HIGH-THROUGHPUT MOLECULAR PROFILING OF THE FETAL INFLAMMATORY RESPONSE IN EXTREMELY LOW GESTATIONAL AGE NEWBORNS

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DEPARTMENT OF EXPERIMENTAL AND HEALTH SCIENCES

Universitat Pompeu Fabra Barcelona

A la meva dona, al meu fill

i a la meva mare

pel seu amor incondicional.

“The two most important days
in your life are the day you are born
and the day you find out why”.
Mark Twain

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Abstract

Preterm birth is the leading cause of neonatal morbidity and mortality worldwide. Preterm newborns require special care for surviving and may develop severe diseases related to prematurity, especially those born very early. The fetal inflammatory response (FIR) to intraamniotic infection is characterized by high levels of cytokines in umbilical cord (UC) blood and it is diagnosed by the identification of pathological vasculitis of vessels of fetal origin in the umbilical cord and the placenta. The FIR jointly with neonatal diseases with systemic inflammation increase the risk of perinatal brain injury. In this thesis we explore the molecular changes associated with FIR in UC and in dried blood spots, collected during the first postnatal week and archived at the newborn screening program, using technologies for high-throughput molecular profiling. The results of this thesis provide new insights into the molecular mechanisms of FIR at birth and postnatally, which can help the identification of biomarkers and therapeutic targets of FIR and FIR-associated disorders.

Resum

El part preterme és una de les principals causes de mortalitat i morbiditat neonatal. Els nadons prematurs requereixen atenció mèdica especialitzada per sobreviure i poden patir freqüentment malalties greus associades a la prematuritat, especialment aquells més prematurs. La resposta fetal inflamatòria (FIR) és caracteritza per l'elevació de citoquines en sang de cordó umbilical i es diagnostica a nivell patològic per la presència de vasculitis dels vasos d'origen fetal en el cordó umbilical i la placenta. FIR conjuntament amb d'altres malalties neonatals associades amb inflamació sistèmica incrementen el risc de dany cerebral perinatal. En aquesta tesis explorem el canvis moleculars associats a FIR en el cordó umbilical i en sang seca de taló, recollida durant la primera setmana postnatal i emmagatzemada en el programa de cribatge neonatal, ulitzant tecnologies de perfilatge molecular d'alt rendiment. Els resultats aquí obtinguts mostren mecanismes moleculars de FIR, els quals poden ajudar a la recerca de nous biomarcadors i dianes terapèutiques per FIR i patologies neonatals associades.

Preface

This thesis is about preterm newborns, the youngest human beings. Many newborns are born too soon every day and require our attention and care to survive and grow healthy. Extremely low gestational age (GA) newborns are the most fragile among preterm newborns. They are frequently exposed to adverse environmental conditions before and after birth, and they require advance medical care for living. Extremely low GA newborns frequently develop severe diseases related to prematurity with short and lasting effects. Intraamniotic infection (IAI), a leading cause of preterm birth (PB), can trigger a fetal inflammatory response (FIR) that damage the fetus and increasing the risk of adverse neonatal outcomes, especially to those born very early. The underlying cellular and molecular mechanisms of FIR are not fully understood, and their knowledge will help to discover new biomarkers and therapeutic targets for FIR and FIR-related neonatal diseases, for which few treatments are currently available.

New high-throughput molecular profiling technologies have been applied in the last decade with success to gain new insight into the molecular mechanisms of human diseases profiling an increasingly broad range number of molecules and concentrations from the transcriptome and proteome, respectively. This has allowed the detection of new molecular biomarkers and therapeutic targets that have been applied with success in clinical practice. Likewise,

advances in sample preparation protocols enable the application of these programs. However, neonatal medicine has been slow to apply these new technologies to the study of neonatal diseases.

In this thesis, we performed high-throughput molecular profiling of FIR in umbilical cord (UC) and archived dried blood spots (DBS) to investigate molecular alterations associated with FIR at birth and during the first postnatal week. We have analyzed for the first time the transcriptome of UC from FIR-affected extremely low GA (ELGANs, less than 28 weeks of gestation) reanalyzing a published gene dataset. Next, after granting access to archived DBS from the neonatal screening, we were able to perform RNA-Sequencing (RNA-Seq) based transcriptomics and proteomics to explore the postnatal blood molecular alterations associated with FIR. Our results in both studies have provided new insights into the molecular pathophysiology of FIR.

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Abbreviations

PB: preterm birth

IAI: intraamniotic infection

UC: umbilical cord

AF: amniotic fluid

IA: intraamniotic inflammation

MIAC: microbial invasion of the amniòtic cavity

PTB: spontaneous PB

PPROM: premature rupture of the membranes

ELGANs: extremely low gestational age newborns

GA: gestational age

HCO: histological chorioamnionitis

FIR: fetal inflammatory response

CCA: clinical chorioamnionitis

PRRs: pattern recognition receptors

IL: interleukin

Th: T helper

TLR: Toll-like receptor

RNA-Seq: RNA sequencing

NEC: necrotizing enterocolitis

GO: Gene ontology

LTF: lactotransferrin

DBS: dried blood spots

NLRs: NOD-like receptors

G-MDSCs: granulocytic-myeloid-derived suppressor cells

1. INTRODUCTION

1.1. Preterm birth and intraamniotic infection

1.1.1. Preterm birth

PB is a significant public health problem and a health-care challenge that annually affects around 15 million preterm newborns worldwide. The World Health Organization defines PB as birth occurring before 37 completed weeks of gestation. PB is the leading cause of neonatal morbidity and mortality, and it is the first cause of death in children under 5 years (Blencowe, Cousens, et al., 2013; L. Liu et al., 2016). PB causes significant burden in rich and emerging countries alike, especially among those preterms born very early. The global PB rate is steadily increasing worldwide, and it is estimated to be of 11%, ranging between 5% in northern European and 18% in sub-Saharan African countries. The majority of PB occur in emerging countries and only around 10% of total PB occur in high-income countries (1.2 million), of which 0.5 million in the United States(Blencowe et al., 2012).

Human fetal viability, defined as the 50% chance of survival with or without medical support, and neonatal mortality vary between the countries by the level of neonatal care(Rysavy et al., 2015). Human fetal viability is around 24-25 and 32-36 completed weeks of gestation in countries with and without neonatal intensive care units, respectively (Blencowe, Lee, et al., 2013). In high-income countries,

neonatal mortality decreases with each new week of gestation, especially from 22 to 26 completed week' (94% to 16%), to 1% observed in late (between 34 and less than 37 completed weeks of gestation) and moderate (between 32 and less than 34 completed weeks of gestation) preterm newborns(Ancel, 2012; Loftin et al., 2010; Stoll et al., 2010). Extremely (less than 28 weeks of gestation) and very preterm neonates (between 28 and less than 32 completed weeks of gestation) are the most vulnerable, but they represent only the 5.2% and 10.4% of all preterm live births, respectively. Moderate and late preterm newborns represent the highest number (84.3%), and they are the most significant contributors of neonatal morbidity and mortality worldwide(Goldenberg, Culhane, Iams, & Romero, 2008).

PB has been divided clinically into spontaneous PB (PTB) and iatrogenic or elective PB. PTB is the most common presentation accounting for the 70% of all cases of PB, and it can be further divided into preterm premature rupture of the membranes (PPROM, 30%) and preterm labor with intact membranes (PTL, 40%). Iatrogenic or elective PB accounts for the remaining cases (30%) and includes diverse adverse maternal and fetal conditions, such as preeclampsia and fetal growth restriction (Goldenberg, Culhane, et al., 2008). PB is a syndrome with diverse causes and phenotypic manifestations(see Figure 1; Kramer et al., 2012; Villar et al., 2012). Regarding physiopathology, many causes of PTB are unknown, and often a pathological mechanism cannot be identified (see Figure 2).

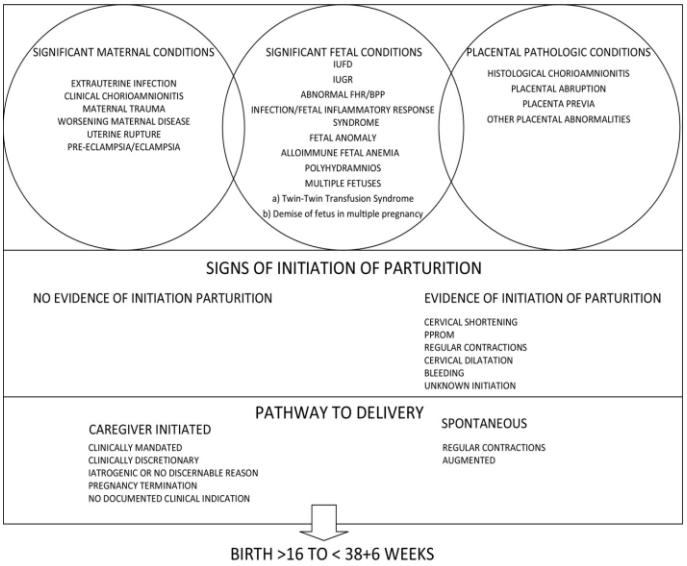

Figure 1. Clinical phenotypes of the preterm birth syndrome. Clinical phenotypes are defined by the presence of one or more established features of biochemical and physical signs of the mother, the fetus, and the placenta, and signals of parturition and pathway to delivery. The figure is taken from(Villar et al., 2012)**.**

Concerning to neonatal consequences of PB, the cost of PB up to 5 years old in the USA was estimated in $26 million annually(Frey & Klebanoff, 2016). Preterm neonates may require critical support for living and may suffer numerous serious diseases related to prematurity (e.g., neonatal sepsis, perinatal brain injury). This is particularly the case among ELGANs, defined as those whose GA is less than 28 weeks. After discharge from the hospital, preterm neonates also may face a lifetime of diseases and disabilities that impart a heavy burden on children, families, and society(Båtsvik et al., 2015; Baumann,

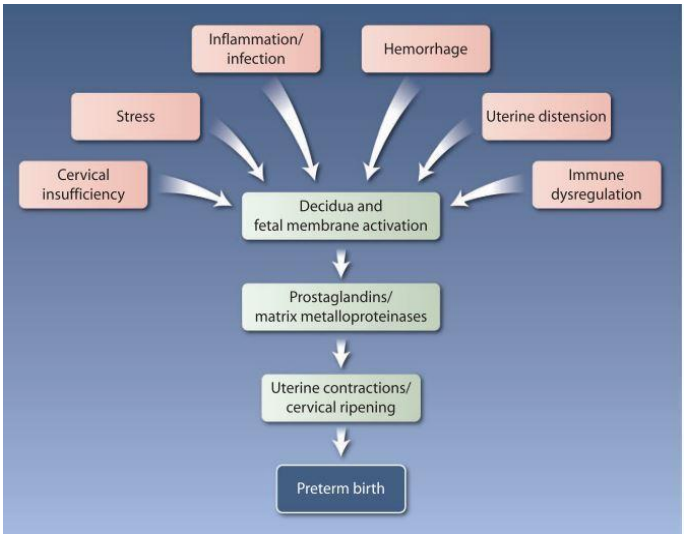

Figure 2. Pathological pathways to preterm birth. Pathological mechanisms can activate fetal and maternal tissues releasing multiple inflammatory mediators that lead to the onset of parturition. Multiple risk factors (e.g., biological, environmental, behavioral and social) can contribute to the onset of pathological pathways in preterm birth. The figure is taken from (Rubens et al., 2014).

Bartmann, & Wolke, 2016; van Lunenburg et al., 2013). Long-term complications of prematurity include neurologic impairment, one of the most frequent sequelae of PB, and other chronic disorders, such as asthma(Haataja et al., 2016), insulin resistance and hypertension(Dalziel, Parag, Rodgers, & Harding, 2007; Hofman et al., 2004; Rotteveel, van Weissenbruch, Twisk, & Delemarre-Van de Waal, 2008)**.**

1.1.2. Definition and epidemiology of intraamniotic infection

IAI is one of the leading cause of PB that affects between 25% and 40% of all PB(Goldenberg, Culhane, et al., 2008). IAI is frequently a subclinical process, as only around of 15% cases of IAI are complicated with clinical chorioamnionitis (CCA; Combs et al., 2014). CCA refers to the clinical signs and symptoms of the maternal systemic inflammatory response to IAI. Recently, an expert panel proposed to replace the term CCA for "Intrauterine Inflammation or Infection or both" (Triple I) defined by several diagnostic criteria(Higgins et al., 2016). On the other hand, Romero et al. (2015) proposed to classify IAI and other related subclinical conditions into four subgroups according to the presence of microbial invasion of the amniotic cavity (MIAC) and intraamniotic inflammation (IA; see Figure 3 and Romero et al., 2015). IAI is also frequently associated with histological chorioamnionitis (HCO) (85%), which refers to the detection of maternal and fetal inflammatory infiltrate of neutrophils in the placenta and the fetal vessels in the UC and chorionic plate, respectively, by placental examination(Redline, 2012).

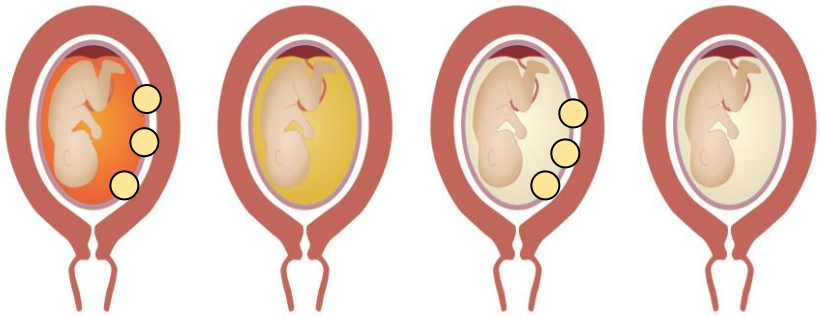

Figure 3. Intraamniotic infectious and inflammatory phenotypes. From left to right:(1) intraamniotic infection (IAI) (microbial invasion of amniotic cavity (MIAC) plus intraamniotic inflammation (IA), (2) sterile IA (IA without MIAC), (3) colonization (MIAC without IA), (4) negative (no MIAC and no IA).

The frequency of IAI and the other subclinical related disorders vary between the published studies, due methodological and technical issues related to the diagnosis and differences in cohort studies. Microbiological and molecular studies of amniotic fluid (AF) have been used in the research of IAI, as AF is considered a sterile fluid(DiGiulio et al., 2008). The prevalence of IAI increases with decreasing GA at birth, and it is higher in PPROM (23%-29%) compared to PTL (11%; Shim et al., 2003; Yoon et al., 2001). Colonization represents nearly 1 in 10 cases of MIAC (1% of all cases PTL; Combs et al., 2014). The frequency of sterile IA compared to IAI is similar in PPROM (23-29%) and higher in PTL (26% vs. 11%; Romero et al., 2015; Romero, Miranda, et al., 2014).

IAI is frequently a polymicrobial infection. More than one microorganism are isolated in AF of 24% to 67% of all IAI cases. Among microbes implicated in IAI, five bacterial phyla and more than 87 different bacterial taxa have been identified. Bacteria of the phylum *Firmicutes* (e.g., *Mycoplasmatales* order) are the most common isolated, followed by bacteria of the phylum *Fusobacteria*. At the level of species *Ureaplasma* spp are the leading cause of IAI (49%), particularly *Ureaplasma urealyticum (*11%). Other relevant microbes are *Streptococcus agalactiae* (11%), *Mycoplasma hominis* (9%), and *Fusobacterium nucleatum* (9%), bacteria belonging to oronasal microbiota(DiGiulio, 2012; Mendz, Kaakoush, & Quinlivan, 2013).

1.1.3. Pathogenesis of intraamniotic infection

IAI is a complex infectious disease evident in the AF and characterized by increases in inflammatory cells and mediators. The amniotic inflammatory response syndrome is a term coined by Combs et al. (2014) to describe the association between IAI and the onset of PB, and adverse neonatal outcomes(Combs et al., 2014). Nowadays, there is no international consensus about which are the best treatment and clinical biomarkers for IAI(Cobo, Kacerovsky, & Jacobsson, 2018). A better understanding of how IAI pathological mechanisms in PTB cause the onset of parturition and induce neonatal damage is mandatory to find new preventive measures, clinical biomarkers, and therapies.

The ascending route is considered the most common way in which microbes reach the amniotic cavity. During gestation, the cervical environment, particularly cervical mucus and vaginal microbiota, protects from ascending infection of the lower genital tract. Recent studies have found that vaginal microbiome in gestation is composed by a stable microbiota dominated by *Lactobacillus* spp., that could protect the fetus from ascending infection. Specifically, *Lactobacillus* spp. can induce a lower expression of pro-inflammatory cytokines (IL-1b, IL-8) and a higher production of anti-inflammatory protein secretory leukocyte protease inhibitor in cervical mucus(Cobo et al., 2018). Microorganisms from the lower genital tract and gut, however, under certain circumstances can ascend and proliferate in the amniotic cavity without rupture of placental membranes leading to IAI (Romero, Dey, & Fisher, 2014).

Under normal conditions, many antimicrobial peptides and proteins and few inflammatory cells are present in the amniotic cavity. The identification of pathogen-associated molecular patterns of microbes by pattern recognition receptors (PRRs), germline-encoded receptors that mediate innate immune responses(Gabay & Kushner, 1999; Medzhitov, 2008), on immune and non-immune cells in gestational tissues (placenta, fetal membranes, and maternal decidua) trigger the downstream activation

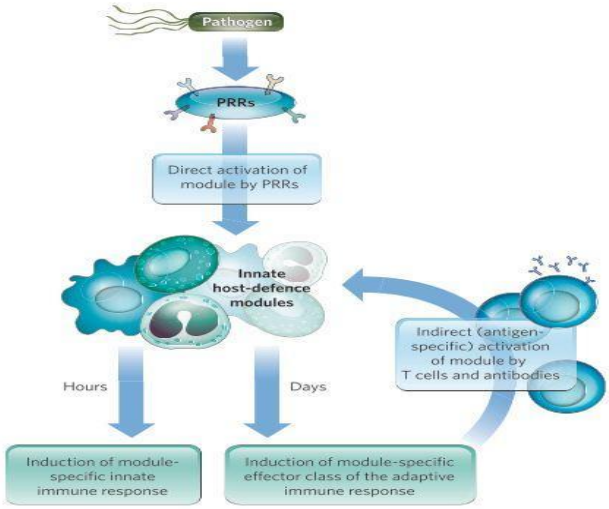

Figure 4. Pathogenesis of intraamniotic infection. Microbial detection by pattern recognition receptors (PRRs) of gestational tissues causes an increase of the amniotic fluid level of antimicrobial peptides and proteins and multiple inflammatory mediators (e.g., cytokines, chemokines, metalloproteases), which trigger the recruitment of innate immune cells, particularly maternal and fetal neutrophils to the amniotic cavity and amniochorionic membranes. The figure is taken from (Medzhitov, 2007).

of inflammatory signaling pathways(see Figure 4; C. S. Buhimschi et al., 2007; I. A. Buhimschi et al., 2013; C. J. Kim et al., 2015). IA correlates with the degree of placental inflammation(S. M. Kim et al., 2014) and it is inversely related with GA at birth(Park, Park, Jun, & Yoon, 2015). Most of AF neutrophils collected in IAI are of fetal origin, and, remarkably, women with abundant and predominant AF fetal neutrophils have frequently extremely PTB(Gomez-Lopez et al., 2017). Many of AF inflammatory mediators and cells associated with IAI can participate in the mechanisms involved in parturition (see Figure 5; Vrachnis et al., 2012).

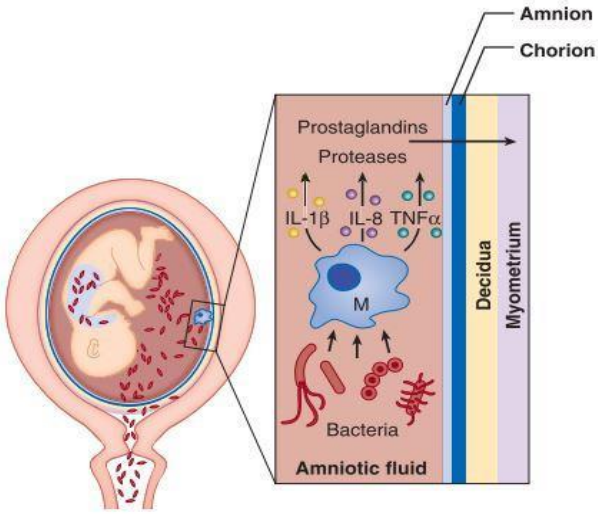

Figure 5. The onset of preterm labor and intraamniotic infection. High level of proinflammatory mediators in the amniotic fluid in intraamniotic infection (IAI), such as cytokines, prostaglandins, and proteases, can activate amniochorionic membranes. This can lead to the onset of preterm parturition. (Romero et al., 2015; Romero, Miranda, et al., 2014b)Both IAI and severe sterile intraamniotic inflammation (IA) have similar and shorter latency than cases without evidence of IA (median 3 days vs. 31 days)(Romero et al., 2015a; Romero, Miranda, et al., 2014). The figure taken from (Romero, Dey, et al., 2014).

