoA in the cell nucleus and is involved in DNA repair following damage using as exposure to ionizing radiation. In the Preterm Birth Transcriptomic Database, NET1 was found to be downregulated in both 3rd trimester maternal blood and 3rd trimester cord blood. This can represent a vicious cycle between the strong proinflammatory immune signatures (discussed later) and lack of DNA repair promoting additional immune recruitment leading to potential preterm birth.

The ALPL gene codes for an enzyme known as tissue-nonspecific alkaline phosphatase (TNSALP). This enzyme is essential for the formation of bones and teeth.[16] It is also found in a variety of other tissues, most significantly in the liver and kidneys. This enzyme functions as a phosphatase, removing clusters of oxygen and phosphorus atoms from other proteins. As stated earlier, ALPL codes for an enzyme abbreviated as TNSALP. TNSALP is required for mineralization, the process in which minerals such as calcium and phosphorus are formed in developing bones and teeth. Mineralization is essential for the production of strong, stiff bones and teeth that can survive eating and grinding. There is a health condition related to the ALPL gene that occurs mainly before birth and early infancy, this condition is known as Hypophosphatasia. Hypophosphatasia is a disorder that affects the development of bones and teeth. The signs and symptoms of hypophosphatasia can occur at any age, from before birth to adulthood. The most severe types of the condition usually appear before birth or in early infancy. Hypophosphatasia weakens and softens the bones, resulting in skeletal deformities similar to rickets, another childhood bone condition.[17] Affected infants have short limbs, an irregularly formed chest, and fragile skull bones at birth. Poor feeding, failure to gain weight, respiratory issues, and excessive levels of calcium in the blood, which can lead to repeated vomiting and kidney problems, are all potential complications throughout infancy. In certain circumstances, these problems are fatal.

The EPM2A gene codes for the development of a protein known as laforin.[18] Although this protein is found in all cells of the body, it appears to be essential for the survival of neurons in the brain. Laforin has numerous functions within cells, it interacts with other various proteins, including malin, which is produced from the NHLRC1 gene. These proteins are components of complex networks that transmit chemical signals and degrade unnecessary or defective proteins. Additionally, Laforin may also operate as a tumor suppressor protein, which means it prevents cells from growing and dividing uncontrollably. We saw this earlier with the H19 gene. Laforin and malin play a critical role in the regulation of glycogen, a complex sugar. Glycogen is the body's primary source of stored energy. This sugar is stored in the liver and muscles and is broken down whenever it's needed for fuel. A condition called Glycogen storage disease type IV can be related to preterm birth.[19] It's a disorder caused by buildup of glycogen in the body's cells, signs for this condition often develop before birth and many infants are diagnosed at birth. Infants with this condition have very low muscle tone and muscle wasting, these infants

unfortunately do not survive past the newborn period due to a weakened heart and muscles used for breathing.

Immune Related Genes

Six pro-inflammatory gene signatures were found to be upregulated in maternal and fetal cord blood including: CLEC4D, TLR4, TREM1, NLRC4, HIF3A, and WDFY3.

Several myeloid related immune genes were upregulated consistent with prior literature showing the importance of myeloid activation in this condition. CLEC4D is involved in cell adhesion, cell-cell signaling, glycoprotein turnover, and roles in inflammation and immune response.[20] TLR4 is a part of the TLR (toll-like receptor) family, which plays an important role in pathogen recognition and innate immune activation.[21] They identify pathogen-associated molecular patterns (PAMPs) expressed on infectious pathogens and mediate the generation of cytokines required for successful immunity development. Inflammation is the central mechanism of preterm birth, and TLR4 is an important mediator of pro-inflammatory triggers. In preclinical studies, TLR4 has been identified as an interesting medicinal target to protect against preterm birth and prenatal inflammatory damage.[22] TREM1 amplifies TLR induced inflammation by increasing the production of inflammatory cytokines.[23] TREM1 forms a compound with the adaptor DAP12 and promotes the release of inflammatory cytokines. TREM1 expression is higher in tumor tissues vs non-tumor tissues, most likely due to myeloid cells invading tumors. Higher levels of TREM1 mRNA in tumor tissues correlate with shorter survival times of patients with colon cancer, breast cancer, and pancreatic cancer highlighting the negative prognostic association with this gene. Finally, NLRC4 plays essential roles in the immune response to a wide variety of pathogenic pathogens, tissue injury, and other cellular stimuli.[24] Overall, these 4 genes emphasize the clear pro-inflammatory microenvironment present in maternal and fetal cord blood in preterm birth patients.

