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THE ROLE OF PROTEOMICS IN THE DIAGNOSIS OF CHORIOAMNIONITIS AND EARLY-ONSET NEONATAL SEPSIS

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SYNOPSIS

Intra-uterine infection is viewed as a unique pathological process which raises considerably the risk for early onset neonatal sepsis (EONS). By acting synergistically with prematurity, EONS increases the risk for adverse neonatal outcomes including intraventricular hemorrhage (IVH) and cerebral palsy which are often encountered as sequelae of sepsis. Although several distinct pathways for the pathogenesis of fetal damage have been proposed, the basic molecular mechanisms that modulate these events remain incompletely understood. Therefore, discovery of clinically and biologically relevant biomarkers able to reveal key pathogenic pathways and predict pregnancies at risk for antenatal fetal damage is a priority. Proteomics provides a unique opportunity to fill this gap. In the short run proteomic biomarkers may aid with medical decision-making including timing of delivery and steroid administration. In the long run proteomic biomarkers may lead to development of targeted therapies against pathogenic variants of EONS. Herein, we aimed to illustrate the richness of the topic and set the stage for the argument that discovery of novel proteomic biomarkers is a critical step in improving outcomes and preventing long-term disability.

Keywords

newborn; sepsis; biomarkers; proteomics; DAMPs; RAGE

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CONFLICT OF INTEREST STATEMENT Dr. Irina and Catalin Buhimschi are co-inventors on patent applications regarding the use of proteomics analysis of amniotic fluid, cord blood and urine to determine the risk of intra-amniotic inflammation, neonatal sepsis and preeclampsia, respectively. The authors have not served as consultants or received any honoraria from any third party as related to the information included in this article.

1. PROTEOMICS

1.1. General principles

Proteomics is a newly developed field of research which has emerged to complement genomics. Although several definitions are available, most scientists agree that proteomics studies provide insight into the functional expression of proteins present in a tissue, cell or organism at a given moment. Moreover, there is a general perception that data derived from proteomics experimentation are more clinically relevant and easier to translate into diagnostic tools and therapies than genomics results which rely on DNA or mRNA studies.

Like other “omics” approaches, proteomics is viewed as a non-reductionist approach to identification of protein biomarkers. In truth, proteomics should be viewed as complementary to traditional approaches with potential to greatly enhance their reach. In hypothesis-driven research the view that one or a group of specific proteins could be diagnostic markers is heavily dependent on prior knowledge which links a precise protein or factor to a specific disease process. Proteomics, however, enables a reverse pathway. Proteomics allows an unbiased perspective by discovering that a group of named intact proteins and/or unnamed proteins fragments are characteristic to a diseased but not to a non-diseased state of the same organism. Such relationships are non-intuitive and draw a picture of the dysregulated biological phenomena that otherwise could not have been predicted through the classical reductionist approach.

Several important lessons have been learned from the human genome project: there are only about 30,000 genes in the human genome which encode for over half a million proteins; 99.9% of humans are identical at gene level; one of the most notable characteristics of the human genome is the startling amount of non-coding DNA it possesses; only 1 to 1.5% of the human genome is coding DNA, devoted to genes encoding proteins [1]. Based on these observations the major advantage of proteomics over DNA-RNA based technologies is that proteomics investigates directly functional molecules and not the source code. Evidence in support of this assertion is that protein abundance and activity do not correlate with mRNA amount [2]. This suggests that post-transcriptional regulation of gene expression which cannot be predicted by linear genetics is a frequent phenomenon in higher level organisms. The recent discovery of small silencing RNAs provides another explanation to the divergence between information coded into the RNA versus proteins. Thus, in the context of this biological complexity one should not be surprised that the results of proteomics versus genomics experimentation could be divergent yet complementary [3].

Proteomics involves a diversity of techniques first aimed to separate and then to identify the proteins of interest. However, practical aspects of proteomics experimentation can be hugely complex. Proteins are heterogeneous and complex entities. They vary widely in concentration, molecular weight, isoelectric point, and hydrophobicity. They differ from individual to individual and change dynamically over time while interacting with each other, with DNA or RNA, metal ions and hormones. Protein fragmentation, association and aggregation can create non-standard endogenous ligands with biological activity that is divergent from that of the precursor encoded by the original messenger RNA [4]. To place everything into perspective, if there is one genome per individual, it is estimated that the number of possible human proteomes is in the thousands [5]. {AUTHOR QUERY: Sentence beginning “To place everything in perspective...” has been revised by editors. Please verify that change preserves original intent.} These proteomes further change dynamically with food and drugs and over time.