The clinical diagnosis of IAI can be challenging and require so far the performance of an invasive amniocentesis to analyze the AF. The traditional culture methods are relatively slow and the diagnostic accuracy of standard techniques for IA (e.g., white blood count and biochemical analysis) is limited. Thus, new methods have been proposed based on detection of AF inflammatory biomarkers, such as cytokines and peptides, but with a lack of clinical translation(I. A. Buhimschi et al., 2013; Polettini et al., 2017). Since the diagnosis of IAI requires the performance of an invasive procedure, there is currently increasing interest in new non-invasive methods of IAI diagnosis in cervical mucus, vaginal fluid (e.g., IL-6) and maternal

blood(Cobo et al., 2018). Regarding the treatment of CCA, it is based on the induction of labor under antibiotic therapy to reduce the rate of maternal and neonatal complications associated with CCA(Gibbs, Dinsmoor, Newton, & Ramamurthy, 1988; Johnson, Adami, & Farzin, 2017).

1.2. Fetal inflammatory response

1.2.1. Definition and epidemiology of fetal inflammatory response

IAI can induce not only a local and systemic inflammatory response in the mother but also one in the fetus that has been named FIR(O. Dammann & Leviton, 2000; see Figure 6). FIR can cause multiple-organ alterations at birth and predispose exposed newborns to multiple adverse neonatal outcomes during the perinatal period(Gantert et al., 2010). FIR is defined by the presence of high levels of inflammatory mediators circulating in UC blood (e.g., Interleukin(IL)-6) (R. Gomez et al., 1998) and by pathological changes in the placenta at birth. The anatomical pathology definition of FIR is based on the presence of neutrophils in the walls of vessels from chorionic plate (chorionic vasculitis) of the placenta and UC (funisitis; Khong et al., 2016; see Figure 7).

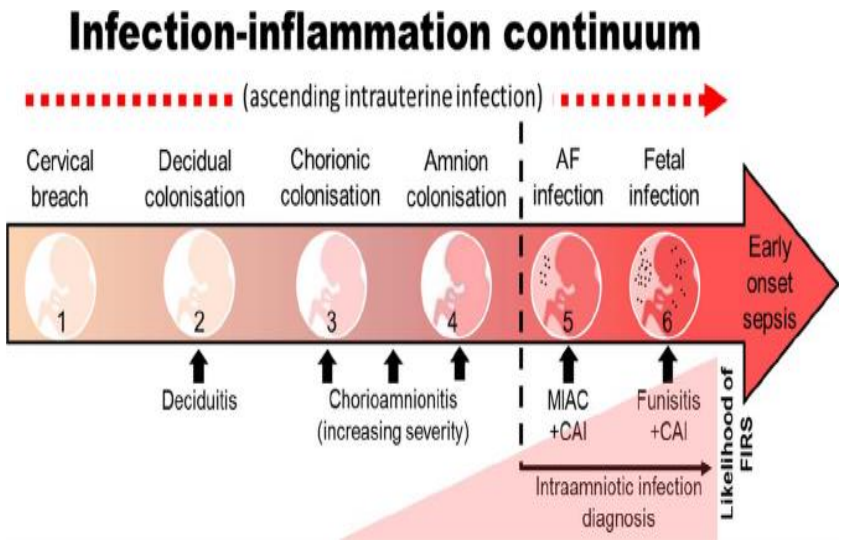

Figure 6. Intraamniotic infection-inflammation continuum. The IAI infection-inflammation continuum has been proposed as a model of the effect of IAI on the fetus. The spectrum stages in the IAI infection-inflammation continuum describe the progression of ascending IAI, together with the fetal response to IAI. At the same time, FIR represents a continuum of illness due to fetal and neonatal systemic inflammation caused by IAI, representing neonatal sepsis and death the most extreme ends of IAI infection-inflammation. The figure is taken from (Keelan, 2018)**.**

The clinical manifestations of a systemic inflammatory response in newborns are nonspecific and usually superimposed on neonatal diseases(Shane, Sánchez, & Stoll, 2017). Thus, the clinical diagnostic of FIR relies on the detection of placental pathology. According to this, the frequency of FIR varies according to diverse conditions, such the GA at birth, the employed diagnostic criteria, and the clinical presentation of PTB. FIR is rarely observed in fetuses with less than 20 weeks of gestation, due to the immaturity of the

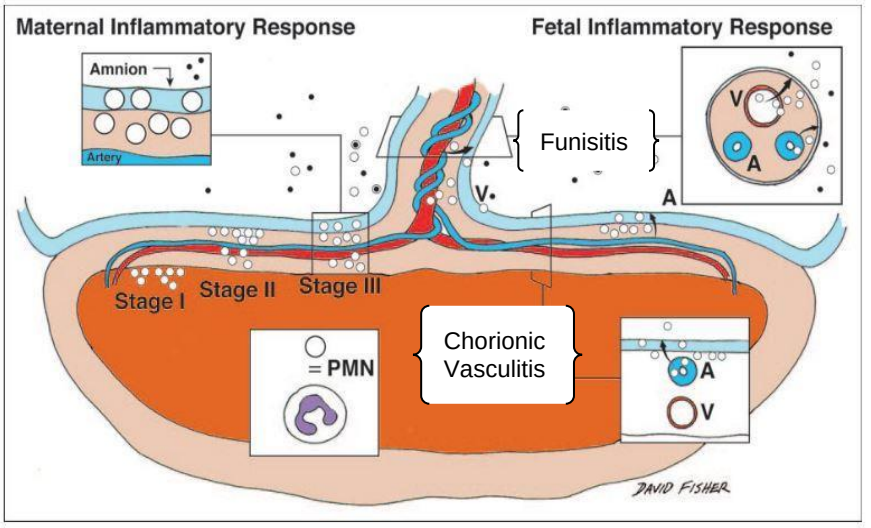

Figure 7. Histological chorioamnionitis (CHO). CHO is divided into maternal (MIR) and fetal inflammatory response (FIR). FIR is classified by increasing levels of stage (duration in time of FIR) and grade (intensity of FIR). FIR Stage 1: chorionic vasculitis or umbilical phlebitis; Stage 2: involvement of the umbilical vein and one or more umbilical arteries; Stage 3: necrotizing funisitis. The figure is adpated from (Fullerton & Gilroy, 2016; Reilly & Faye-Petersen,2008).

fetal immune system. However, FIR is more common in preterm infants (35%), especially in the preterm newborns with less than 26 weeks of gestation (52%), than in term newborns (5%). The detection of FIR also increases with the grade of placental inflammation and it is frequently associated with PTB, particularly with CCA, PTL, and PPROM (O. Dammann et al., 2004; Redline, 2006).

1.2.2. The neonatal immune system

Understanding the neonatal immunity is crucial for elucidating pathological molecular mechanisms of FIR. Traditionally, it has been accepted that the neonatal immune system (innate and adaptive immune arms) is immature and deficient compared to adults, particularly in preterm newborns. Deficiencies in both innate and adaptive immunity are thought to underlie the increased susceptibility to infections with decreasing GA at birth. However, new evidence from recent studies has shown that newborns can mount adult-like immune effector responses in response to infection. This means that the neonatal immune system is not merely immature, but rather functionally different compared to the adult immune system(Kollmann & Marchant, 2017).

The study of the ontogeny of early life immunity has shown that the neonate can mount immune responses early in gestation according to the development stage at which the insult takes place(Marchant & Kollmann, 2015). The expression and functionality of PRRs is age-dependent and follow an "inside-out" pattern between 24 weeks and 33 weeks of gestation(Kan, Razzaghian, & Lavoie, 2016; see Figure 8). Major immune cell subsets and humoral factors also change during neonatal development, and these variations coincide with a specific period of infectious and inflammatory-related disorders susceptibility(Kollmann, Kampmann, Mazmanian, Marchant, & Levy, 2017; see Figure 9). Moreover, the functionality of immune

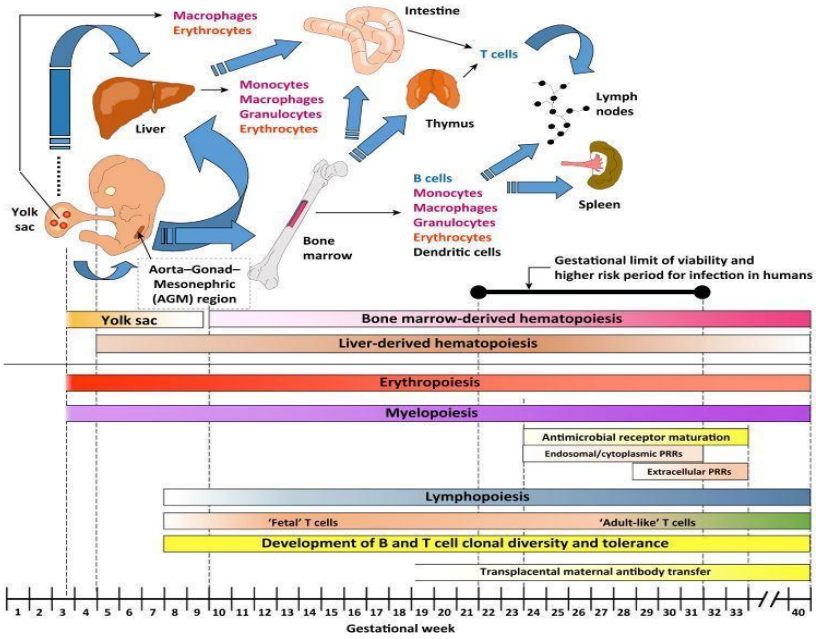

Figure 8. Ontology of the immune system. Hematopoiesis begins early in the embryonic period in the yolk sac and Aorta-Gonad Mesonephric region and continues throughout the lifespan. Fetal hematopoiesis is characterized by the colonization of different tissues by hematopoietic stem cells, the development of immune organs and cells, and simultaneous fetal hepatic and bone marrow hematopoiesis. The earliest pattern recognition receptors (PRRs) activity in monocytes is observed in cytosolic-endosomal PRRs, such as endosomal Toll-like receptors (TLRs, TLR7, TLR8, TLR9) and intracytoplasmic NOD-like receptors (NLRs), followed by the activity of extracellular PRRs (e.g., TLR1-2, TLR4-5, and the dectin-1 receptor). Around 10% and half of the maternal IgG immunoglobulin levels are transferred at 17-22 and 28-32 weeks of gestation, respectively. The figure is taken from (Kan et al. 2016).

cellular components in vitro studies vary according to the GA at birth, and between newborns and adults(Brook, Harbeson, Ben-Othman, Viemann, & Kollmann, 2017) (see Supplemental Table 1).

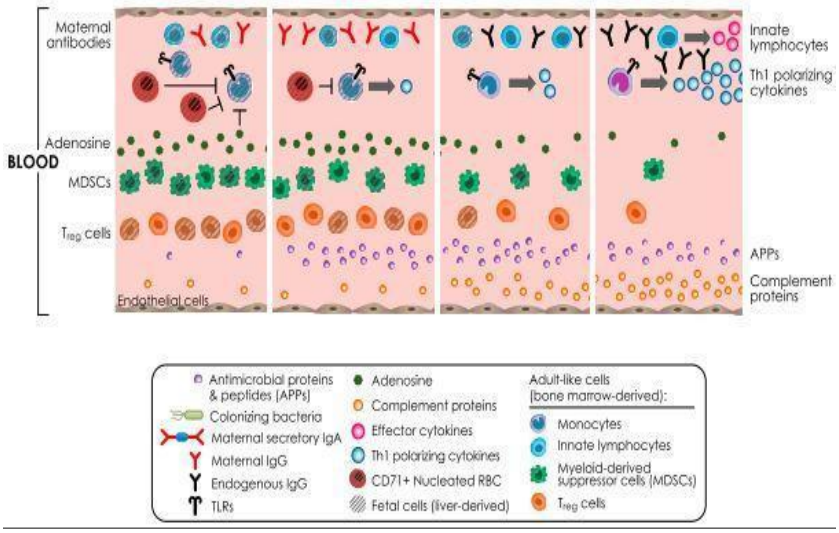

Figure 9. Neonatal blood immune composition. The blood composition of immune cell subset and humoral factors changes during development. Immune regulatory responses predominate in the fetus, and preterm neonate including myeloid-derived suppressor cells, T and B regulatory and CD71+ erythroid cells and blood adenosine predominate. In contrast, humoral immunity, including antimicrobial peptides, immunoglobulin and complement proteins, and effector immune responses predominate in the adult. Figure adapted from (Kollmann et al. 2017).

The cytokine production by immune cells after PRR activation, pivotal in the induction of cell-mediated and humoral immunity¸ also changes during the immune development. Toll-like receptors (TLRs) are the main PRRs in microbial detection and their function varies with GA(Strunk, Currie, Richmond, Simmer, & Burgner, 2011). Newborns, especially those born very early, have a diminished and enhanced in vitro TLR-dependent pro-inflammatory (TNF-alpha, IL1beta, IL-12/23p40, IFN-alpha) and anti-inflammatory (IL-10) cytokine responses, respectively, compared to adults (see Supplemental Table 1). This neonatal cytokine polarization has a prominent role in the neonatal anti-inflammatory T cell responses bias -the propensity of neonatal naïve CD4+ T cells to differentiate

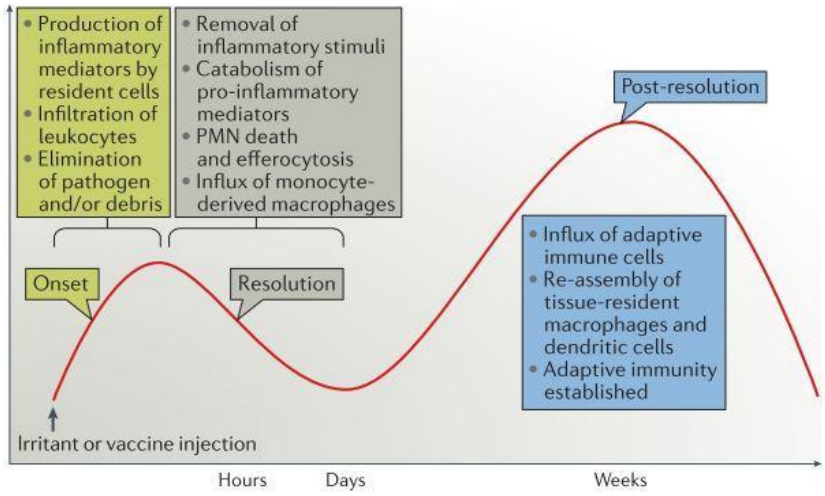

Figure 10. Resolution of inflammation. The onset of acute inflammation drives to a resolution phase that is followed by an immune adaptive response to the insult. Impaired resolution mechanisms and altered adaptive immune responses in preterm newborns may participate in the molecular mechanisms of excessive inflammation and immunopathology. The figure is taken from (Fullerton & Gilroy, 2016).

into T helper(Th)2 and Treg cells-, which results in a diminished neonatal pro-inflammatory Th1, Th17, and Th follicular cell immunity(Debock & Flamand, 2014).

New findings, however, support the view that newborns can mount effective pro-inflammatory T cell immune responses. Diverse pro-inflammatory T cells have been detected in fetus and newborns, such as circulating CD31+CXCL8+ (Gibbons et al., 2014) and CD31-TNF+ CD4+ Th cells(Scheible et al., 2018), and neonatal effector memory T cells with Th1, Th2 and Th17 function (Zhang et al., 2014; Zhivaki & Lo-Man, 2017). UC blood from preterm newborns also has higher counts of effector inflammatory CD8+ T cells with lower CD31 expression(Scheible et al., 2015). In vivo studies of neonatal

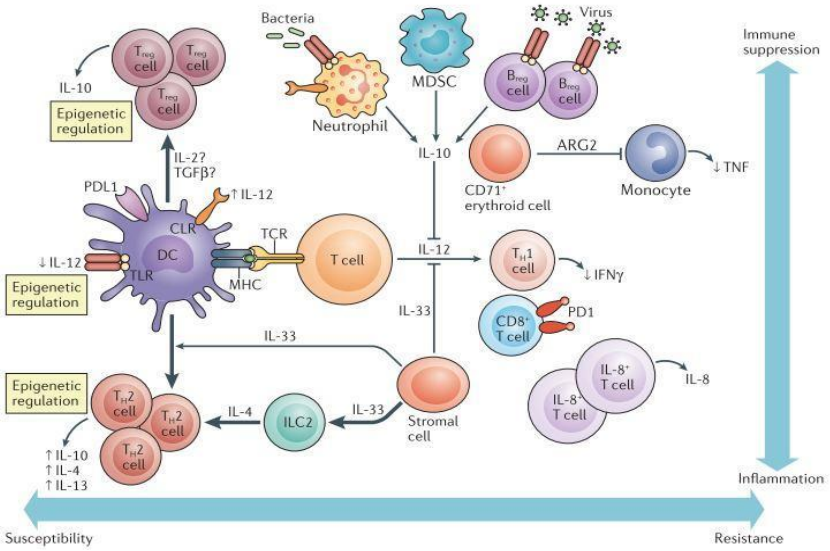

Figure 11. Neonatal immune regulation. Neonatal regulation of pro-inflammatory immune responses is complex and occurs at different levels. Diverse immune regulatory cells modulate immune responses by different mechanisms, such as the production of anti-inflammatory cytokines (e.g., IL-10, TGF-beta), the inhibition of dendritic cells and T cells, and the depletion of L-arginine by arginase 1 and 2 actions. Epigenetic mechanisms in dendritic cells and naïve CD4+ T cells favor the development of T regulatory T-helper 2 cells. The perinatal activation of IL-33-ST2 axis regulate thermogenesis during the neonatal transition and promote the development of T-helper 2 T cells. The figure is taken from (Zhang, Zhivaki, & Lo-Man, 2017)

sepsis have observed high levels of pro-inflammatory cytokines and other inflammatory mediators in neonates affected by sepsis(Sugitharini, Prema, & Berla Thangam, 2013).

Altogether, the new paradigm of the neonatal immune system suggests that newborns may have an increased risk of severe infections not because of their defects in immune function, but rather due to their immune capacity to mount robust and pro-inflammatory responses that may become dysregulated and lead to inflammatory

related-disorders (immunopathologies). This implies, on the one hand, that newborns must tightly control immune responses to avoid the damage of excessive inflammation (see Figure 10). On the other hand, once inflammation occurs neonates should have the capacity to resolve the inflammatory response, otherwise it can also lead to excessive inflammation and immunopathology. The molecular and cellular mechanisms involved in the control and resolution of inflammation are complex, and only partially known in newborns (see Figure 11).

1.2.3. Pathogenesis of fetal inflammatory response

FIR can cause several molecular and cellular alterations that can lead to multi-organ injury. The pathophysiology of FIR is common to other neonatal diseases associated with systemic inflammation, such as neonatal sepsis and necrotizing enterocolitis (NEC). It involves the activation and interaction of the innate and adaptive immune defenses in response to the rupture of homeostasis in a vulnerable preterm newborn exposed to adverse intrauterine and extrauterine environment.