HIF3A is a protein encoding gene as well. According to our Preterm Birth Transcropictic Database, the HIF3A gene is upregulated. This gene encodes the alpha-3 subunit of one of several alpha/beta-subunit heterodimeric transcription factors that influence several adaptive responses to low oxygen tension (hypoxia). The transactivation domain found in factors containing the alpha-1 or alpha-2 subunits is missing from the alpha-3 subunit.[25] Factors containing the alpha-3 subunit are considered to be negative regulators of hypoxia-inducible gene expression. This gene has many alternatively spliced transcript variants. The hypoxia-inducible factors family, which includes the HIF3A gene, are believed to play a key role in formation of new blood vessels, metabolism, obesity, immunity, and variation in DNA methylation. A study found that in children, early BMI predicts later HIF3A methylation in blood. New evidence suggests that gestational diabetes and maternal pre-pregnancy BMI have an effect on cord blood methylation at a second HIF3A promoter site.[26]

WDFY3 is an upregulated gene. The WDFY3 gene plays a key role in the growth of brain cells. Since this gene is most closely related to the growth of brain cells, many people with a WDFY3 related syndrome have an intellectual disability, autism spectrum disorder, or a speech delay. This gene is not directly related to preterm birth, however, fetuses, infants, and children can all develop a syndrome related to the gene anytime throughout their childhood. This doesn't depend on their parents and whether they have a syndrome related to this gene or not. Most of

the time, parents pass on identical copies of the gene to their children. However, the process of copying genes isn't perfect. An alteration in the genetic code might result in physical, developmental, or both problems. A random alteration might occur in the sperm or egg. This new modification to the genetic code is known as a 'de novo' change. The child could be the first in the family to be affected by the gene mutation.[27]

Discussion

The number of preterm births per year is estimated to be around 15 million. Preterm birth is classified by a baby being born 37 weeks or earlier. After screening maternal and fetal cord blood in the Preterm Birth Transcriptomic Database, 12 genes were found to be associated with preterm birth. NET1, EPM2A, and SERPINI1 were the only genes shown as downregulated. WDFY3, ALPL, H19, CLEC4D, TREM1, NLRC4, HIF3A, TLR4 and THBS1, were upregulated compared to healthy controls. (Figure 4)

Downregulated Genes	Upregulated Genes
NET1	WDFY3
SERPINI1	ALPL
EPM2A	H19
	CLEC4D
	TREM1
	NLRC4
	HIF3A
	THBS1

Figure 4 - The list of 12 genes stating whether the gene was Upregulated or Downregulated based on the Preterm Birth Transcriptomic Database

Interestingly, 4 of the 12 genes (25%) were related to proinflammatory myeloid pathways (TREM1, TLR4, CLEC4D, and NLRC4). All of these genes were upregulated highlighting the potential role of myeloid-dependent inflammation on preterm births. Several genes have been studied in preterm births before however a deeper exploration of the role of genes needs to be conducted. When considering further vetting of these drugs for potential clinical trials, we must consider the presence of drug inhibitors currently on the market that may be repurposed for the treatment of preterm births circumventing certain FDA guidelines and hastening “time to clinic.”

There are inhibitors found for each gene, these inhibitors prevent or slow down the process of the chemical reaction occurring which reduces the activity in a particular area. LP17 was found as a TREM1 inhibitor and TAK-242 was found to be a TLR4 inhibitor.[28][29] There are currently no

available drugs targeting CLEC4D and NLRC4. There are commercially available TREM1 and TLR4 inhibitors making these an interesting and feasible drug target for preclinical studies. LP17, a TREM1 inhibitor, has only been studied in preclinical murine models and therefore the patient safety of this drug needs to be tested prior to further investigation. However, TAK-242 has been studied in the context of cancer and according to ClinicTrial.gov, 3 clinical trials have been registered using Resatorvid (TAK-242) with good patient tolerance.[30] This fact combined with our screening results reveal that inhibition of TLR4 is a strong potential gene target for prevention of preterm births. TAK-242 should be studied in murine preterm birth models to validate its role in preterm birth and subsequently, given the positive safety profile of the drug, Phase 1/2 clinical trials should be considered.

Limitations of the study include the retrospective nature of the screening process and lack of validation of these targets in in vitro and in vivo assays. Additionally, changes in gene regulation might be secondary to the preterm birth and in fact assisting with prevention and therefore validation of these targets in preclinical platforms is required. Finally, this data was collected and analyzed through the Preterm Birth Transcriptomic Database and there included any limitations and biases present in the primary data.

In summary of the 12 genes screened, TLR4 is our top gene candidate for preterm birth prevention. TLR4 is a part of the TLR (toll-like receptor) family, which plays an important role in pathogen recognition and innate immune activation. This gene and its inhibitor, TAK-242 would serve as a potential causative gene in preclinical mice models and testing of this drug needs to be conducted in various preclinical platforms with the end target of a Phase 1/2 clinical trial.

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