In contrast to genomics which relies mostly on standardized and automated techniques, proteomics requires experimental fine-tuning which has to be optimized depending on tested

hypotheses and goals. It is important to recognize that the more complex a sample the more challenging it will be to reveal the differentially expressed proteins, especially if these proteins are less abundant. The nature of the human body fluid proteome, with its large dynamic range of protein concentrations, presents problems with both detection and with quantitation. Seeing or not seeing a polypeptide from the realm of signals will depend on the copy number, quantity of sample loaded and “white noise” of the method of detection [6]. In human plasma, the ratio of albumin to signaling molecules is in the order of 10^{12} . For instance, albumin and glucagon have a 9 order of magnitude difference. A significant experimental proteomic challenge is that regular silver stained two dimensional gel electrophoresis can only display 4 orders of magnitude in plasma proteins. In this context, most proteomic techniques display just the tip of the iceberg. This challenge becomes especially important when studying disease processes given that not all biological compartments are expected to be equally affected.

Faced with such challenges, our group has advocated the idea of “hypothesis-driven proteomics”. We proposed that 3 experimental design choices should be made *a priori* of proteomics experimentation. These are: **1)** choice of the disease, **2)** choice of the biological sample; **3)** choice of proteomics technique/data analysis. All three choices are critical and necessary to maximize the likelihood that the biomarkers extracted at the end of the *exploratory phase* have the required biological and clinical relevance to allow them to pass the *challenge phase* against the clinically implemented “gold standard” (Fig. 1). The principles of experimental design required for successful completion of proteomic research in reproductive sciences have been recently reviewed [7,8].

1.2. Proteomics techniques

Protein separation from complex protein mixtures is possible through a number of high-throughput technologies. These experimental techniques make use of intrinsic properties of proteins: molecular weight (gel electrophoresis and mass spectrometry), isoelectric point (isoelectric focusing, ion exchange chromatography), hydrophobicity (reverse-phase or hydrophobic interaction chromatography), or unusual affinities for metals and specific antibodies (affinity chromatography). It is generally recognized that there is a trade-off between using multiple complementary techniques successively, thus being able to visualize a larger part of the proteome, and the excessive manipulation of the biological sample with potential of introducing experimental artifacts. Therefore, emphasis has been placed on proteomics platforms that simultaneously combine at least two of the previously enumerated separation modalities. Some of these are 2-Dimensional PolyAcrylamide Gel Electrophoresis (2D-PAGE), Surface Enhanced Laser Desorption Ionization Time-of-Flight (SELDI-TOF) and Multidimensional Protein Identification Technology (MudPIT). Use of different labels applied in complex biological mixtures at the proteome level raises the sensitivity and accuracy for identification of differentially expressed targets. Two such technologies are 2D-differential gel electrophoresis (2D-DIGE, developed on a 2D-PAGE platform) and Isotope Coded Affinity Tag (ICAT, developed on a mass spectrometry platform).

In essence, two rather opposing approaches to proteomics discovery are available. At this time most proteomics platforms enable just one. One approach relies on generating proteomic patterns from biological samples using high-throughput mass spectrometry platforms. This approach minimizes the need to know the identity of the discriminatory biomarkers (proteomics pattern-centered approach) [9]. The second proteomic strategy focuses on protein identification by digesting them into peptides. This process is followed by sequencing using tandem mass spectrometry and database searching (proteomics identification-centered approach) [10]. As both have advantages and limitations, understanding each method in the context of the disease of interest, of the biological sample available for analysis and of the expectations is important and the essence of our third choice (of proteomics technique/data analysis). For instance in

pattern-centered proteomics additional bioinformatic tools and experimentation are needed to determine identity of the extracted protein biomarkers. Although this information is not needed for correct classification of cases for diagnostics purpose, the identity of dysregulated proteins may provide powerful insight into novel therapeutical targets. Conversely, identification-centered proteomics approaches are at best semi-quantitative and recently, extensive emphasis has been placed on algorithms such as Multiple Reaction Monitoring (MRM) to improve quantification in high resolution mass spectrometry approaches [11]. More important, however, is the limitation resulting from the data output. Most often this represents a list of protein identities found increased or decreased over an arbitrary cut-off. As these identities are converged into unique identifiers, they are ultimately matched to genes indexed in databases. Thus this approach annuls the advantage of proteomics in providing an accurate snapshot of the proteome. In point, it would entirely miss biomarkers derived through proteolytic cleavage of a precursor since both the fragment and the precursor would be converged into the same unique identifier.

1.3. Principles of proteomics approaches in our laboratory

Our laboratory employed a combined pattern-centered and identification-centered approach placing SELDI-TOF at the front-end as choice of technique for the proteomics *exploratory phase*. SELDI-TOF is mostly a pattern-centered proteomics technique which combines chromatography with mass spectrometry. This offers technical advantages such as ease and speed of screening, ability to use very small amounts of crude biological fluids and to rapidly screen large numbers of biological samples. By using this approach we aimed to minimize the biological noise and errors in clinical classification due to imperfect diagnostic gold standards or arbitrary cut-offs. The separation ability of SELDI-TOF is enhanced by the use of the various active surfaces placed on aluminum-based ProteinChip® arrays (Bio-Rad, Hercules, Calif.). The active surfaces contain chemicals that allow the capture of subsets of proteins while repelling others. The bound proteins are laser desorbed and ionized for mass spectroscopy analysis. The differential mass spectral patterns reflect the protein expression bound on the chip surface and allow the comparison between various samples [12,13,14]. At the end of the *exploratory phase*, a bioinformatic algorithm (Mass Restricted scoring) is applied and a combination of protein biomarkers that fulfill all set criteria are extracted from the SELDI-TOF tracings [7,8,15,16,17]. Proteins of interest are next identified using peptide mass fingerprinting or tandem mass spectrometry in conjunction with the PCI 1000 ProteinChip® Interface (Bio-Rad).