Researchers have used diverse animal species, from mice to non-human primates, to investigate the effects of IAI on the fetus. Much of this research has focused on the short-term effects that intra-amniotic inflammatory stimuli have on different fetal organ systems(Matthew W. Kemp, Musk, & Saito, 2013). These studies

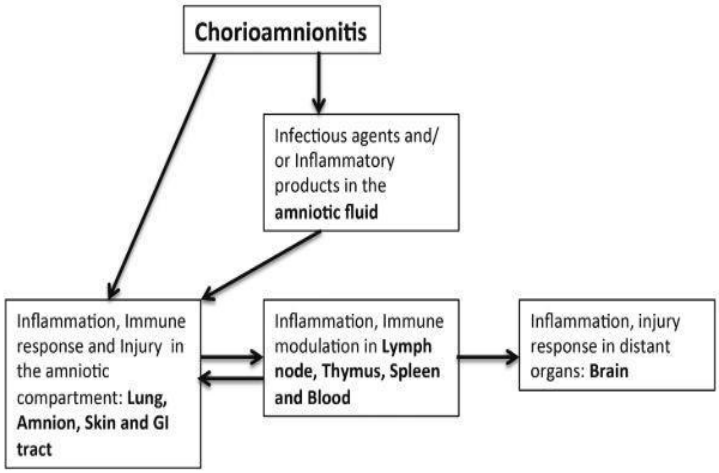

Figure 12. Pathogenesis of FIR in animal studies. The exposure of the fetus to microbes and inflammatory mediators in the amniotic cavity triggers fetal immune response in the amniotic compartment. This can lead to a fetal systemic inflammation that can result in injury to distant organs, such as the brain. The figure is taken from (Kallapur, Presicce, Rueda, Jobe, & Chougnet, 2014).

have shown that fetal organs in contact with AF and amniochorionic membranes can produce an array of inflammatory mediators and trigger the recruitment of inflammatory cells in response to intra-amniotic inflammatory stimuli. These inflammatory mediators and cells can then circulate into the amniotic and the systemic compartment (e.g., blood, brain, liver and lymphoid organs) leading to IA and systemic inflammation, which can play a significant role in mechanisms of disease in perinatal neonatal diseases (see Figure 12).

Human fetus just like in animal models are mainly exposed to IAI at epithelial surface of organs in close contact with AF. Fetal

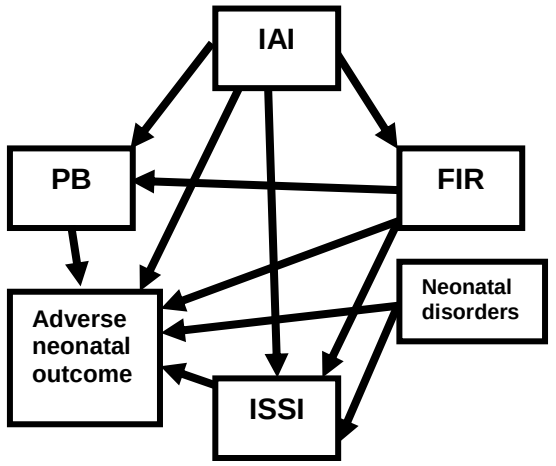

Figure 13. Pathogenesis of FIR in preterm newborns. Intraamniotic infection (IAI) can trigger fetal inflammatory response (FIR). Preterm birth (PB), intermittent or persistent systemic inflammation (ISSI), IAI, FIR and postnatal disorders (e.g. neonatal sepsis, necrotizing enterocolitis) can cause diverse adverse neonatal outcomes, such as perinatal brain injury, by itself or due the interaction with other inflammatory conditions. ISSI is defined as the elevated concentration of inflammation-related proteins on two separate times in the neonatal period in ELGANs.

bacteremia occurs only in 23 % of cases of IAI in preterm newborns (Carroll et al., 1995; Goldenberg, Andrews, et al., 2008). During IAI, the fetus can swallow and breathe and have skin contact with infected AF, which can trigger FIR. The most active period of fetal swallowing (between the 17 and 30 weeks of gestation), and the transition from fetal hiccups to fetal breathing (around 26 weeks of gestation) seem to correlate with the risk of FIR in preterm newborns(Miller, Sonies, & Macedonia, 2003; Pillai & James, 1990).

In preterm newborns, a continuum exists from low-moderate systemic inflammation, such as FIR and other perinatal inflammation-initiating illnesses, to severe systemic inflammation associated with neonatal sepsis. In ELGANs intermittent or sustained

systemic inflammation (ISSI) is defined as the elevated concentration of inflammation-related proteins on two separate times in the neonatal period in ELGANs(Olaf Dammann & Leviton, 2014). Likewise, severe systemic inflammation, low-moderate systemic inflammation, and ISSI may induce systemic organ injury in preterm newborns, particularly perinatal brain injury (see Figure 13).

Systemic cellular and molecular perturbations of FIR

FIR can induce alterations in various biological systems of the systemic inflammatory network that can lead to organ injury (see Figure 14). However, the molecular mechanisms underlying FIR and driving organ injury are only partially known. FIR produces a robust blood inflammatory cytokine response detectable in UC blood that consists in the elevation of IL1-b, IL-6, and CXCL8 pro-inflammatory cytokines (D'Alquen et al., 2005; Ricardo Gomez et al., 1998; Mestan et al., 2009; Tasci et al., 2006). Blood elevation of anti-inflammatory IL-10 and IL-19 cytokines have also been observed in association with FIR, which suggests the activation of inflammatory counter-regulatory mechanisms(Savasan et al., 2012). Elevated levels of cytokines associated with FIR have also been detected in gastric (of IL-1b, CXCL8, CXCL5, and CXCL1) and tracheal aspirates (IL-6) of preterm newborns exposed to FIR (Bry, Jacobsson, Nilsson, & Bry, 2015; Iwatani et al., 2014). Fetal dermatitis with leucocytic infiltrates, and stronger TLR-2 immunoreactivity was found in fetal autopsies of ELGANs exposed to CHO(Y. M. Kim et al., 2006).

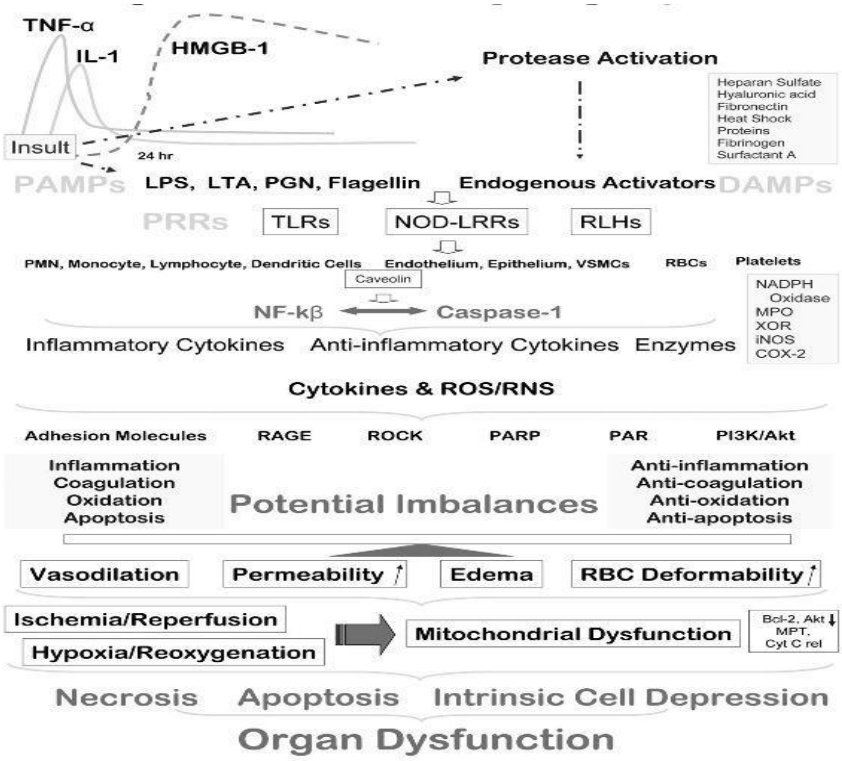

Figure 14. The inflammatory network in systemic inflammation. Systemic inflammation can rupture the balance between the diverse biological systems of the inflammatory network. The production of pro-inflammatory after the recognition of damage-associated molecular patterns (DAMPs) and or microbial-associated molecular patterns (PAMPs) by immune cells can cause numerous cellular and tissue alterations that can lead to organ dysfunction. The production of anti-inflammatory mediators serves as counterregulatory function. PRRs, pattern recognition receptors; TLRs, toll-like receptors; NOD-LRR, nucleotide- oligomerization domain leucine-rich repeat protein receptors; RLHs; retinoic-acid-inducible gene I (RIG-I)-like helicases; TNF-□, tumor necrosis factor alpha; IL-1, interleukin 1; HMGB-1, high mobility group box-1;LPS, lipopolysaccharide; LTA, lipoteichoic acid; PGN, peptidoglycan; VSMCs, vascular smooth muscle cells; RBC, red blood cell; MPO, myeloperoxidase; XOR, xanthine oxidoreductase; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase; RAGE, receptor for advanced glycation end products; ROCK; RhoA/Rho kinase; PARP-1, poly(ADP ribose) polymerase-1; PAR, protease activated receptor; ROS/RNS, reactive oxygen and nitrogen species; NF, nuclear factor; NADPH, nicotimanide adenosine dinucleotide phosphate. The figure is taken from (Cinel & Opal, 2009).

Local and circulating proinflammatory cytokines are potent mediators of inflammation and immune responses that produce multiple cellular and tissue alterations in different organs (see

Supplemental Table 2). Effects of systemic inflammation in FIR include the elevation of C-reactive protein, a potent acute-phase protein produced by the liver(Sorensen et al., 2007; Yoon et al., 2003), and the increased expression of adhesion molecules (ICAM-1, E-selectin and VCAM-1) in UC and serum concentration of preterm newborns with funisitis(D'Alquen et al., 2005). Funisitis also induces the elevation of circulating white blood cells and the activation of neutrophils and monocytes (e.g., enhanced intracellular production of reactive oxygen species; S. K. Kim et al., 2009). Funisitis may also regulate the Th17 cell responses in preterm newborns increasing the UC blood counts of progenitor and mature Th17 cells(Rito, Viehl, Buchanan, Haridas, & Koenig, 2017).

High levels of inflammatory cytokines present at birth induced by FIR can persist during the neonatal period and participate in ISSI. CHO and funisitis are both associated with a systemic inflammatory response during the first three postnatal days following a dose-response pattern in preterm newborns(Hecht et al., 2011; Nishimaki et al., 2013). Leviton et al. (2011) also found that the concentrations of seven inflammation-related proteins tended to be elevated on the seventh postnatal day in funisitis(Leviton et al., 2011).

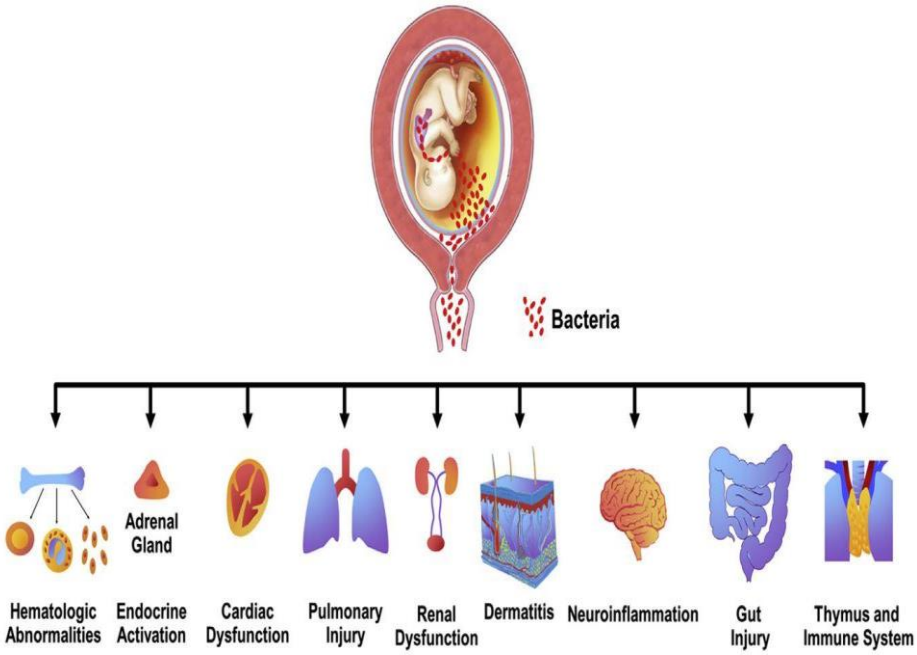

Figure 15. FIR multiple organ disease. FIR can cause multiple alterations in the fetus and the neonate in organs exposed to amniotic fluid and distant organs. These alterations at birth can cause and predispose preterm newborns to multiple adverse neonatal outcomes. The figure is taken from (C. J. Kim et al., 2015).

Organ injury of FIR

FIR can cause a multiple-organ disease (see Figure 15) that predisposes to multiple adverse neonatal outcomes. The majority of published studies that have investigated the adverse neonatal outcomes associated with IAI have been done in preterm newborns by using different predictor variables, such as CCA, HCO, and FIR. In clinical studies, they are strongly associated with neonatal sepsis and increase the risk of suffering other neonatal diseases, particularly of perinatal brain damage(Bracci & Buonocore, 2003; Galan Henriquez, Henriquez, & Rodrigo, 2017; Roescher, Timmer, Erwich, & Bos, 2014; see Supplemental Table 3). However, the association

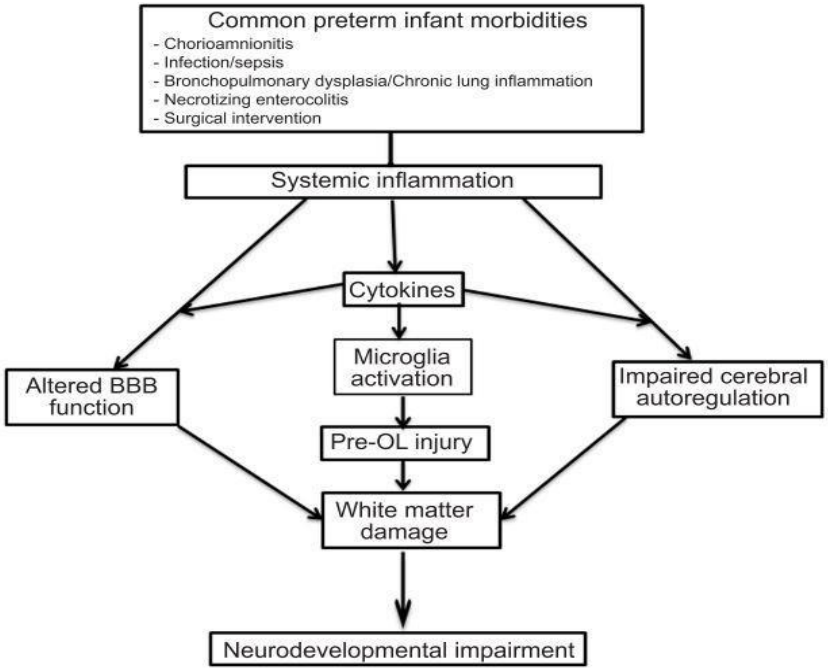

Figure 16. Pathogenesis of perinatal brain injury. The elevation of blood cytokines and inflammatory cells in fetal and neonatal circulation induced by FIR and other neonatal disorders have been implicated in perinatal brain injury. These inflammatory molecules and cells can alter and cross the blood-brain barrier (BBB) and lead to neuroinflammation without microbial invasion of the brain(Olaf Dammann & Michael O`Shea, 2008; Strunk et al., 2014). Microglial activation, a hallmark of neuroinflammation, is characterized by the production of an array of inflammatory mediators that can damage the brain of preterm newborns, particularly the immature oligodendrocytes (Hagberg, Mallard, &Sävman, 2016). The damage of oligodendrocytes can cause white matter injury, a common manifestation of perinatal brain injury. The figures is taken from (Patra, Huang, Bauser & Giannone, 2017).

is still controversial, as some investigators failed to replicate it(Thomas & Speer, 2011). The presence of FIR can increase the risk of adverse neonatal outcomes compared with IAI without evidence of FIR, and it can also interact with postnatal inflammatory conditions further increasing the risk of neonatal complications, particularly of perinatal brain injury. The underlying molecular mechanisms linking perinatal brain injury and systemic inflammation

remain to be fully understood (Leviton, Gressens, Wolkenhauer, & Dammann, 2015; see Figure 16).

1.2.4. Clinical diagnosis and treatment of fetal inflammatory response

The clinical diagnostic of FIR is rarely done in the neonatal unit despite the accumulated scientific evidence, and there are no clinical criteria and "bedside" biomarkers for FIR diagnosis. Clinical and common laboratory alterations are unspecific, and the FIR gold standard of placental diagnosis may take several days after parturition, affecting clinical decision making. The clinical application of the laboratory methods used in research for quantitative determination of blood cytokines and in AF is also hampered by different issues(Bonadio, 2016). Thus, prompt diagnosis and the appropriate management of preterm newborns affected by FIR could improve neonatal outcome(Boyle, Rinaldi, Norman, & Stock, 2017).

Specific effective treatment options for the FIR are still lacking, and recent therapeutic approaches for preventing and treating perinatal brain injury in preterm newborns are based on targeting systemic inflammation and neuroinflammation(Ranchhod et al., 2015). Antenatal maternal corticosteroids are the most important common available therapy to improve fetal and neonatal outcome. They reduce the risk of most severe neonatal complications(M. W. Kemp, Newnham, Challis, Jobe, & Stock, 2016; D. Roberts, Brown,

Medley, & Dalziel, 2017), due inhibitory and regulatory effects on maternal and fetal immune system. Maternal antibiotic therapy can diminish the AF bacterial burden but have little effects on ongoing maternal and fetal inflammation. New specific treatments targeting fetal inflammatory response could protect the neonate from adverse effects of fetal systemic inflammation(Boyle et al., 2017; Johnson et al., 2017; P. Y. Ng, Ireland, & Keelan, 2015).

1.3. High-throughput molecular profiling in perinatal medicine

1.3.1. High-throughput molecular profiling technologies

Advances in molecular high-throughput technologies in the last decade have allowed the characterization and quantification of whole cellular transcriptomes and proteomes in cells and tissue at a given moment under certain conditions. Transcriptomics explore the total RNA expression, while proteomics can quantify the abundance of proteins. Proteogenomics refers to the integration of both transcriptomics and proteomics techniques, and it has been recently used in research to gain novel insights into the molecular mechanisms underlying human diseases.

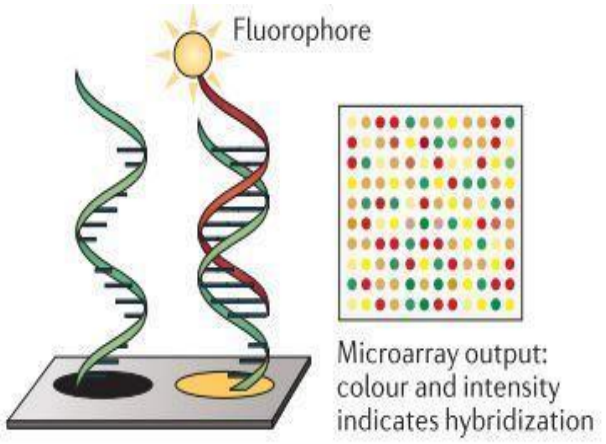

Figure 17. DNA microarrays. DNA-microarrays contain thousands of DNA sequences attached to a solid substrate (DNA spots or probes), which hybridize the cDNA or cRNA (targets) from biological samples(Sobek, Bartscherer, Jacob, Hoheisel, & Angenendt, 2006). Probe-target hybridization intensity, then, is quantified capturing the fluorescent emission after laser light excitation of the samples, which gives an estimate of the relative expression of RNA transcripts. Exist different microarray platforms available that differ in the array fabrication (e.g., spotted and in-Situ synthesized arrays) and dye choice. Affymetrix high-density oligonucleotide expression arrays are one of the most popular DNA microarray platforms. The statistical microarray analysis of DNA microarray raw data from gene expression experiments is a complex task that has evolved rapidly during the last decade. The figure is taken from(Goodwin, McPherson, & McCombie, 2016).

Transcriptomics

The transcriptome is the whole set of RNA transcripts in a cell for a given moment. A single mammalian cell contains between 50,000 and 300,000 transcripts, which consists of diverse RNA molecules including coding and non-coding, and small mRNAs(Marinov et al., 2014). The technology for profiling RNA had evolved rapidly since the 1980s, when low-throughput Sanger sequencing was initially used to sequence individual transcripts (expressed sequence tags). In the mid-1990s and 2000s, new high-throughput technologies were developed based on hybridization (microarrays) and massive parallel

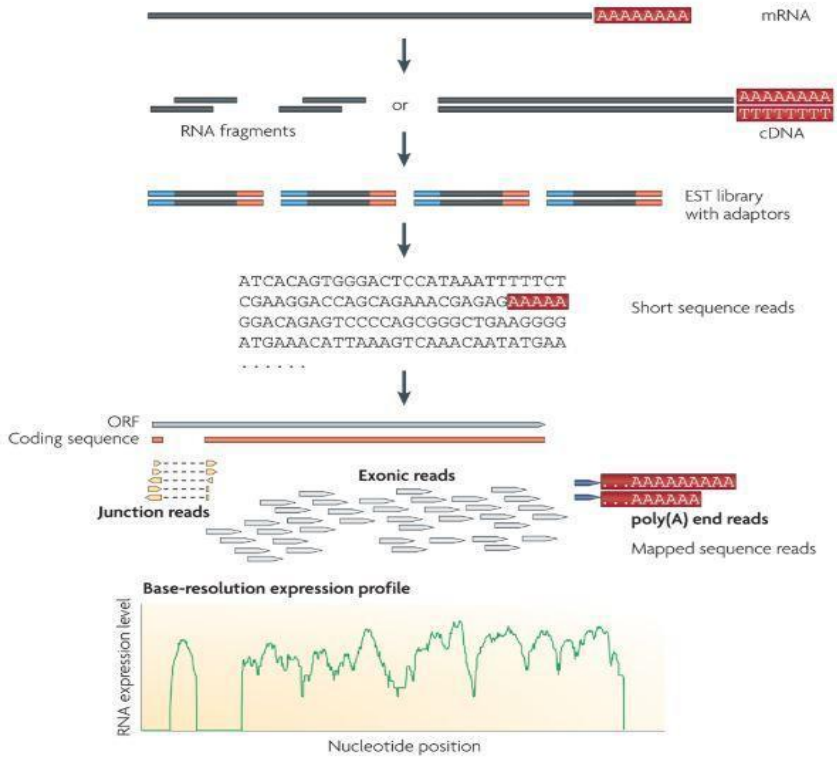

Figure 18. RNA-Seq. A typical RNA-Seq experiment includes different steps. First, the fractionated (e.g., poly A) RNA is transformed into a cDNA library with an adaptor attached. Then, these molecules are sequenced massively with NGS technologies to get short sequences (30-400 bp in length), which are then aligned to a reference genome to acquire a gene expression profiling signature. This is obtained by counting the number of mapped reads per genes, which is an indicator of gene expression level. The resulting sequence reads are also classified into different types (e.g., axonic reads, junction reads). The sensitivity and accuracy of RNA-Seq technology for gene expression analysis are dependent on the RNA-Seq sequence coverage (number of reads) and genome complexity. The figure is taken from (Z. Wang et al., 2009).

cDNA sequencing. These new technologies have allowed the identification and quantification thousands of cellular transcripts(Lowe, Shirley, Bleackley, Dolan, & Shafee, 2017), which

has enabled the genomic investigation of the molecular basis of human development and disease.

Microarray technology is based on the detection of biological material deposited in a two-dimensional array on a solid substrate using high-throughput screening technology (see Figure 17). DNA microarrays are reliable and reproducible but have several limitations that may limit their clinical application (Sobek, Bartscherer, Jacob, Hoheisel, & Angenendt, 2006). The introduction of high-throughput next-generation DNA sequencing technologies has allowed the massive scale sequencing of RNA transcripts. RNA-Seq is a transcriptomic quantitative method that allows the investigation in the same experiment of a diverse biological phenomenona, such as gene and transcript expression, transcript discovery, allele-specific expression or RNA editing (see Figure 18; Z. Wang, Gerstein, & Snyder, 2009). RNA-Seq has a wide dynamic range (of 5 orders of magnitude) and requires lower RNA amounts (nanogram quantity) compared to DNA microarray (microgram quantity). Disadvantages of RNA-Seq include bioinformatics challenges, and its higher cost compared to DNA microarrays(Lowe et al., 2017; Ozsolak & Milos, 2011).

Proteomics

The science of proteomics is the study of all proteins in a tissue or cell (proteome). Proteomics has evolved rapidly over the last decade thanks to the development of new methods that allowed the large-scale quantitative analysis of proteins, such as soft protein ionization

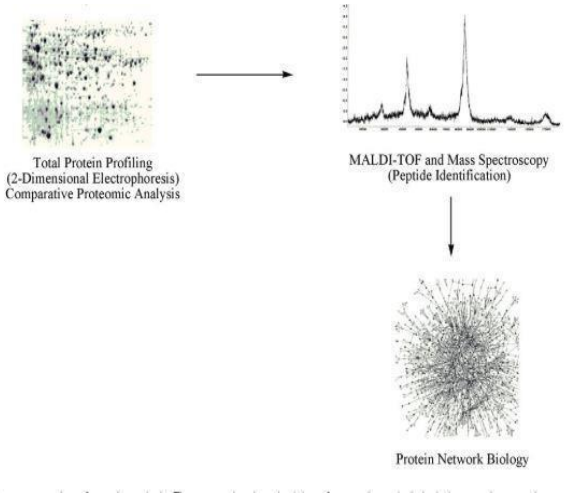

Figure 19. Proteomics. The identification of proteins is generally done by mass spectrometry. First, the proteins are isolated by laser capture microscopy or by resolution of extracted proteins by a gel. Then, they are injected into HPLC (the liquid chromatography of liquid chromatography) for quantification and identification by tandem mass spectrometry. The latter can be a complex task due the dynamic characteristics of proteins. As an example, a cell can contain from zero to six orders of magnitude copies of a single protein (Hood et al., 2012). Figure is taken from(Aslam, Basit, Nisar, Khurshid, & Rasool, 2017).

methods applied in mass spectrometry methods (Domon, 2006; Schulze & Usadel, 2010) (see Figure 19). There is now evidence from the Human Proteome Project for protein expression from over 17,000 human genes covering >84% of the human genome(Legrain et al., 2011). Proteomics data have multiple applications including structural, and clinical proteomics (Bachi & Bonaldi, 2008; Tyers & Mann, 2003), and provides information on the relative concentration of proteins, post-translational modifications and protein-protein interactions. However, the cellular expression of RNA and protein correlates poorly, due to diverse biological (e.g., protein translation, protein half-life) and technical conditions(Maier, Güell, & Serrano, 2009).

1.3.2. Applications of gene expression molecular profiling in perinatal medicine

Different transcriptomic and proteomic approaches have been applied in the last decade for understanding molecular mechanisms in perinatal medicine moving forward in precision medicine (Vora & Hui, 2018; see Figure 20). On the one hand, proteomics has been used to identify biomarkers of different pregnancy-related disorders, such as IAI, preeclampsia and gestational diabetes mellitus (Kolialexi, Mavreli, & Papantoniou, 2017), and in neonatal sepsis and NEC(S. Ng et al., 2018). On the other hand, transcriptomics of placenta using DNA microarray have been extensively used for studying pregnancy disorders, particularly preeclampsia(Eidem, Ackerman, McGary, Abbot, & Rokas, 2015). However, the molecular profiling of FIR has been seldom attempted; therefore, gene expression molecular profiling of FIR is partially known. To date, only one published study investigated the gene expression profiling associated with FIR using

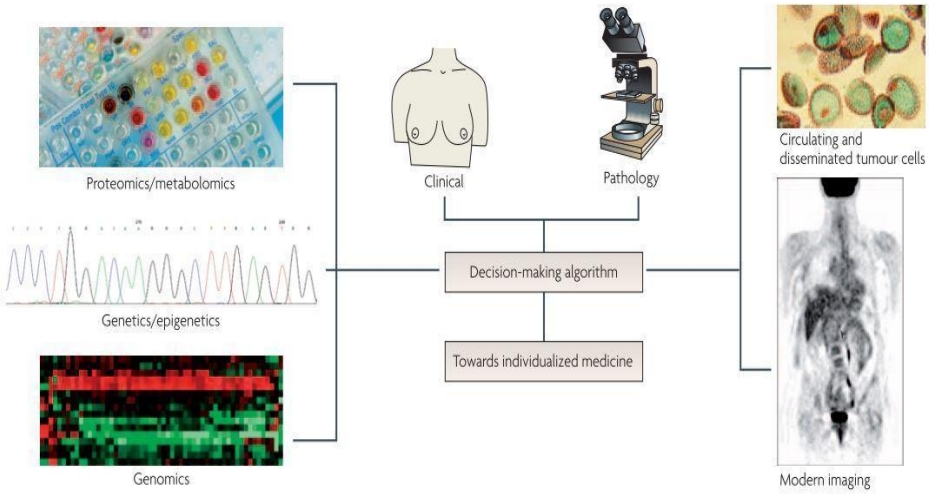

Figure 20. Precision Medicine. Precision medicine refers to a personalized approach to medicine in which the biological information of the patient is employed for medical diagnosis and treatment. New high-throughput technologies in molecular biology (genomic, transcriptomic, proteomic and metabolomics) are integrated with the traditional clinic-pathological practices into clinical decision-making algorithm. The figure is taken from(Sotiriou & Piccart, 2007).

RNA microarrays in UC leukocytes of preterm newborns(Madsen-Bouterse et al., 2010), while no attempts exist using mass-spectrometry proteomics. Thus, gene and protein expression molecular profiling of different tissues and conditions affecting the preterm newborn exposed to FIR may provide an improved understanding of the underlying molecular mechanisms of FIR.

1.3.3. Blood transcriptomics and proteomics

Blood is a specialized bodily fluid that consists of a mixture of plasma (55%) and cells, including red blood cells (40-45%) and leukocytes (1%). Plasma has a very high concentration of proteins; however, only 22 proteins account for 99% of their protein content (e.g., albumin, immunoglobulins, complement and coagulation factors). The reaming 1% consists of lower abundance circulatory proteins and proteins from tissue leakage. These proteins can have a difference of more than ten orders of magnitude in concentration concerning albumin, which represents around 1/2 of the total protein mass; Veenstra et al., 2005; Anderson, Leigh Anderson, & Anderson, 2002). On the other hand, blood cells share more than 80% of the transcriptome of many tissues, including the brain. They also can express organ-specific genes and respond to external stimuli regulating blood gene expression levels (e.g., blood insulin) (Mohr & Liew, 2007).

The changes in transcript and proteomic abundance in peripheral blood are complex due blood dynamics. Blood changes in RNA and protein abundance can vary according to intra-subject and inter-subject variation due to diverse constitutional conditions, host exposure, and changes in the relative abundance of blood cell populations. Alterations in blood transcript and protein abundance can also follow many human diseases, reflecting molecular changes in disease. High-throughput molecular profiling technologies can detect these changes as a molecular blood signature. Many human

diseases associate a peripheral blood transcriptomic signature, such as neurologic (e.g., Alzheimer's disease, schizophrenia), cancer (e.g., breast cancer) and inflammatory diseases (e.g., systemic lupus erythematosus). Proteomic biomarkers have also been identified in blood for different human diseases, such as heart disease, cancer, and neurodegenerative disorders(Lippolis & De Angelis, 2016). These molecular signatures and biomarkers have been used for advancing the understanding of the molecular pathophysiology of disease, diagnosis, and therapeutics (Mohr & Liew, 2007; Touzot et al., 2015).

Molecular profiling of peripheral blood can capture the systemic complex immune network activated at a given moment. Blood transcriptomic signatures have been associated with many microorganisms, including bacteria (e.g., Mycobacterium tuberculosis, Staphylococcus aureus), viruses (e.g., respiratory syncytial virus, influenza) and parasites (e.g., *Plasmodium*). Alterations in the blood transcriptome, associated with infectious diseases, provide an insight into the ongoing systemic immune reaction mounted by the host in response to microbial invasion (Chaussabel, Pascual, & Banchereau, 2010). These host blood molecular signatures can help in the diagnosis of several infectious diseases (Ramilo & Mejías, 2009).

1.3.4. Dried blood spots

The so-called Guthrie filter cards, made out of absorbent filter paper, are used to preserve fresh blood spot punches, commonly known as DBS, obtained from a heel/finger stick (see Figure 21). DBS have been used in the measurement of small (e.g., antibodies, viruses, drugs of abuse) and large molecules (e.g., proteins) in many applications, such as newborn screening, HIV testing and therapeutic drug monitoring(Grüner, Stambouli, & Ross, 2015; McDade, (Sharon Ann Allanson), & Josh. Snodgrass, 2007). Interestingly, the stability of unstable analytes, such as RNA, cytokines, and drug metabolites, is preserved in DBS compared with liquid samples because of blood enzymatic, which allow the molecular investigation of human disease(Freeman et al., 2018; Lehmann, Delaby, Vialaret, Ducos, & Hirtz, 2013). Recent technological advances have favored the development of new automated processess in DBS bioanalyses, such as direct mass spectrometric analysis and online extraction technology(Meesters & Hooff, 2013).

DBS differ in many aspects from the traditional plasma o serum samples. DBS have a small volume of blood (less than 50 microliters per blood spot) and consist of open-air dried uncentrifuged whole capillary blood. Compared to plasma samples (liquid medium), the drying process can affect the cellular blood composition due to the cellular rupture and release of intracellular components, such as hemoglobin, which can interfere with the analytic procedures. Drying

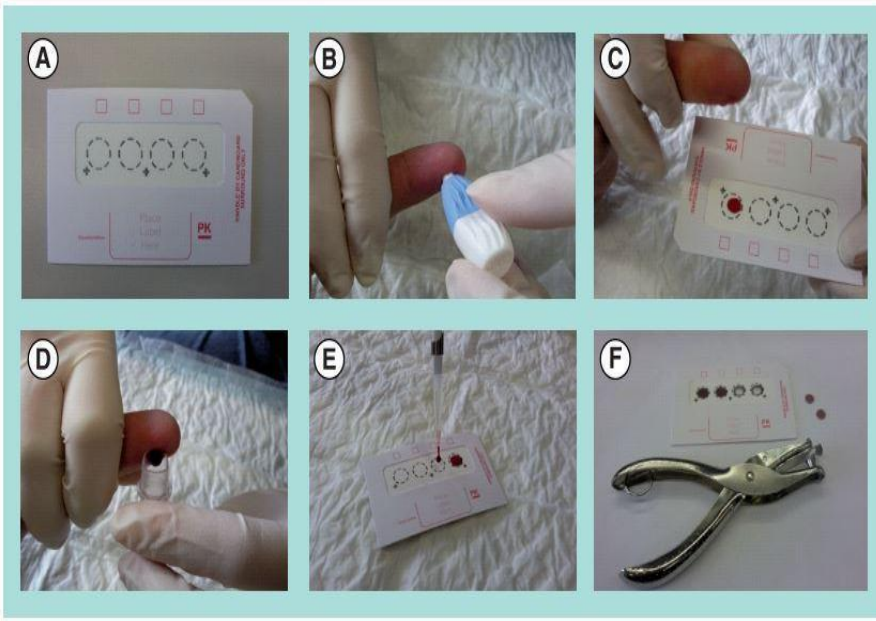

Figure 21. Dried blood spots (DBS). DBS collection is a minimally invasive technique as less than 0,5 ml of blood is obtained, without venipuncture, to fill five dried blood spots of the filter paper (75-80 microliters of blood per spot). This is very advantageous in neonatal screening, in addition to, ease of DBS storage (room temperature) and transport (e.g., regular mail). The quality of DBS is dependent upon the blood specimen collection, which depends on the hematocrit level (blood dispersal on filter paper) and proper blood extraction techniques(Timmerman et al., 2011). The figure is taken from (Meesters & Hooff, 2013).

can also inactivate most pathogens and alter the function of blood enzymatic proteins. The detection of blood proteins is also maintained during long periods of years in archived DBS, which may be of utility for proteomic investigations(Björkesten et al., 2017; Ignjatovic, Pitt, Monagle, & Craig, 2014).

2. OBJECTIVES

The main objective of this thesis is to investigate and characterize the molecular mechanisms underlying FIR. Our starting hypothesis is that FIR induces molecular alterations in various tissues evident at birth and during the first postnatal week in ELGANs. These changes may provide rationales for the molecular mechanisms underlying FIR and can aid to identify new potential biomarkers, moreover, therapeutic targets for FIR-related disorders. To investigate this hypothesis, we pursued the following specific aims:

1. Explore the UC gene expression response to FIR in ELGANs. Our working hypothesis for this aim is that FIR has specific effects on RNA expression in UC, which may participate in the pathogenesis of FIR.

2. Characterize the peripheral blood RNA gene expression to FIR in ELGANs in the first postnatal week. We will assess whether FIR is associated with differential RNA expression profile in peripheral whole blood.

3. Characterize the peripheral blood proteome associated with FIR in ELGANs in the first postnatal week. We will assess whether FIR is associated with differential protein abundance in peripheral whole blood.

These aims should allow us to explore for the first time using high-throughput molecular profiling technologies the molecular expression profiles of FIR in UC and whole peripheral blood. These molecular expression profiles can provide a new comprehension of the molecular mechanisms associated with FIR, knowledge currently incomplete. They also constitute a valuable resource for the identification of novel biomarkers and therapeutic targets for FIR-related disorders. We also will prove the utility of archived DBS for RNA and protein measurement, which may encourage future researches using archived dried blood spots samples.

3. RESULTS

3.1. Umbilical cord gene expression reveals the molecular architecture of the fetal inflammatory response in extremely preterm newborns

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Full text URL:

https://www.nature.com/articles/pr2015233

Supplementary Material:

http://functionalgenomics.upf.edu/supplements/FIRinELGANs

Abstract:

The fetal inflammatory response (FIR) in placental membranes to an intrauterine infection often precedes premature birth raising neonatal mortality and morbidity. However, the precise molecular events behind FIR still remain largely unknown, and little has been investigated at gene expression level. We collected publicly available microarray expression data profiling umbilical cord (UC) tissue derived from the cohort of extremely low gestational age newborns (ELGANs) and interrogate them for differentially expressed (DE) genes between FIR and non–FIR-affected ELGANs.We found a broad and complex FIR UC gene expression signature, changing up to 19% (3,896/20,155) of all human genes at 1% false discovery rate. Significant changes of a minimum 50% magnitude (1,097/3,896) affect the upregulation of many inflammatory pathways and molecules, such as cytokines, toll-like receptors, and calgranulins. Remarkably, they also include the downregulation of neurodevelopmental pathways and genes, such as Fragile-X mental retardation 1 (*FMR1*), contactin 1 (*CNTN1*), and adenomatous polyposis coli (*APC*).The FIR expression signature in UC tissue contains molecular clues about signaling pathways that trigger FIR, and it is consistent with an acute inflammatory response by fetal innate and adaptive immune systems, which participate in the pathogenesis of neonatal brain damage.

Umbilical cord gene expression reveals the molecular architecture of the fetal inflammatory response in extremely preterm newborns

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ABSTRACT

BACKGROUND: The fetal inflammatory response (FIR) in placental membranes to an intrauterine infection often precedes premature birth raising neonatal mortality and morbidity. However, the precise molecular events behind FIR still remain largely unknown and little has been investigated at gene expression level.

METHODS: We collected publicly available microarray expression data profiling umbilical cord (UC) tissue derived from the cohort of Extremely Low Gestational Age Newborns (ELGANs), and interrogate them for differentially expressed (DE) genes between FIR and nonFIR affected ELGANs.

RESULTS: We found a broad and complex FIR UC gene expression signature, changing up to 19% (3,896/20,155) of all human genes at 1% false discovery rate (FDR). Significant changes of a minimum 50% magnitude (1,097/3,896) affect the upregulation of many inflammatory pathways and molecules, such as cytokines, toll-like receptors and calgranulins. Remarkably, they also include the downregulation of neurodevelopmental pathways and genes, such as Fragile-X mental retardation 1 (*FMR1*), contactin 1 (*CNTN1*) and adenomatous polyposis coli (*APC*).

CONCLUSION: The FIR expression signature in UC tissue contains molecular clues about signaling pathways that trigger FIR and it is consistent with an acute inflammatory response by fetal innate and adaptive immune systems, which participate in the pathogenesis of neonatal brain damage.

INTRODUCTION

FIR in histological chorioamnionitis (HCA) is defined by the detection of polymorphonuclear cells within the wall of vessels in UC (funisitis) and chorionic plate. HCA is a surrogate of intra-amniotic infection and intrauterine inflammation and its frequency increases with lower GA at birth (1). FIR is considered an advanced stage of HCA and increases the risk for perinatal mortality and morbidity, particularly of neonatal brain damage (2,3). The latter is one of the most severe short and long term complications of preterm birth (PB), particularly in ELGANs—preterm infants born before 28 weeks of gestation (4).

Here, we describe the results of a gene expression analysis of FIR in UC tissue using data from a previously published study by Cohen (5) et al. (2007). In that study, the authors profiled gene expression using microarrays in UC tissue at birth from a cohort of n=54 ELGANs. Their goal was to search for gene expression changes in bronchopulmonary dysplasia and, in fact, did not find any with FDR < 5%, corresponding to an uncorrected $p < 10^{-6}$. However, exploring the extensive clinicopathological data from the ELGAN cohort (4) (Supplemental Tables S1 to S3 (online)) and the molecular metadata from the microarray chips (Supplemental Table S4 (online)), we have been able to obtain an appropriate experimental design that has allowed us to interrogate these data for DE genes between FIR and nonFIR affected ELGANs.

RESULTS

Umbilical Cord Tissue Carries a FIR Gene Expression Signature in ELGANs

Using state-of-the-art statistical techniques and software for processing and analyzing microarray gene expression data we discarded 6 samples, from the initial n=54, that did not meet specific quality control criteria (Supplemental Figures S1 to S6 and Supplemental Table S5 (online)). Because FIR status was also missing in some of the samples, the final data set analyzed in this paper consisted of n=43 samples where 18 were derived from FIR-affected infants and 25 from non-affected ones. After background correction and normalization of the microarray samples, a differential expression analysis with linear models (6), in which batch and other sources of FIR-unrelated variation were adjusted (7), showed that up to 19% of genes (3,896 out of 20,155) changed their expression at 1% FDR.

The magnitude of change among these 3,896 genes ranged from 1.12 to 32.7-fold difference between FIR and nonFIR affected ELGANs. Using a minimum 1.5-fold change cutoff, we called 1,097 DE genes. From these, 592 were upregulated and 505 were downregulated (Figure 1 and Supplemental Tables S6 and S7 (online)). A functional enrichment analysis of these genes with the Gene Ontology (GO) database (8) yielded 542 and 35 significant GO terms (FDR < 10%

and OR > 1.5) by up and downregulated genes, respectively (Figure 2 and Supplemental Tables S8 and S9 (online)).

Upregulation of Innate and Adaptive Immune Pathways in FIR

We found that genes involved in the innate immune response were among the top-10 upregulated genes with largest fold-change (Supplemental Table S6 (online)), such as calgranulins (S100A8, S100A9, S100A12), the lyzome (LYS), chemokines (CXCL8, CXCL1, CXCL5, CCL2) and the pentraxin 3 (PTX3). The GO enrichment analysis of upregulated genes also showed multiple significant GO terms (FDR < 10%, OR > 1.5) of the innate immune system. Among the top-10 enriched GO terms with largest OR (Figure 2 and Supplemental Table S8 (online)) we found terms related to the activation of neutrophils (e.g., neutrophil extravasation), monocytes (e.g, mononuclear cell migration) and oxidative stress (OS) (respiratory burst). We also tested the enrichment of a targeted collection of 17 immune pathways (16 innate (9) and one T helper type 17 (TH 17) response (10)) by the subset of significant 592 upregulated genes at 1% FDR with a minimum 1.5-fold change. Using one-tailed Fisher's exact tests we found 12 of these 17 immune pathways to be significantly enriched at 10% FDR with OR > 1.5. More concretely, we found enrichment of DE genes in the TH 17 response pathway and in 11 innate response pathways (Table 1). Next to the enrichment of DE genes in

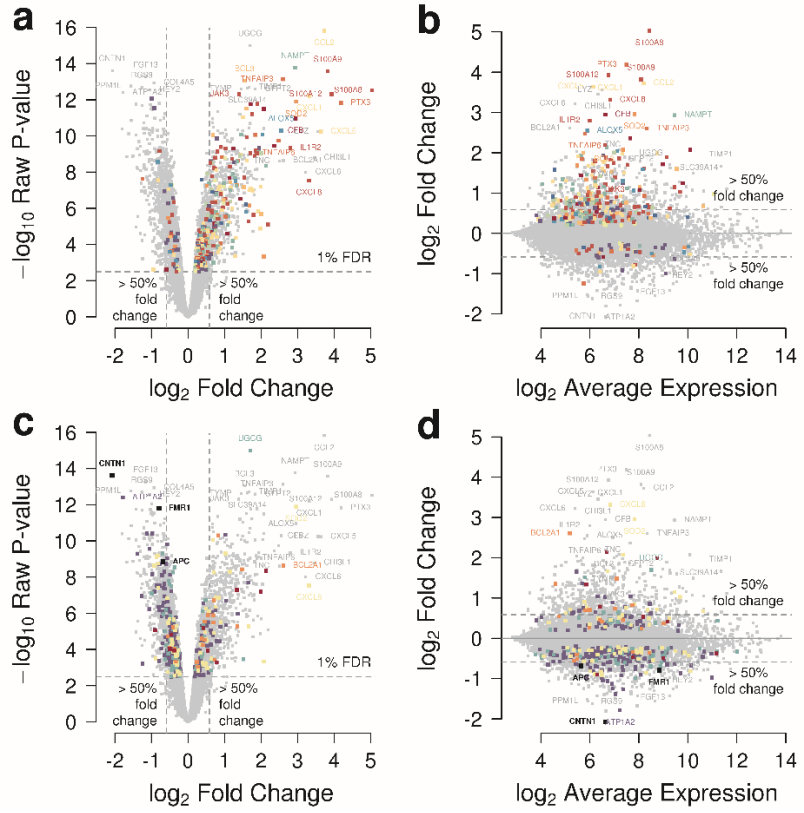

Figure 1 Differential gene expression caused by FIR. (**a, c**) Volcano plot of the significance level of gene expression changes between FIR and nonFIR infants in $-\log_{10}$ scale (*y*-axis), as function of their fold-change (*x*-axis) in $\log_2$ scale. The horizontal dashed line indicates the threshold for a multiple-test correction above which 3,896 genes change significantly their expression at FDR < 1%. Vertical dashed lines indicate a minimum magnitude of 1.5-fold change in expression, met by 1,097 genes with FDR < 1%. Genes highlighted in colors enrich significantly the following gene sets: (**a**) Complement Cascase (carmine), Cytokine Signaling (dark red), TNF Superfamily Signaling (red), ROS/Glutathione/Cytotoxic granules (salmon), Th17 cell response (orange), Adhesion/Extravasation/Migration (yellow), NF-kB Signalling (yellow-green), Innate pathogen detection (light green), Leukocyte Signaling (green), Eicosanoid Signaling (turquoise), Phagocytosis/Ag presentation (blue), MAPK Signaling (violet); (**c**) MSC genes (carmine), Apoptosis genes (salmon), Senescence genes (yellow), APC targets (green), FMRP targets (violet). (**b, d**) MA-plot of the magnitude of gene expression changes (*y*-axis) as function of the average expression (*x*-axis), both in $\log_2$ scale. Horizontal dashed lines indicate a minimum magnitude of 1.5-fold change in expression. Colors highlight the same gene sets as in (**a, c**).

the TH 17 response pathway, we found additional evidence of an adaptive immune response in FIR by enriched GO terms such as positive regulation of T cell migration (OR=8.18) and positive regulation of B cell activation (OR=3.58).

As a result of the activation of the innate and adaptive immune systems, the inflamed tissue may show cellular adaptations such as apoptosis and cellular senescence. We found enrichment of upregulated DE genes in apotosis (9) and senescence (11) gene sets (FDR < 10% and OR > 1.5); see Table 2. Finally, we also interrogated the Human Phenotype Ontology (HPO) database (12) and found a significant enrichment of upregulated genes in HPO terms (FDR < 10% and OR > 1.5) related to skin, dental and respiratory infections (Supplemental Table S10 (online)). This suggests that some of the upregulated gene expression changes associated with FIR, are similar to those caused by infection of surface tissues.

Downregulation of Neurodevelopmental Pathways in FIR

We found enrichment of downregulated DE genes in GO terms associated with neurodevelopment (see Figure 2 and Supplemental Table S9 (online)), such as *neural crest cell development* (OR=6.25), *astrocyte differentiation* (OR=5.51), *forebrain development* (OR=2.33) and *synaptic transmission* (OR=1.90). Among the most significantly downregulated genes (Figure 1 and Supplemental

Table S7 (online)) we found *CNTN1*, *FMR1* and *APC*, where *CNTN1* is a cell adhesion molecule that plays a role in the orderly progression of cortical development (13), *FMR1* encodes the Fragile-X mental retardation RNA-binding protein (FMRP) whose loss of function causes Fragile-X syndrome (FXS) and which is essential for cognitive development by regulating translation in neurons (14), and *APC* encodes an RNA-binding protein implicated in RNA localization and required for human neuron and axon migration (15). We mapped the FMRP and APC target genes found by HITS-CLIP (14,15) to our data set and observed a significant enrichment of downregulated genes at FDR < 1% in these two gene sets (one-tailed Fisher's exact $p=1.08\cdot10^{-6}$, OR=1.49 and $p=0.01$, OR=1.44, respectively); see Figure 1.

These findings were unexpected because there is no neuronal tissue in UC. Interestingly, we observed enrichment of the GO terms *stem cell development* (OR=1.89) and *stem cell differentiation* (OR=1.75) and, in a gene signature of 19 selective markers for UC mesenchymal stem cells (MSCs) that mapped to our data set (16), we also detected a significant enrichment by upregulated genes (one-tailed Fisher's exact $p=0.001$, OR=11.64, Figure 1).

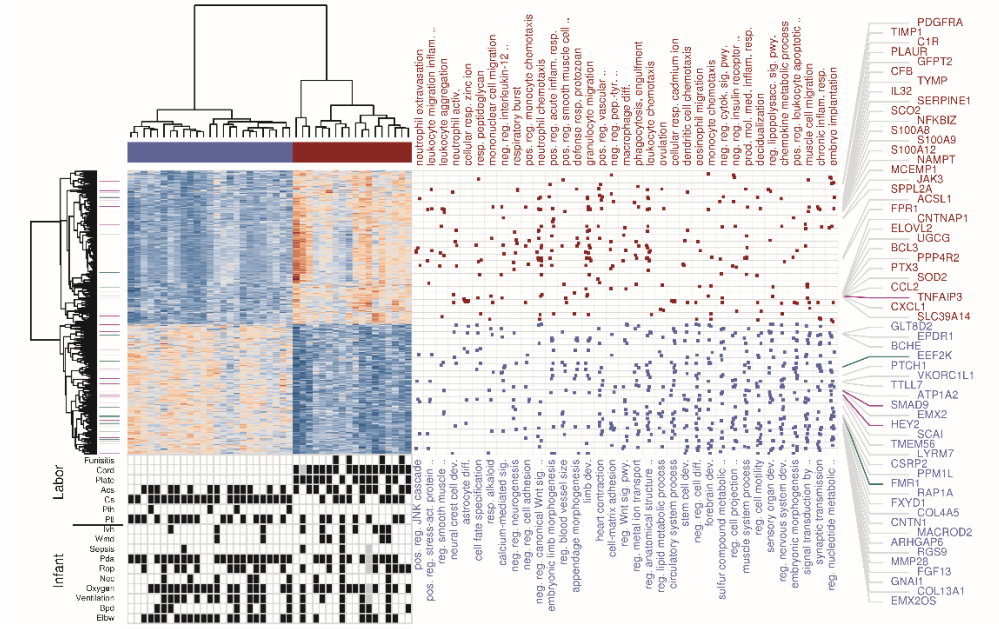

Figure 2 Functional enrichment analysis of the FIR expression signature. Heatmap of expression values for 1,097 DE genes with FDR < 1% and minimum 1.5-fold change between FIR and nonFIR infants, obtained after removing FIR-unrelated variability. Dendrograms on the *x* and *y*-axes represent the hierarchical clustering ofsamples and genes, respectively. Leaves on the gene dendrogram are color-coded according to whether genes encode for transcription factors (violet) or RNA-binding proteins (green), while those on the sample dendrogram are color-coded according to FIR status where red indicates samples derived from FIR-affected infants and blue nonaffected ones. The right dot-matrix represents DE genes (*y*-axis) belonging to GO terms (*x*-axis) significantly enriched (FDR < 10% and OR > 1.5) by upregulated (top) and downregulated (bottom) DE genes. Only the top-35 GO terms with highest OR from left to right, are reported. The top 60 DE genes with largest fold-change are on the right-hand side. At the bottom-left a dot-matrix representation of infant phenotypes is provided including Ivh, Wmd, Pda, Rop, Nec, Oxygen (> 27 days of oxygen), Ventilation (> 7 days of ventilation), Bpd, Elbw (weight < 1,000 gr.), and labor conditions including Cord (neutrophils in UC), Plate (neutrophils in chorionic plate), Acs, Cs, Pih and Ptl.

A Molecular Signature of Severe FIR

Advanced histological patterns of FIR are associated with perinatal brain damage (17), therefore gene expression profiles of UC tissue from infants with ”severe FIR” can potentially shed light on molecular mechanisms leading to adverse neurological outcome. Despite the limited sample size of n=43, we observed in our data an increasing fraction of intraventricular hemorrhage (IVH) and white matter disease (WMD) cases among FIR-affected over non-affected infants (Supplemental Table S2 (online)), which grows markedly when considering only the n=17 infants who were not administered antenatal glucocorticoids (ACS), and even significantly in the case of IVH. (two-tailed Fisher's exact p=0.005 and p=0.1 for WMD; see Supplemental Table S3 (online)).

Using the joint IVH and WMD condition as surrogate for severe FIR, we compared the expression profiles of infants affected by all three conditions FIR, IVH and WMD (n=4) with those not affected by any of them (n=22) and found 183 DE genes with FDR < 1%, 151 of them with a minimum 1.5-fold change (Supplemental Table S11 (online)). These figures increased more than 3-fold up to 570 and 566 genes, respectively, when restricting the comparison to infants who were not administered with ACS (n=3 FIR/IVH/WMD vs. n=10 nonFIR/nonIVH/nonWMD); see Supplemental Table S12 (online). In this comparison, we observed that calgranulin genes remain among the top-10 upregulated genes.

However, many of the expression changes occurred in genes that either did not change significantly their expression, or did it below a 50% magnitude, in the comparison between FIR and nonFIR infants from the whole data set of n=43 samples. For instance, the nerve injury-induced protein 2 gene (*NINJ2*) that is involved in nerve regeneration after nerve injury, is significant at FDR < 1% in both cases but its significance level and fold-change increase, respectively, from $p=2.45\cdot10^{-3}$ to $p=9.79\cdot10^{-5}$ and from 1.3-fold to 3.7-fold.

A GO functional analysis among the 310/566 upregulated DE genes with FDR < 1% and minimum 1.5-fold change yielded 70 significantly enriched GO terms (FDR < 10% and OR > 1.5), 4 of which were exclusively enriched by upregulated genes called DE only in the joint FIR/IVH/WMD comparison: *oxygen transport*, *hydrogen peroxide catabolic process*, *tetrapyrrole metabolic process* and *homeostasis of number of cells* (Supplemental Table S13 (online)). Downregulated genes (256/566) enriched only two GO terms (FDR < 20% and OR > 1.5) associated with neurogenesis (*negative regulation of neurogenesis*) and morphogenetic processes in central nervous system development (*negative regulation of cytoskeleton organization*); see Supplemental Table S14 (online). These results show that expression changes in UC tissue, occurring under a joint FIR/IVH/WMD condition, convey a molecular signature of severe

FIR concealing additional clues on molecular mechanisms associated with neonatal brain damage.

DISCUSSION

We have shown that FIR is characterized by a broad and complex acute inflammatory molecular expression signature in UC tissue of ELGANs. Previously, Madsen-Bouterse (18) et al. (2010) explored gene expression changes in UC blood leukocytes of preterm infants with fetal inflammatory response syndrome (FIRS) and funisitis (FIR) (n=10) vs. a control group (n=10), and they reported a total of 541 genes changing significantly at 5% FDR.

Among the 20,155 genes analyzed in our study, 483 from the 541 genes reported by Madsen-Bouterse (18) et al. (2010) mapped to our data, overlapping significantly with our FIR signature (one-tailed Fisher's exact $p < 2.2 \cdot 10^{-16}$ with OR=2.67 for DE genes with FDR < 1% and $p < 2.2 \cdot 10^{-16}$ with OR=3.58 for DE genes with FDR < 1% and minimum 1.5-fold change). The comparison of the $\log_2$-fold changes (Supplemental Figure S7 (online)) of these overlapping genes shows that even though there are important differences in the RNA source and microarray technology employed in both studies, the magnitude of expression change shows a positive and significant correlation in every subset of genes derived at different significance levels ($0.39 < \rho < 0.51$ with $p < 3.16 \cdot 10^{-4}$).

There is, however, an important discrepancy in the extent of the expression changes reported by Madsen-Bouterse (18) et al. (2010)

and ours. We found a total of 3,896 DE genes with FDR < 1% between the UC tissue of FIR and nonFIR infants, a figure that increases up to 5,461 with FDR < 5%, one order of magnitude above the 541 DE genes found by the study of Madsen- Bouterse (18) et al. (2010) at the same significance level. To rule out the possibility that this discrepancy is due to our larger sample size (n=43 vs. n=20) we carried out a simulation study matching the FIRS group sample composition, showing that on average one expects 4,532 DE genes with FDR < 5% (Supplemental Figure S8 (online)). Therefore, we can conclude that the main difference between the Madsen-Bouterse (18) et al. (2010) study and ours has a biological explanation, and more concretely, on the origin of biological samples, that points to the participation of UC cells, apart from UC blood leukocytes, in the FIR molecular signature.

The immune cells that trigger the production of inflammatory mediators in UC blood in FIR are not yet fully characterized (19). Madsen-Bouterse (18) et al. (2010) did not find differential expression of the *IL6* gene in UC blood leukocytes. Diverse studies (20,21) have failed to detect differences in the concentration of the mRNA encoding for interleukin (IL)-6 in UC blood leukocytes between infants with elevated concentration of IL-6 in UC blood vs. control, and the authors have proposed that other cells than UC blood leukocytes participate in the production of inflammatory mediators. We did find differential expression in the *IL6* gene and

in multiple other inflammatory genes that participate in the innate immune response (Supplemental Table S6 (online)). We found DE genes related to neutrophil activation and migration (e.g, *CXCL8*, *CXCL1*, *CXCL2*); activation of endothelium (e.g, *ICAM1*, *ICAM3*, *SELE*, *ITGB2*); production of reactive oxygen species (ROS) and OS (e.g, *SOD2*, *GPX3*, *MGST1*); secretion of cytokines (e.g., *IL1B*, *NAMPT*, *MMP9*); synthesis of leukotrienes (e.g., *ALOX5*) and prostaglandins (e.g, *PTGES*). On the other hand, Hecht (22) et al. (2011) recently demonstrated that ELGANs with funisitis (FIR) had elevated blood concentrations of multiple inflammatory mediators during the first 3 days after birth. Thus, altogether it suggests the hypothesis that UC cells participate in the onset of FIR, which can result in a systemic neonatal inflammation after birth.

The origin of HCA in preterm birth has been traditionally attributed to an ascending intrauterine infection. However, recent evidence (23) suggests that one of the initial events of HCA could be a microbial invasion of the amniotic cavity. Gillaux (24) et al. (2011) have shown the capacity of amniotic epithelial cells to trigger an immune response through TLR recognition. We found upregulation of *TLR2* and *TLR8* genes, and genes encoding proteins that participate in the downstream signaling pathways associated with TLR activation (e.g., *MYD88*, *CD14*, *IRAK3*, *NFKB1*, *NFKB2*). In addition, genes encoding for S100 calgranulin proteins (*S100A8*, *S100A9* and *S100A12*), which can act as damage-associated molecular patterns (”alarmins”), are

among the most upregulated genes in FIR and severe FIR (Supplemental Tables S6 and S12 (online)). Among the body of evidence consistent with these results, Buhimschi (25) et al. (2007) has shown that the calgranulin S100A12 is associated with clinically significant funisitis in amniotic fluid. On the other hand, Kim (26) et al. (2006) has demonstrated that skin samples from fetal autopsies, which had chorioamnionitis with fetal vasculitis (FIR) on placental examination, showed perivascular inflammatory cell infiltration by neutrophils, lymphocytes and histiocytes in the superficial dermis, and TLR-2 immunoreactivity in the skin. Interestingly, we found a significant enrichment of upregulated genes in HPO terms related to skin infections (Supplemental Table S10 (online)). In summary, it is biological plausible that microbial invasion of the amniotic cavity triggers the onset of downstream molecular pathways by UC epithelial cells and TLR recognition, which initiate the production of inflammatory mediators and the recruitment of blood leukocytes to UC tissue (Figure 3). Afterwards, the production of calgranulins and ROS can act as alarmins, enhancing FIR. These results support the role of calgranulins as biomarkers of intra-amniotic inflammation in preterm birth (25).

The role of the adaptive immune system has not been fully investigated in comparison to the innate system in FIR. In preterm infants, Duggan (27) et al. (2001) and Luciano (28) et al. (2011) have demonstrated that neonatal brain damage and PB were respectively associated with T-cell activation. In premature infants,

these cells have a bias to differentiate in T$_H$17 cells, which play a prominent role in the body defense against extracellular microbes at epithelial barriers. In our study, we found a significant enrichment of DE genes in the T$_H$17 response pathway (e.g., *HIF1A*) and in GO terms related to T and B cell activation (Table 1 and Supplemental Table S8 (online)). Interestingly, *HIF1A* encodes for a transcription factor protein that can induce the differentiation of T$_H$17 vs. T-regulatory cells. Thus, our results corroborate the activation of the fetal adaptive immune system in FIR, concretely of T$_H$17 cells, and support the evidence of an adaptive immune response in intrauterine inflammation during PB (Figure 3). This has been shown to occur through the induction of a T$_H$17 cell response by the activation of the IL-1/IL-17 axis (29).

The cellular adaptation of UC tissue to FIR is of pathogenic interest, yet it has been poorly investigated. Senescence is one such cellular adaptations in response to noxious stimuli such as OS, and it is defined by an irreversible cell-cycle growth arrest, and by the synthesis of multiple inflammatory mediators (SASP). We found endogenous enzymatic antioxidant encoding genes (*SOD2*, *GPX3*, *MGST1*) and matrix metalloproteinases (*MMP9*, *MMP10*, *MMP19*) upregulated in FIR (Supplemental Table S6 (online)) and a significant enrichment of multiple OS-related GO terms (e.g., *superoxide anion generation*; Supplemental Table S8 (online)), senescence pathways and of a UC MSCs expression signature (Table 2). OS damage and the activation of senescence pathways

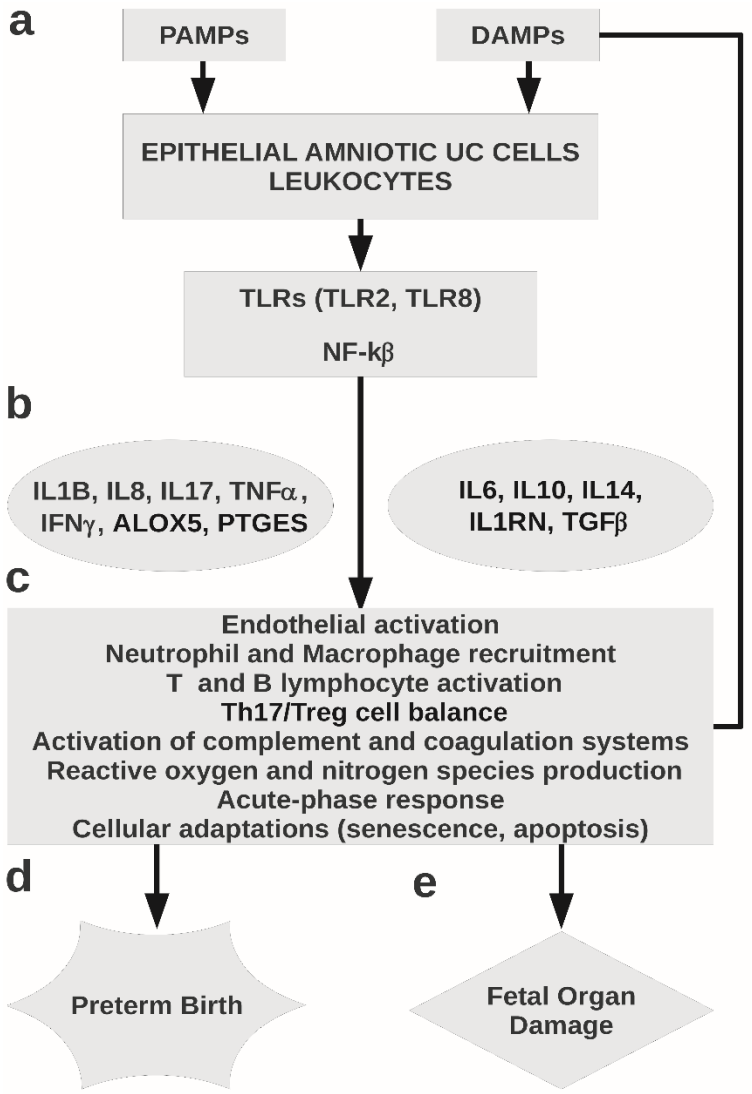

Figure 3 Molecular architecture of FIR. (**a**) UC microbial invasion is detected by epithelial amniotic cells in UC tissue. TLRs identify PAMPs and DAMPs, producing and enhancing FIR and participating in the activation of the NF-kB signaling pathway. (**b**) The transcriptional response of NF-kB activates pro-inflammatory mediators and anti-inflammatory ones. (**c**) The state of multiple cell types and tissues is affected by the acute inflammatory cascade. (**d**) The activation of pro-inflammatory pathways can trigger the production of labor intermediates and promote parturition onset. (**e**) The acute inflammatory response can damage fetal organs through its mediators and immune cells.

have been proposed as candidate pathogenic mechanisms in PB and neonatal diseases (30,31). Thus, the acute inflammatory cascade and OS generated during FIR may damage MSCs, which could enter into a senescence state with SASP that in turn may also participate and enhance FIR (Figure 3).

Accumulated evidence indicates that intrauterine inflammation participates in the genesis of encephalopathy of prematurity. While epidemiological studies have demonstrated that FIR increases the risk of brain damage, few investigations have explored pathogenic mechanisms behind this association. We found that downregulated genes enriched significantly GO terms related to fetal development, particularly of brain (Supplemental Table S9 (online)). We observed downregulation of neurodevelopmental genes such as *FMR1*, *APC* and their targets, and *CNTN1*, which are essential for cognitive development (13-15). Despite its limited sample size (n=43), the data set analyzed in this article recapitulates the significant association between FIR and IVH after adjusting by ACS (Supplemental Table S3 (online)), reported in larger studies (32). Using the perinatal neurological status (IVH and WMD), we found a molecular signature of severe FIR and evidence of the effect of the ACS therapy at molecular level. Overall, these findings are consistent with the hypothesis that expression changes in UC caused by strong intrauterine inflammation conceal clues on neurological damage.

One possible molecular mechanism for this hypothesis is the presence of MSCs in UC, which have the capacity to differentiate into neurons and could be damaged by FIR. Given that we observed the differential expression of key genes and pathways associated with MSCs in UC tissue, we postulate that UC MSCs could constitute a useful system for modeling perinatal brain damage in ELGANs. In fact, several studies (33) have already used human pluripotent stem cells, obtained from tissue of patients affected by diverse disorders, including neurological diseases, for investigating mechanisms of disease. In turn, hypotheses on precise pathogenic molecular mechanisms, such as the loss of function of neurodevelopmental genes (e.g., *CNTN1*, *FMR1* and *APC*), provide an array of potential therapeutic targets (34) to fight against such an adverse neonatal outcome.

METHODS

The bioinformatic and statistical analyses described in this section were performed using R version 3.1.3 and multiple add-on software packages deposited at the Comprehensive R Archive Network (CRAN) and at the Bioconductor project (35) release 3.0. Scripts reproducing the analyses described in this section are available in the Supplemental Source File (online).

Subjects and clinical data

The data analyzed in this article were generated by Cohen (5) et al. (2007) using UC samples derived from preterm newborns at 23 to 28 GA weeks enrolled in the ELGAN study (4), and therefore, informed consent was not required to conduct the bioinformatic and statistical analyses presented in this article. A full description of the ELGAN study, sample collection and preparation is provided elsewhere (4, 5). Phenotypic and clinical data were obtained through the ELGAN Publication and Data Analysis (PAD) committee for the purpose of this research and are described in Supplemental Tables S1 to S3 (online). The clinical record of each infant included the presence or absence of neutrophils in umbilical cord and chorionic plate. From these two factors we built a binary variable indicating the activation of FIR when neutrophils were present in either the umbilical cord or the chorionic plate and, conversely, indicating the lack of FIR when neutrophils were absent from both, umbilical cord and chorionic plate. For six infants, FIR status could not be determined due to missing values occurring in these two factors and that precluded using the previous rule.

Data Import, Quality Assessment and Normalization

Raw data CEL files corresponding to the $n=54$ samples of RNA in UC, hybridized on Affymetrix HG-U133 Plus 2.0 gene expression microarray chips and initially published by Cohen (5) et al. (2007),

were downloaded from the Gene Expression Omnibus (National Center for Biotechnology Information, Bethesda, MD) using the accession GSE8586. Batch processing information was derived from the scanning timestamp stored in CEL files as previously described (36), creating a batch indicator variable that divided the samples into three different batches. The cross-classification of infants by FIR status and batch indicator showed no correlation between the primary outcome of our analysis, FIR status, and sample batch processing (Supplemental Table S4 (online)).

We applied seven quality assessment diagnostics on the whole set of n=54 samples and flagged by visual inspection those showing potentially problematic features (see Supplemental Figures S1 to S6 (online)). We ranked samples by the number of flagged diagnostics (Supplemental Table S5 (online)) and discarded six that failed in the majority of them. Raw data from the remaining n=48 samples were normalized into Affymetrix probesets using fRMA (37) leading to an expression data set of p = 54,675 probesets by n=48 samples. At this point, we also excluded from further analysis five samples for which the FIR status was missing, ending with a final set of n=43 samples.

Nonspecific Filtering and Differential Expression Analysis

A first nonspecific filtering step was performed to remove probesets of little interest for the rest of the analysis. Concretely, we removed probesets without annotation to *Entrez* gene identifiers, probesets

annotated to a common *Entrez* gene keeping the one with highest variability measured by the IQR, and Affymetrix control probesets. This resulted in an expression data set of p = 20,155 probesets in one-to-one correspondence with *Entrez* genes, by n=43 samples.

Using the top-10% most variable (IQR) genes, we estimated covariates that capture variability unrelated to FIR status and batch, with surrogate variable analysis (SVA) (7) that yielded seven such covariates. For each gene g we defined the following linear model,

$$y_g \sim x_F + x_B + x_{S1} + \cdots + x_{S7},$$

where the expression profile y_g of gene g is a linear function of the FIR status variable x_F, the batch indicator variable x_B and the seven covariates $x_{S1}, \ldots, x_{S7}$ estimated by SVA. Using limma (6) we fitted this linear model to the every gene expression profile, and calculated moderated *t*-statistics for the coefficient estimating the effect between FIR and nonFIR affected infants and their corresponding *p*-values for the null hypothesis of no-differential expression.

We considered discarding nine fractions, from 10% to 90%, of genes with lower variability (IQR). In the remaining genes from each fraction, we adjusted the previously calculated *p*-values by FDR and tallied the number of genes with FDR < 1%. In a second nonspecific filtering step, we removed from further

analysis the fraction of genes with lower variability that maximized the amount of detected differential expression (38) at 1% FDR. Supplemental Figure S9 (online) shows that this fraction was 40% (8,062 genes from 20,155), which enabled adjusting by FDR on a reduced data set of 12,093 genes providing a set of 3,896 genes changing significantly at 1% FDR, 1,097 of them with a minimum 1.5-fold change.

When analyzing the subset of n=17 infants who were not administered with ACS we introduced two changes to this procedure. First, we used a factorial design without intercept term, estimating all the 3-way effects of FIR, IVH and WMD. Then, we calculated the moderated *t*-statistic based on the contrast that compared infants affected by all three FIR, IVH and WMD effects against those not affected by any of them. Second, due to the small sample size, we removed from the model the xB term corresponding to the batch indicator variable and let SVA to estimate any effects unrelated to the contrast of interest.

Differential Expression Analysis with Smaller Sample Size

From the entire data set of n=43 infants we generated 100 bootstrapped data sets with a sample-group composition identical to the study by Madsen-Bouterse (18) et al. (2010). Infants in each bootstrapped data set were randomly sampled with replacement ensuring that there were n=10 FIR-affected infants, n=10 nonFIR ones, and at least one sample per batch. In each bootstrapped data

set we carried out a differential expression analysis identical to the one performed on the whole data set. The number of genes, *p*-values and absolute $\log_2$-fold changes shown in Supplemental Figure S8 (online) correspond to those with FDR < 5% to match the significance level employed by Madsen-Bouterse (18) et al. (2010).

Functional Enrichment Analysis

Functional enrichment analyses with the GO and the HPO databases were performed using a conservative implementation of the conditional hypergeometric test (39) in the GOstats Bioconductor package (40), where a significant conditioning term was identified not only on the basis of a *p*-value cutoff, but also using minimum OR and gene-set size thresholds. In all GO and HPO enrichment analyses we used a *p*-value cutoff of 0.01, a minimum OR of 1.5 and a minimum and maximum gene set sizes of 5 and 300 genes, respectively. Among the previously identified GO or HPO terms, we discarded those whose average IQR across their respective genes was below the 90% of such values in a simulated distribution with random genes sets of matching size. On the remaining GO or HPO terms passing these nonspecific filters, *p*-values were adjusted by FDR and we reported as significantly enriched those GO or HPO terms with FDR < 10%, OR > 1.5 and at least 5 genes enriching them. When testing functional enrichment separately by up and downregulated genes, the gene universe was restricted to the subsets of 10,868 genes with positive $\log_2$ fold-change and 9,287 genes

with negative $\log_2$ fold-change, respectively. Sources for the targeted gene sets shown in Figure 1 and Tables 1 and 2, are described in Supplemental Table S15 (online).

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TABLES

Table 1 Significantly upregulated genes classified into inflammatory pathways. Inflammatory pathways significantly enriched (FDR < 10% and OR > 1.5) by genes upregulated in FIR with FDR < 1% and minimum 1.5-fold change.

Pathway	OR	P-	Counts	Size	Genes
Complement Cascase	8.40	3.3e-06	10	31	*C1R, C1S, C3, C3AR1, C5AR1, CD55, CFB, CFP, SERPINA1, SERPINE1*
Cytokine Signaling	8.10	2.4e-20	41	135	*CEBPB, CSF1R, CSF2RB, CSF3, CSF3R, CXCL8, CXCR2, IFNAR2, IFNGR1, IFNGR2, IL10RA, IL17RA, IL18R1, IL18RAP, IL1A, IL1B, IL1R1, IL1R2, IL1RL1, IL1RN, IL32, IL4R, IL6, IL6ST, IRF1, JAK3, LIF, NFIL3, NMI, OSM, OSMR, PDGFRA, PTPN2, S100A12, S100A8, S100A9, SOCS1, SOCS3, STAT3, TGFBR2, VEGFA*
TNF Superfamily Signaling	7.50	2.0e-05	9	30	*PTX3, TNFAIP3, TNFAIP6, TNFRSF11B, TNFRSF1B, TNFSF13B, TNFSF15, TNIP1, TRAF3*
ROS/Glutathione/Cytotoxic granules	7.30	1.7e-03	5	17	*ANPEP, CYBB, GPX3, NCF2, SOD2*
Th17 cell response	7.00	1.1e-08	17	60	*BCL3, BCL6, CCL20, HIF1A, IFNGR1, IL1R1, ITGB2, JAK3, LIF, NFKB1, NFKBIZ, PTGER4, SOCS1, SOCS3, STAT3, TGFBR2, VDR*
Adhesion-Extravasation-Migration	6.80	1.1e-14	32	117	*ACKR1, ADAM8, CCL2, CCL20, CCL4, CCL5, CCL8, CCR1, CCR2, CD36, CD48, CEACAM1, CXCL1, CXCL2, CXCL3, CXCL5, ICAM1, ICAM3, ITGA2, ITGAM, ITGAX, ITGB2, MMP10, MMP19, MMP9, PLAUR, PPBP, RASSF5, SELE, SELL, SELPLG, VCAM1*
NF-kB Signaling	6.80	2.8e-04	7	25	*BCL3, BCL6, NFKB1, NFKB2, NFKBIA, NFKBIE, RELB*
Innate pathogen detection	6.10	3.2e-05	10	39	*CD14, IRAK1, IRAK3, LY96, MYD88, NLRC4, NOD2, PYCARD, TLR2, TLR8*
Leukocyte Signaling	5.60	1.1e-09	23	96	*CD37, CD44, CD52, CD53, CD86, FCER1G, FCGR2A, FCGR2B, LCP2, LILRA2, LILRA3,*
Eicosanoid Signaling	4.80	3.4e-03	6	28	*ALOX5, ALOX5AP, PLA2G2A, PTGER4, PTGES, PTGS2*

Phagocytosis-Ag presentation	3.20	2.8e-02	5	32	*CD1D, CTSS, PSMB9, TAP1, TAPBP*
MAPK Signaling	2.40	2.6e-02	8	66	*IFI16, LYN, MAP3K5, MYC, PRKCD, RAC2, RPS6KA1, SHC1*

Table 2 Significantly upregulated genes classified into apoptosis and senescence pathways. Apoptosis and senescence pathways significantly enriched (FDR < 10% and OR > 1.5) by genes upregulated in FIR with FDR < 1% and minimum 1.5-fold change.

Pathway	OR	P-	Counts	Size	Genes
Senescence-Associated Secretory Phenotype (SASP)	4.70	8.4e-04	8	38	*CDKN1A, CEBPB, CXCL8, IL1A, IL6, NFKB1, RPS6KA1, STAT3*
Apoptosis	4.30	1.4e-03	8	41	*BCL2A1, BID, BIRC3, CFLAR, MCL1, TNFRSF10B, TNFRSF10D, TNFRSF21*
Cellular Response Stress	3.30	2.0e-04	15	96	*CDKN1A, CEBPB, CXCL8, ERO1L, GPX3, HIF1A, IL1A, IL6, KDM6B, MAP3K5, NFKB1, RPS6KA1, SOD2, STAT3, VEGFA*
Cellular Senescence	2.80	5.4e-03	10	72	*CDKN1A, CEBPB, CXCL8, IL1A, IL6, KDM6B, MAP3K5, NFKB1, RPS6KA1, STAT3*

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3. 2. Molecular profiling of neonatal dried blood spots reveals changes in innate and adaptive immunity following fetal inflammatory response

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Abstract: The fetal inflammatory response (FIR) increases the risk of preterm neonates to adverse neonatal outcomes, such as perinatal brain injury, particularly in extremely low gestational age newborns (ELGANs, < 28 weeks of gestation). One of the mechanisms contributing to such adverse neonatal outcomes is a postnatal intermittent or sustained systemic inflammation (ISSI) following FIR. This link between fetal and neonatal systemic inflammation is supported by the presence of well-established inflammatory biomarkers in umbilical cord (UC) and peripheral blood. However, the extent of molecular changes contributing to this association is unknown. Using RNA sequencing and mass spectrometry proteomics, we have profiled the transcriptome and proteome of archived dried blood spots (DBS) from 21 ELGANs. Comparing ELGANs who were exposed to FIR (n=10) against those that were not (n=11), we identified, respectively, 783 and 27 gene and protein expression changes, with a minimum 50%-fold change and an experiment-wide significance level below 5% false discovery rate. These expression changes confirm the persistent and robust activation of the innate immune system in FIR-affected ELGANs and provide new insights into the molecular mechanisms underlying sustained systemic inflammation and perinatal brain injury. Interestingly, 21 out of the 27 differentially expressed proteins did not match transcriptomic changes, thereby concealing clues on the link between FIR and ISSI that could not be uncovered by either transcriptomics or known biomarkers alone. These findings contribute to the understanding of the association between FIR and ISSI, provide DBS biomarkers of these two systemic inflammatory conditions and may help to identify future therapeutic targets for the associated perinatal disorders.

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4. DISCUSSION

The rapid technological advances in high-throughput technologies for biological molecules (e.g., DNA, RNA, proteins) and the completion of the Human Genome Project have allowed scientists to investigate molecular mechanisms in human diseases at large-scale molecular level. New high-throughput technologies, such as DNA microarray and next-generation sequencing technologies, have been extensively used for studying genomics, transcriptomics and other-omics approaches in multiple human diseases. By doing so, omics approaches have uncovered relevant molecular mechanisms of human diseases pertinent to disease management, diagnosis, treatment and prevention(Hasin, Seldin, & Lusis, 2017). However, the current evidence of the health benefits of, for instance, genomic tests, is limited and the application of omics technologies to routine clinical care face multiple challenges(M. C. Roberts, Kennedy, Chambers, & Khoury, 2017).

Exist different transcriptomics approaches for understanding molecular mechanisms of placental function and fetal development under normal and pathological conditions(Vora & Hui, 2018). Cell-free fetal RNA collected from AF supernatant allow the study of gene expression profiling of multiple fetal organs(Zwemer & Bianchi, 2015). Single-cell RNA-Seq investigations of pre and post-implantation human embryos can explore the transcriptional

landscape of fetal development(Gerrard et al., 2016; Petropoulos et al., 2016). DNA microarray technologies have allowed the investigation of placental transcriptomics associated with several pregnancy disorders, particularly preeclampsia(Eidem, Ackerman, McGary, Abbot, & Rokas, 2015). Circulating placenta RNAs in maternal plasma and single-cell RNA-Seq of placenta opens up new avenues in placenta transcriptomics(Tsang et al., 2017; Whitehead, Walker, & Tong, 2016). However, only a few contributions using transcriptomics have explored the molecular landscape of UC associated under disease. To best of our knowledge, we investigated for the first time gene expression changes in UC of FIR.

The implementation of new omics technologies in neonatal medicine has been slow up in comparison to other medical fields. Neonatologist use commonly comparative genomic hybridization methods to detect chromosome imbalances in congenital anomalies and abnormal fetal growth. Researchers have used microarray-based technologies for examining the differential expression levels of RNA and micro RNA in neonatal sepsis; yet, they have not applied RNA-Seq technology to the genomic characterization of neonatal sepsis. RNA expression signatures of neonatal sepsis have revealed molecular mechanisms underlying neonatal sepsis and candidate biomarkers. Metabolomics and proteomics investigations in neonatal sepsis and NEC have also identified potential protein biomarkers.

To the best of our knowledge, no previous published research has investigated both, proteomics and transcriptomics alterations of neonatal disorders in peripheral blood from ELGANs using DBS. Our integrated transcriptomic and proteomic approach to understanding the molecular pathophysiology of FIR may help the discovery of new systemic inflammatory biomarkers in preterm newborns(Kumar et al., 2016).

4.1. Umbilical cord gene expression reveals the molecular architecture of the fetal inflammatory response in extremely preterm newborns

The UC is an essential structure that connects fetal and maternal circulation. It contains three umbilical vessels (two arteries and one vessel) within the mucoid connective tissue, Wharton's jelly, surrounded by amniotic epithelium. Within Wharton's jelly, there are only mesenchymal cells, and no other cell types and vascular or nervous tissue have been isolated, except those from umbilical vessels UC (endothelial and smooth muscle cells). Wharton's jelly-derived mesenchymal stromal cells have been isolated and used in different clinical trials due to their immune modulatory and anti-inflammatory capacities(Davies, Walker, & Keating, 2017). The pathological changes in UC associated with FIR are well known and are consequently reflected in various classifications. However, the

molecular mechanisms underlying FIR in UC are mostly unknown, including the tissues and cell types involved in FIR.

The mother, the fetus and gestational tissues could participate in the high level of inflammatory mediators in UC blood associated with FIR. At birth, the UC blood cytokine concentration is related to the presence or severity of CHO and not with maternal blood cytokine concentration(Salafia et al., 1997). The cessation of placental-derived cytokine transfer after birth can cause the progressive decrease of high UC blood levels of pro-inflammatory cytokines (IL-1, IL-6, CXCL8, TNF-alpha) during the first postnatal days(O. Dammann et al., 2001). A recent investigation in very preterm sheep fetuses demonstrated that LPS intra-amniotic injection triggers a fetal lung, skin and a systemic inflammation from the first 2 to 48 hours. The latter was characterized by absence and mild increase of RNA inflammatory mediator expression in UC blood and spleen, respectively. The authors speculated that fetal systemic inflammation was driven by AF-exposed tissues and not by fetal blood cells(Matthew W. Kemp et al., 2016).

We found for the first time that FIR induces substantial changes in UC gene expression in ELGANs that involve up to the 19% of human genes (3,896 out of 20,155 at FDR < 1%). FIR transcriptional profile is most strongly enriched with an innate and adaptive immune response. Previously, gene expression changes associated with FIR have been investigated in UC blood leukocytes from preterm

newborns by Madsen-Bouterse et al. (2010). In UC blood leukocytes FIR is associated with gene expression changes on 541 with FDR < 5%, representing about a 2% of all human genes (296 upregulated and 252 downregulated). Such a discrepancy in the magnitude of gene expression changes between UC and UC blood leukocytes (about one order of magnitude) suggest differences in the ability to mount immune responses.

The gene expression profile of inflammatory mediators varies substantially between in UC and UC blood leukocyte. We observed in UC from FIR-affected ELGANs differential expression of multiple genes encoding for various inflammatory proteins, such as *IL6, CXCL8, and IL1b*. In contrast, high levels of UC blood concentrations of pro-inflammatory cytokines (IL-6, CXCL8, TNF-alpha, CXCL10 and CCL2) in FIR-affected preterm were not correlated with transcriptional up-regulation of pro-inflammatory mediators in Madsen-Bouterse et al. (2010) study.

Similarly, other authors investigating early onset neonatal sepsis could not detect differences in UC blood mRNA concentrations of several cytokines, despite their high UC blood concentration. Diminished innate immune responses at birth in preterm newborns (Marchant et al. 2015) may underlie the low correlation between UC blood inflammatory mediator level and transcriptional expression of pro-inflammatory mediator genes. Overall, our findings support the

hypothesis of FIR driven by AF-exposed tissues, particularly by UC in ELGANs.

The molecular alterations in UC associated with FIR remain unexplored. According to our findings, UC amniotic epithelial cells could detect pathogen-associated molecular pattern by PRRs and trigger fetal innate and adaptive immune responses, including Th17 reactions. In animal studies, Kallapur et al. (2013) found that CHO may induce the fetal activation of the IL-1/IL-17 axis, which may be associated to the development of fetal intrauterine inflammation(S. G. Kallapur et al., 2013). We found significant enrichment of DE genes in the IL-1 and Th17 cells immune pathways. Supporting our results, Ito et al. (2010) demonstrated the association of CHO with Th17 cells in amniochorionic membranes(Ito et al., 2010). Black et al. (2012) also found that naive neonatal CD4+ Th cells, particularly at lower GA, express higher levels of genes related with Th17 function (IL23R, RORC, STAT3; Black, Bhaumik, Kirkman, Weaver, & Randolph, 2012).

Aging, detected by reductions in telomere length of fetal membrane chromosomes, of the amniochorionic membranes can also participate in the molecular mechanisms of FIR. Aging in human tissues is characterized by the increased expression of S100A8 and S100A9 proteins (Swindell et al., 2013). Menon et al. (2017) have proposed that oxidative stress generated in placental tissue during gestation and other pathological conditions can induce aging senescence changes

in amniochorionic cells that could be involved in the onset of parturition(Menon, Mesiano, & Taylor, 2017). Senescence cells are characterized by the production of senescence-associated secretory proteins and the release of damage-associated molecular patterns (DAMPs) (Muñoz-Espín & Serrano, 2014). These molecules can reach gestational tissues via exosomes (30–100 nm spherical microvesicles) participating in the molecular mechanisms of parturition.

The UC contains mesenchymal stem cells, which are highly resistant to apoptosis and frequently undergo senesce induced by oxidative stress (Turinetto, Vitale, & Giachino, 2016). We found significant enrichment of DE genes in senescence, oxidative stress pathways and a gene set of mesenchymal stem cells from UC. We also found that the fold changes of those genes coding for S100A8, S100A12, and S100A9 proteins were at least of 32.7, 15,2 and 14.1, respectively. Calgranulins (S100A8, S100A9, S100A12) are calcium-binding proteins with multifunctional that belong to S100 protein family. They are constitutively expressed in neutrophils and during acute inflammation in other cells with immune properties, such as epithelial cells. Calgranulins have an essential role during fetal implantation, pregnancy, and labor and they related to adverse pregnancy outcomes such as preeclampsia (high maternal S100A8/S100A9 blood concentrations) and IAI (Kostakis, Cholidou, Kallianidis, Perrea, & Antsaklis, 2010).

Calgranulins have been associated with inflammation in diverse diseases, such as sepsis, inflammatory diseases, cancer and atherosclerosis(Averill, Kerkhoff, & Bornfeldt, 2012; Gebhardt, Németh, Angel, & Hess, 2006; S. Wang et al., 2018). High level of AF S100A8 and S100A12 proteins are associated with IAI, funisitis and early-onset sepsis(C. S. Buhimschi et al., 2007). They are potent chemotactic for neutrophils, and they can activate TLR4/RAGE signaling pathways and downstream effector molecules. Therefore, calgranulins can amplify an initial inflammatory response by increasing the recruitment and activation of leukocytes and the production of inflammatory mediators (inflammatory feedback loop). The release of a high amount of calgranulins by inflamed tissues can result in distant organ damage due to their leakage into systemic circulation (Ehrchen, Sunderkötter, Foell, Vogl, & Roth, 2009; Foell, Wittkowski, Vogl, & Roth, 2007; Goyette & Geczy, 2011).

To explore the molecular pathogenesis of perinatal brain injury, we defined a surrogate of severe FIR, and we compared the gene expression profile of ELGANs with harsh FIR with those not affected with the three conditions. Among DE upregulated genes we found those encoding for calgranulins, PTX3, and CXCL1 proteins. These proteins can induce neutrophil activation and recruitment, endothelial alterations and trigger neuroinflammation. CXCL1/CXCR2 signaling is essential to neural cell development and neutrophil recruitment(Yellowhair et al., 2018). PTX3 is an acute phase protein

that facilitates bacterial engulfment and regulates vascular inflammation. Central nervous disorders increase PTX3 expression in brain and blood(Rajkovic, Denes, Allan, & Pinteaux, 2016). Thus, our findings suggest that neutrophils jointly with T cell activation (Th17 cells) and high levels of circulatory inflammatory mediators can contribute to perinatal brain injury associated with FIR.

Overall, our findings suggest that UC can mount a robust inflammatory response mediated by microbial detection through PRRs, which can be amplified by the release of alarmins, such as calgranulins, and senescence-like changes induced in UC mesenchymal cells. We speculate that high level of UC calgranulins may participate in UC pathological changes, such as vasculitis(Viemann et al., 2005) and atherosclerosis(Rafferty et al., 2017), and subacute necrotizing funisitis (Ohyama et al., 2002). The leakage of inflammatory mediators and calgranulins into fetal circulation, either directly or packaged in UC inflammatory exosomes, may also damage distant fetal organs, such as the brain.

4.2. Molecular profiling of neonatal dried blood spots reveals changes in innate and adaptive immunity following fetal inflammatory response

Our investigation provides new insights into the relationship between FIR and ISSI in ELGANs, and into the contribution of both to

perinatal brain injury and developmental disorders in ELGANs(Korzeniewski et al., 2014; Yanni et al., 2017). Leviton et al. (2011) found an association between funisitis and high blood concentrations of inflammatory mediators at postnatal day 7 and postulated the existence of unknown molecular mechanisms that sustain systemic inflammation in FIR-affected ELGANs. Investigators of the ELGAN study have demonstrated that ELGANs are at increased risk of ISSI during the first postnatal month, which predisposes to perinatal brain injury and developmental disorders. Among the diverse prenatal and postnatal conditions associated with ISSI, investigators of the ELGAN study could not detect unique inflammatory signatures(Olaf Dammann et al., 2016). In this study, we have used archived DBS to conduct gene and protein expression molecular profiling of FIR in the first postnatal week in ELGANs.

DBS constitute a practical alternative for molecular investigations due to the limitation of blood sampling in preterm newborns. DBS are stored for long periods of time in biobanks and allow retrospective cohort molecular studies of diseases. Blood sampling can impact significantly the neonatal circulating blood volume, which is estimated to be around 85-105 mL/kg in neonates(Ward et al., 2017). WHO guidelines recommend for clinical research in children a maximum allowable total blood volume collection of 1-5% of total blood volume in one single blood draw(Heidmets et al., 2011; Howie, 2011; Veal, 2014). ELGANs can weight between 0.5-1.5 kg, and therefore, a maximum of 1-2 mL can be obtained for

research purposes. Strategies to overcome these limitations in preterm newborns are thus using residual blood from clinical studies, remaining DBS and UC blood(Ward et al., 2017).

DBS are a source of genomic DNA and RNA molecules, which could be preserved during longs period in archived DBS. In 1987 for the first time, good quality and quantity of DNA were extracted from DBS for electrophoresis and hybridization(McCabe, Huang, Seltzer, & Law, 1987). Since then, DNA obtained from DBS (around 60 nanograms of gDNA per spot; Hannelius et al., 2005; Sjöholm, Dillner, & Carlson, 2007) have been used for genotyping studies(Hardin et al., 2009; Hollegaard, Thorsen, Norgaard-Pedersen, & Hougaard, 2009), whole genome microarray analysis(Hardin et al., 2009), whole-genome and exome-targeted next-generation sequencing(Hollegaard et al., 2013), and methylome profiling(Ghantous et al., 2014).

The suitability of RNA extracted from DBS for genomic studies has been demonstrated later compared to DNA studies, due to the lability of RNA molecules in serum because of enzymatic degradation (ribonucleases and micro-RNAs). Matsubara et al. (1992) extracted for the first time good quality RNA from archived DBS stored at room temperature for one year(Matsubara, Ikeda, Endo, & Narisawa, 1992; McCabe et al., 1987). Later, long-term RNA stability studies in archived DBS obtained good quality RNA after long years of storage, up to 20 years(Gauffin, Nordgren, Barbany, Gustafsson, &

Karlsson, 2009). The main factor of RNA preservation in archived DBS seems to be mostly due to storage conditions (frozen vs. unfrozen) than to the length of the storage period(Grauholm et al., 2015; Wei et al., 2014).

To date, although, DBS enable the storage of peripheral blood for long periods of time, allowing for the molecular characterization of different cellular states, few published studies have used RNA from DBS for genome-wide expression studies in human disease(Haak et al., 2009). Ho et al. (2013) reported a set of blood DE genes in newborns, who later suffered cerebral palsy, from residual archived unfrozen DBS using a DNA gene expression microarray(Ho et al., 2013). Recently, Bybjerg-Grauholm et al. (2017) obtained good quality RNA for RNA-Seq analysis from neonatal archived DBS. The authors could classify the samples according to the gender using gene expression values of sex-specific genes(Bybjerg-Grauholm et al., 2017).

In this thesis, we performed an integrated transcriptomic and proteomic approach for molecular profiling of whole peripheral blood from archived DBS of the neonatal screening program. The DE analysis of transcriptomic and proteomic profiles identified 783 DE genes and 27 DE proteins, respectively, at 5% FDR with a minimum 50%-fold change between FIR-affected vs. unaffected ELGANs. The magnitude of the differences we observed in gene and protein expression between FIR-affected vs. unaffected ELGANs is similar

to those found in sepsis transcriptomics and proteomics investigations. The number of DE genes across the published neonatal sepsis studies varies from 292 to 2,573 (Hilgendorff et al., 2017; Wynn et al., 2015). Similarly to neonatal sepsis, upregulated DE genes represent the dominant group of expression change in FIR-affected ELGANs (487 upregulated vs. 295 downregulated DE genes).

Previously to our work, Wynn et al. (2016) investigated the differential transcriptomic responses in whole peripheral blood obtained within first 24 hours between uninfected preterm neonates with and without HCO(Weitkamp et al., 2016) The analysis of total RNA gene expression found a total of 488 DE genes ($p < 0.001$; 331 upregulated and 157 downregulated) between uninfected neonates with and without HCO. The authors found among most DE genes, those related to the inflammatory response, such as *OLFM4, C5AR1,* and *S100A12*. However, the authors did not report a differential expression of significant pro-inflammatory cytokine genes, such as *IL6, IL1b,* and *IL18*. In this study, the investigators did not differ between FIR-affected vs. unaffected newborns, and they included newborns with a wide range of GA in their analysis.

The transcriptional analysis of FIR affected vs. unaffected ELGANs uncovers significant differences in gene expression levels of key immune genes during the first postnatal week. We found the upregulation of NLCR4 and IL-18 gene expression in whole

peripheral blood from FIR-affected ELGANs. Supporting these findings, we found a significant overlap of DE genes of NLCR4-infantile-onset Macrophage Activation Syndrome (MAS) transcriptional profile. NLRC4-inflammasome hyperactivity causes macrophage MAS and enterocolitis, which are characterized by extraordinary serum IL-18 elevation. NLRC4 belongs to the family of NOD-like receptors (NLRs), which are PRRs localized in the cytoplasm that detect microbial invasion and trigger innate immune responses(Kanneganti, Lamkanfi, & Núñez, 2007).

The NLRP1, NLRP3, NLRC4 are NLRs that oligomerize to form multiprotein inflammasome complexes, which induce the cleavage and activation of caspase-1 that leads to the cellular cytoplasmic processing and secretion of IL-1b and IL-18. The functionality of NLRs in newborns is partially known, and the role of NLRC4-inflammasome in the neonatal immune system remains to be fully investigated. NRLs are one of the first functional PRRs in preterm newborns (Kan et al. 2016). Concretely, the expression and functionality of cytosolic NOD1 and NOD2 belonging to the NLCR family in monocytes of very preterm newborns is well established at birth(Granland et al., 2014). The inflammasome NLRP3/caspase-1 activation during fetal life is impaired, but it maturates to adult levels within two weeks in extremely preterm newborns(Sharma et al., 2015). Thus, our results suggest that the activation of NLRC4 inflammasome and the IL-1b/IL-18 axis can play the main role in FIR-ISSI.

Among top upregulated DE genes in FIR-affected vs. unaffected ELGANs we also found *S100A8, S100A9, S100A12, LTF, MPO, OLFM4, MMP8, RETN,* and *CD177*. These genes are involved in neutrophil function and their expression is also elevated in neonatal sepsis (Cernada et al., 2014; Hilgendorff et al., 2017; Wynn et al., 2015). *MMP8* and *OLFM4* blood gene expression levels, which are highly expressed in neutrophils, also correlate with decreased survival and increased organ failure in pediatric septic shock(Alder, Opoka, Lahni, Hildeman, & Wong, 2017; Solan et al., 2012). The gene expression of these genes in UC, except for the MMP8 gene, was also upregulated in FIR affected ELGANs. In contrast, only genes coding for RETN (FC 9.5), MMP8(FC 2.3), fS100A12(FC 3), S100A9 (FC 2.5), S100A8 (FC 1.7) were upregulated in UC leukocytes from preterm newborns with FIR. Thus, the encoded proteins of these genes may act as immune modulators of innate immune responses at birth and in the first postnatal week.

The functional enrichment analysis of DE genes in FIR yielded the enrichment of pathways related to the innate immune system among upregulated genes, while among downregulated genes we found genes associated with the adaptive immune system. The downregulation of genes associated with the adaptive immune system is further supported by the lower number of the copies of T-cell receptor excision circles in DBS from FIR-affected ELGANs. Our findings resemble those reported in the functional enrichment

analysis in neonatal sepsis (Cernada et al., 2014; Smith et al., 2014). However, these results are different from those we observed associated with FIR in UC, which suggest the activation of the adaptive immune system in FIR-affected ELGANs. One possible explanation the previous observation may be that FIR induces initially an adaptive immune response at birth that is posteriorly suppressed to avoid adverse effects of their activation.

The neonatal adaptive immune system is active at birth in preterm newborns(Luciano, Yu, Jackson, Wolfe, & Bernstein, 2011) and there is evidence of the association fetal T cell activation with perinatal brain injury(Duggan et al., 1999; Leviton, Dammann, & Durum, 2005). We found in FIR-affected ELGANs the overexpression of the *OLR1* gene, which is a marker of granulocytic-myeloid-derived suppressor cells (G-MDSCs), and a significant overlap (one-tailed Fisher's exact $P < 2.2e\text{-}16$, OR = 26.4) between FIR upregulated genes and a G-MDSC expression signature(Condamine et al., 2016). He et al. (2018) reported that the highest neonatal concentration of MDSCs was during the first three days of life, although their inhibitory action on the adaptive immune system was observed only after day 4 of life(He et al., 2018). Thus, it is possible that during the first days FIR can mount an effective adaptive immune response that posteriorly becomes suppressed by the expansion and action of G-MDSCs.

Our results further suggest an essential role of calgranulins and

LTF in FIR-ISSI. LTF is an iron-binding glycoprotein with multiple relevant biological properties including immunoregulatory, anti-inflammatory, antioxidant and tissue repair activities(Kruzel, Zimecki, & Actor, 2017). Genes encoding these proteins are among the most upregulated ones induced by FIR in UC at birth and in whole peripheral blood in the first postnatal week. Interestingly, the *S100A8*, *S100A9*, and *S100A12* genes were among those most highly upregulated in NLRC4-MAS(Canna et al., 2014). Proteomic analysis of DBS also revealed the differential expression of S100A8, S100A9 and LTF proteins in FIR-affected ELGANs.

On the one hand, the release of calgranulins can induce the synthesis of new pro-inflammatory (e.g., IL1-b, IL-18) cytokines, which in turn, trigger the production of more calgranulins in neutrophils and monocytes leading to an autoinflammatory loop(Kessel, Holzinger, & Foell, 2013). Inhibition of the adaptive immune system and other inflammatory cells can also promote the inflammatory response. On the other hand, the action of anti-inflammatory mediators, such as LTF and G-MDSCs (see Figure 22), could counterbalance the activation and reinforcement of the inflammatory cascade.

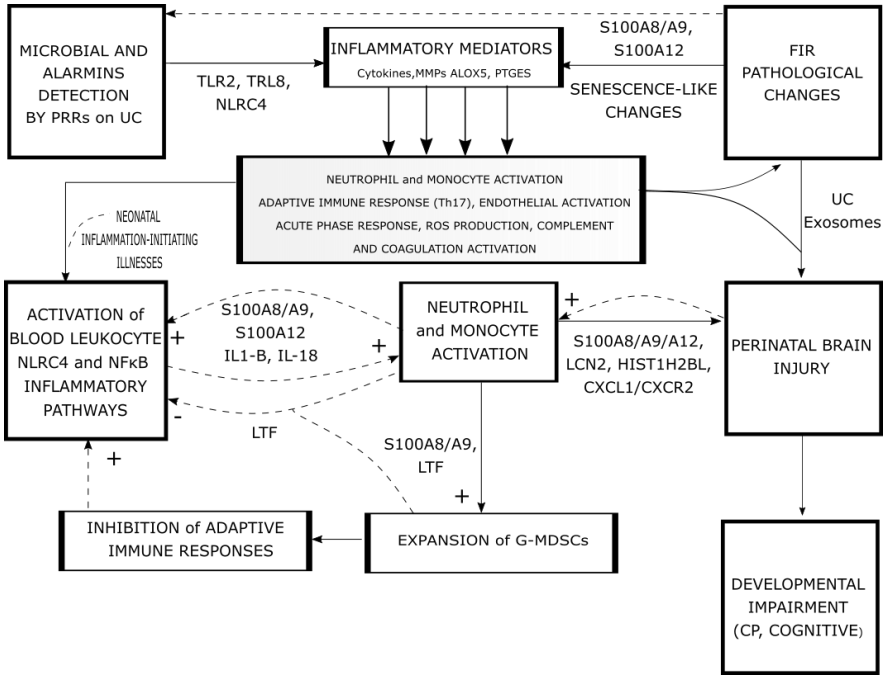

Figure 22. Model for the association between FIR, ISSI and perinatal brain injury. Umbilical cord (UC) triggers the production of multiple inflammatory mediators after the recognition of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) by pattern recognition receptors (PRRs), this leading to FIR. Locally FIR can induce UC senescence-like changes including the production of calgranulins and senescence-associated proteins, which can be released in inflammatory exosomes reinforcing FIR and reaching the fetal brain. Postnatally, FIR and neonatal inflammation-initiating illnesses can create a positive loop for neutrophil and monocyte recruitment and activation mediated by the overactivation of NLRC4 inflammasome/IL1b/IL-18 axis and calgranulin release. This inflammatory loop can be counterbalanced by the action of lactotransferrin (LTF) and granulocytic myeloid-derived suppressor cells (G-MDSCs), induced by S100A8/A9/LTF axis. The great expansion of G-MDSCs can inhibit neonatal adaptive immune responses, which can enhance the inflammatory process. The blood monocytic neutrophil-associated inflammation-boosting loop can cause perinatal brain injury mediated by inflammatory mediators and cells.

The underlying molecular mechanisms of perinatal brain injury are only partially known, and there are few biomarkers and effective therapeutic options for perinatal brain injury(Leviton et al., 2015). The elevation of blood cytokines and inflammatory cells in the fetal circulation in response to IAI can alter and cross the blood-brain barrier and lead to neuroinflammation without microbial invasion of the brain (Olaf Dammann & Michael O'Shea, 2008; O. Dammann & Leviton, 2000; Malaeb & Dammann, 2009; Molloy et al., 2007; Strunk et al., 2014). Microglial activation, a hallmark of neuroinflammation, is characterized by the production of an array of inflammatory mediators that can damage preterm brain cells, particularly immature oligodendrocytes(Hagberg, Mallard, & Sävman, 2016). Damage in oligodendrocytes can cause white matter injury, a common manifestation of perinatal brain injury in preterm newborns(Back, 2017).

The so-called two-hit model of brain damage postulates that an initial insult can sensitize the brain to a next insult, that will cause more brain damage than without the initial. There is substantial evidence of the association between brain inflammatory sensitization and neonatal encephalopathy(Fleiss et al., 2015). Leviton et al. (2012) found that ELGANs have a higher frequency of neurodevelopmental disorders if they had a postnatal systemic inflammation(Leviton et al., 2012). Preterm newborns with placental inflammation and subsequent postnatal inflammation had an increased risk of white matter disease (*OR* 3.6; 95%CI 1.5–8.3; Korzeniewski et al., 2014).

Yanni et al. (2017) also found that placental inflammation and a subsequent postnatal inflammation increased the risk of white matter damage on cranial ultrasound, cerebral palsy, microcephaly and neurodevelopmental disorders in ELGANs(Yanni et al., 2017).

Our results suggest that the persistent systemic activation of neutrophils and monocytes by the IL-1b/IL-18 axis and calgranulins can lead to organ injury, particularly of perinatal brain injury in ELGANs. We observed upregulated in FIR-affected ELGANs neutrophil-related genes coding for proteins associated with brain damage, such as LCN2(Song & Kim, 2018), CXCL1, CXCR2(Yellowhair et al., 2018b), MPO, calgranulins, and histones (e.g., HIST1H2BL; Gilthorpe et al., 2013). The S100A8, S100A9, myeloperoxidase and histones proteins were also differentially expressed in DBS. Extracellular histones can act as damage-associated molecular patterns, such as calgranulins, activating inflammatory signaling pathways and inducing many organ-specific damages(Silk, Zhao, Weng, & Ma, 2017). Excessive myeloperoxidase production is associated with oxidative stress by the production of ROS, which can cause brain damage. The expression of S100A8 and S100A9 increases in the brain of human patients who die of sepsis. In an animal model of sepsis, S100A9 expression was required for cerebral recruitment of neutrophils and microglia activation(Denstaedt et al., 2018).

Overall, our investigations of gene expression profiling in UC and peripheral blood from archived DBS in FIR-affected ELGANs have shown that FIR is a complex inflammatory disorder. FIR is characterized by well-known pathological changes in placenta and UC and by the elevation of inflammatory mediators in UC blood at birth and during the first postnatal week. We demonstrated that UC mounts a robust inflammatory molecular signature, which may play an essential role in the pathophysiology of FIR and perinatal brain damage. Further, the results of transcriptomic and proteomic analysis of archived DBS confirm the association between FIR and ISSI, and uncover inflammatory mechanisms associated with ISSI, which may contribute to FIR-related diseases, particularly of perinatal brain damage. We propose that calgranulins could be important molecular mediators of the FIR at birth and during the perinatal period.

Future directions

Future directions in PB will focus on identifying biomarkers and therapeutics target for prematurity-related diseases. Many efforts have been destined to investigate the molecular and biochemical mechanisms of perinatal brain injury and finding new blood biomarkers and neuroprotective therapies for its evaluation and treatment. Currently, hypothermia is an accepted neurotherapeutic in term newborns, and there is evidence that antenatal maternal administration of magnesium sulfate and delayed UC sampling can protect the preterm newborns from perinatal brain injury. However, new therapeutics for the treatment of perinatal brain injury is required to improve the neurological outcome of preterm newborns, particularly to those born very early. New neuroprotective approaches under study are based on targeting systemic inflammation and neuroinflammation. In this context, the investigation of the molecular pathophysiology of FIR and ISSI can provide essential clues to the underlying causes of perinatal brain injury.

The early prenatal and postnatal diagnosis and treatment of the newborn with suspected FIR and ISSI are essential to prevent severe and life-threatening outcomes. Translational research in FIR and ISSI can result in advances in the comprehension of its pathophysiology and offer new promising biomarkers and therapeutics in perinatal medicine. Many key open questions remain in the study of FIR and

ISSI that are worthy of future investigation. On the one hand, the identification in AF and neonatal peripheral blood of FIR and ISSI biomarkers and of molecular mechanisms that promote brain injury in preterm newborns with FIR and ISSI. On the other hand, some open questions remain with respect the role of mesenchymal cells in UC senescence-like changes and the release of fetal inflammatory exosomes, as well as, the individual role of the different cell types in UC, UC and peripheral blood leukocytes, and other fluid-exposed organs, such as the lungs in FIR and ISSI.

New advances in high-throughput technologies in molecular biology offer an important new avenue for the investigation on the molecular basis of FIR and ISSI in preterm newborns. Advances in NSG-based technologies for genomics, transcriptomics, and epigenomics allow the characterization of single cells(Hwang, Lee, & Bang, 2018). Single-cell RNA sequencing could uncover the transcriptional state of epithelial, endothelial and mesenchymal cells from UC, and of UC and peripheral leukocytes in FIR. New exosome technology based on high-throughput mass spectrometry-based proteomic analyses could investigate the role of UC inflammatory exosomes in FIR(Choi, Kim, Kim, & Gho, 2014). The advances in human induced pluripotent stem cell technology have allowed the development new human model based on pluripotent stem cells that mimic in vivo tissues and organs(C. Liu, Oikonomopoulos, Sayed, & Wu, 2018), which can be implemented for investigating FIR associated diseases.

4. CONCLUSIONS

The objective of this thesis was to investigate the transcriptome and proteome of FIR in ELGANs to provide new insights into its molecular pathophysiology and discover candidate biomarkers and therapeutic targets of FIR and FIR-related diseases. The main contributions of our investigations are:

- FIR associates a complex and broad UC gene expression signature in ELGANs.

- Changes in UC gene expression associated with FIR involve up to 19% (3,896/20,155) of all human genes at 1% FDR. Functional analysis of these changes revealed the overexpression of numerous innate and adaptive immune response genes and the underexpression of genes associated with neurodevelopmental pathways in UC of ELGANs. These findings suggest a principal role of UC in FIR pathophysiology.

- FIR is associated with peripheral blood gene and protein expression signatures involving 783 and 27 DE genes and proteins, respectively, at 5% FDR in archived DBS from ELGANs in the first postnatal week.

- Gene and protein expression changes confirm the persistent and robust activation of the innate immune system and suggest the inhibition of the adaptive immune system in FIR-affected ELGANs.

- FIR associated expression changes in DBS point to NLRC4/IL1-b/IL-18 and calgranulin driven autoinflammatory loop enhanced by the adaptive immune inhibition and counterbalanced by LTF and G-MDSCs actions. These molecular alterations can participate in perinatal brain injury.

6. ANNEX

6.1 Supplemental Tables

Table 1 (next page)**. Comparison of neonatal versus adult in vitro and in vivo immune function**. Table adapted from (Brook et al. 2017).

Immune cell type	Observation	Neonate vs. Adult	Biological context	Tissue
T cells	Absolute count	+	Steady state (in vivo)	Umbilical and peripheral blood
	Functional effector response	~	TLR9 agonist in vitro stimulation	
Naïve T cells	Relative proportion	+	Steady state (in vivo)	Umbilical and peripheral blood
	cytokines production (IL7, IL15)	+		
Treg cells	Absolute counts	+	Steady state (in vivo)	Umbilical and peripheral blood
B cells	Antibody responses	~	Vaccines response (in vivo)	Serum
Neutrophils	Absolute counts	–	Infections (in vivo)	Peripheral blood
	Absolute counts	+	Steady state (in vivo)	Peripheral blood
	Phagocytosis	+/~/–	Various in vitro stimulations	Purified neutrophils
	Chemotaxis	Reduced	In vitro FMLP induced chemotaxis	Umbilical and peripheral blood
	Antimicrobial peptides and proteins production	Reduced	E. coli in vitro stimulation	Umbilical and peripheral blood
	Rective oxgyen species production	~/–	In vitro stimulation with group B, Strep., zymosan/ S.	Purified neutrophils

			aureus, E. coli	
	NETosis	~/–	In vitro stimulation with β-glucan, or C. albicans/LPS	Purified neutrophils
	Bacterial killing	~	In vitro stimulation with *S. aureus or E. coli*	Purified neutrophils
	Transmigration	–	In vitro IL-1 stimulation	Purified neutrophils
Monocytes/macrophages	Phagocytosis	~/–	In vitro; pre opsonized E. coli/Candida albicans, E coli in vitro stimulation	Purified, monocytes/monocyte-derived, macrophages (MDM)
	Killing	Reduced	In vitro (group B *Streptococcus)*	MDM
	Chemotaxis	~/–	In vitro (zymosan-A simulation)	Monocytes purified from cord blood/newborn postnatal peripheral blood
	T cells antigen presentation	–	In vitro (tetanus toxoid,M. tuberculosis)	Purified monocytes
	Anti-inflammatory cytokine production (IL-10)	+	In vitro	Peripheral blood
	TNF-α production	–	Toll-like receptor or heat killed Listeria, E. coli in vitro stimulation	Purified monocytes
	Pro-inflammatory cytokine production (IL-6. IL-23, IL-1β)	–	TLR in vitro stimulation except TLR8	Peripheral blood

Dendritic cells	Phagocytosis	+	In vitro uptake of fluorescent B cells	Lymphoblastoid B cell line
	Cytokines and co-stimulatory molecules expression	–	In vitro (TLR ligands)	Peripheral blood
Natural killer (NK) and NKT cells	Absolute counts	+	Steady state (in vivo)	Peripheral blood
	Cytotoxic response	–	In vitro	Peripheral blood
	Granzyme B and perforin expression	~/+	Ex vivo	Peripheral blood

Table 2 (next page)**. Principal cytokines in immune responses.** Table adapted from (Zhang and An 2007).

Cytokines	Principal Source	Primary Activity
GM-CSF	T helper (Th) cells	Growth and differentiation of monocytes and dendritic cells
IL-1α	Macrophages and other antigen	Costimulation of APCs and T cells, inflammation and fever, acute phase
IL-β	presenting cells (APCs)	response, hematopoiesis
IL-2	Activated Th1 cells, NK cells	Proliferation of B cells and activated T cells, NK functions
IL-3	Activated T cells	Growth of hematopoietic progenitor cells
IL-4	Activated T cells	B cell proliferation, eosinophil and mast cell growth and function, IgE and, class II MHC, expression on B cells, inhibition of monokine production
IL-5	Th2 and mast cells	Eosinophil growth and function
IL-6	Activated Th2 cells, APCs, other somatic cells	Acute phase response, B cell proliferation, thrombopoiesis, synergistic with IL-1 and TNF on T cells
IL-7	Thymic and marrow stromal cells	T and B lymphopoiesis
IL-8	macrophages, somatic cells	Chemoattractant for neutrophils and T cells
IL-9	T cells	Hematopoietic and thymopoietic effects

IL-10	Activated Th2 cells, CD8+ T and B cells, macrophages	Inhibits cytokine production, promotes B cell proliferation and antibody production, suppresses cellular immunity, mast cell growth
IL-11	Atromal cells	Synergistic hematopoietic and thrombopoietic effects
IL-12	B cells, macrophages	Proliferation of NK cells, IFN production, promotes cell-mediated immune functions
IL-13	Th2 cells	IL-4-like activities
IL-18	Macrophages	potent inducer of interferon by T cells and NK cells. Neutrophil activation and promotes differentiation of myeloid-derived supperssor cells
IFN-α	Macrophages, neutrophils and	Antiviral effects, induction of class I MHC on all somatic cells, activation of
IFN-β	some somatic cells	NK cells and macrophages
IFN-γ	Activated Th1 and NK cells	Induces of class I MHC on all somatic cells, induces class II MHC on APCs and somatic cells, activates macrophages, neutrophils, NK cells, promotes cell-mediated immunity, antiviral effects
MIP-1α	Macrophages	Chemotaxis
MIP-1β	Lymphocytes	Chemotaxis
TGF-β	T cells, monocytes	Chemotaxis, IL-1 synthesis, IgA synthesis, inhibit proliferation
TNF-α	macrophages, mast cells, NK, cells, sensory neurons	Cell death, inflammation, pain
TNF-β	Th1 and Tc cells	phagocytosis, NO production, cell death

Table 3 (next page)**. Neonatal outcomes of clinical chorioamnionits, histological chorioamnionitis and fetal inflammatory response.**

Authors	Study Design	Population	Phenotype	Adverse neonatal outcome	Assocation (95% CI)
Been et al. 2013	Systemic review and meta-analysis	< 37 wks GA	CCA	NEC	OR 1.24 (1.01 – 1.52)
		< 37 wks GA	HCO		OR 1.39 (0.95 – 2.04)
		< 37 wks GA	FIR		OR 3.29 (1.87 – 5.78)
Been et al. 2010	Prospective cohort	< 32 wks GA	FIR+surfactant treatment	BPD	OR 3.40 (1.02 – 11.3)
Hartling et al. 2012	Systemic review and meta-analysis	VLBW infants	CHO, CCA	BDP	OR 1.89 (1.56 – 2.3)
Dessardo et al. 2014	Prospective cohort	< 32 wks GA	FIR	BDP	OR 31.05 (10.7-87.7)
Park et al. 2015	Systemic review and meta-analysis	< 37 wks GA	CCA	PDA	OR 1.28 (1.00 – 1.79)
		< 37 wks GA	HCO		OR 1.54 (1.10 – 2.15)
Shatrov et al. 2010	Meta-analysis	All GA, most <34 weeks	CCA	CP	OR 2.42 (1.52 – 3.84)
			HCO		OR 1.83 (1.17 – 2.89)
Wu and Colford et al. 2000	Meta-analysis	All GA, mostly VLBW infants or <32 wks GA	CCA	CP	RR 1.9 (1.4 – 2.5)
				PVL	RR 3.0 (2.2 – 4.0)
			HCO	CP	RR 1.6 (0.9 – 2.7)
				PVL	RR 2.1 (1.5 – 2.9)
Levtion et al. (1999)	Multicenter prospect	VLBW infants	FIR	WMD	OR 10.8(1.03 – 114)

	ive cohort				
Yoon et al. (2000)	Prospective cohort	<35 wks GA	FIR	CP	OR 6.6 (1.4-31.8)
Rovira et al. (2011)	Prospective cohort	VLBW or <32 wks GA,	FIR	IND	OR 4.07 (1.10-15.09)
Mitra et al. 2014	Systemic review and meta-analysis		CHO, CCA	ROP	RR 1.33 (1.14 – 1.55)
Soraisham et al. 2009	Multicenter prospective cohort	<33 wks GA	CCA	EOS	OR 5.54 (2.87 – 10.69)
				Severe IVH	OR 1.62 (1.17 – 2.24)
García-Muñoz Rodrigo et al. 2014	Multicenter prospective cohort	VLBW <32 wks GA,	CCA	EOS	OR 3.10 (2.30 – 4.17)
				LOS	OR 0.84 (0.72 – 0.98)
				NEC	OR 1.30 (1.02 – 1.65)
Caissutti et al. 2017	Meta-analysis	< 37 wks GA	CHO; CCA	small thymus	OR 16.0 (4.18-61.4)
Cobo et al. 2013	Prospective cohort	< 37 wks GA	FIR	EOS	OR 13.(2.2–81.6)
Yoon et al. (2000)	Prospective cohort	<35 wks GA	FIR	EOS	OR 7.2 (1.8-29.0)

Abbreviations: CCA - clinical chorioamnionitis; HCO – histological chorioamnionitis; FIR – fetal inflammatory response; EOS – early onset sepsis; LOS - late onset sepsis; RDS - respiratory distress syndrome; BPD - bronchopulmonary dysplasia; GA - gestational age; PDA - patent ductus arteriosus; ROP - retinopathy of prematurity; NEC - necrotizing enterocolitis; IVH – intraventricular hemorrhage; CP– cerebral palsy; IND- impaired neurodevelopment.

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