When successful, the *exploratory phase* was followed by a proteomics *challenge phase* on a different set and larger number of biological specimens from consecutively enrolled patients with varying grades of disease severities and confounding morbidities [16,18]. The purpose of the *challenge phase* was to validate the initial biomarker combination and to provide a robust diagnostic tool with potential to improve classification of cases in the current state of clinical practice. This tool is provided in the form of a *proteomics score* based on relative abundance of select biomarkers as identified by their mass on SELDI-TOF tracings. In parallel, we pursued the *translational phase* which begins with strategies aimed to identify the biomarkers composing the proteomics score. As deemed necessary for each project, we used other complementary proteomics approaches (2D-PAGE, 2D-DIGE or SELDI-immunocapture). Our choice of proteomics platform for identification was based on the type of biological sample and the abundance of the biomarkers of interest at the initial SELDI-TOF screen [15,16,17]. Once identification was unambiguously established, a wide range of mechanistic experiments were undertaken to determine the biological relevance of the newly identified markers. A schematic representation of the proteomics approach in our laboratory is illustrated in Fig. 1 [7,8].

As “choice of disease” our group has mainly focused on entities responsible for preterm birth and neonatal morbidity such as spontaneous preterm birth, intrauterine infection and inflammation as well as preeclampsia. These conditions are especially difficult to tackle with respect to development of new diagnostics due to patient heterogeneity, difficulty in obtaining serial biological samples from the fetus and imperfect “gold standards” for presence and/or absence of disease. By using the approach detailed above, we were able to extract several proteomic profiles with both biological and clinical significance. Two of the profiles have been prospectively validated and because of their high accuracy in delineating specific subgroups of preterm birth are currently used in our laboratory as research “gold standards”. The two prospectively validated profiles are the amniotic fluid proteomic fingerprint, the **Mass Restricted (MR) score** [18,19,20,21,22] and the urine proteomic profile (**UPS score**) of preeclampsia [16]. In addition to the UPS profile, we reported another SELDI-TOF profile composed of 4 peaks in cerebrospinal fluid which carried the ability to identify sub-clinical intracranial bleeding in women with severe preeclampsia (**PPB profile**) [23]. Its analogous presence in amniotic fluid was indicative of decidual hemorrhage related to abruption [24]. The presence of the same biomarkers in different biological fluids related to different diseases speaks for the compartmentalization of biomarkers in each disease process and to the importance of choosing a relevant biological sample for proteomics analysis. By using 2D-DIGE in conjunction with SELDI-TOF and a hierarchical bioinformatics algorithm we identified a distinct subgroup of patients at risk for preterm birth in the absence of intra-amniotic inflammation or bleeding (**Q-profile**) [25]. We thus provided first hand evidence of an alternative pathway leading to prematurity for which no other marker existed. The differentially expressed proteins associated with the Q-profile were determined to be involved in non-inflammatory processes such as protein metabolism, signal transduction and transport [25]. Lastly, we recently provided the first comprehensive mapping of human cord blood and identified potential markers which can be used to characterize at birth presence and susceptibility to EONS [17]. This work is still ongoing.

2. Proteomics biomarkers for improved diagnosis of intrauterine infection, inflammation and early onset neonatal sepsis (EONS)

2.1. Clinical relevance of intrauterine infection, inflammation and EONS

Neonatal sepsis is a global public health challenge and a significant contributor to morbidity and death. Both early- and late-onset sepsis occur with increased frequency in neonates born prematurely [26,27,28]. Because the mortality rate of untreated sepsis can be as high as 50%, most clinicians believe that the hazard of untreated sepsis is too great to wait for confirmation based on positive culture results. Therefore, treatment is commonly begun in the absence of documented infection. In data published by the Yale neonatal intensive care unit, which holds the longest running, single-center database of neonatal sepsis started in 1928, mortality attributable to sepsis remains at 11% [29]. This mortality rate is unchanged in recent years despite advances in neonatal care. It is extremely important to make a prompt diagnosis of sepsis, because earlier initiation of antimicrobial therapy improves outcomes.

Intrauterine infection and subsequent inflammation are important risk factors for EONS and poor neonatal outcome [30,31]. During the last two decades it has become clear that intrauterine infection has a strong association with preterm birth [32,33,34]. The problem of quantifying the causal role of infection in determinism of preterm birth and EONS results from the lack of a proper “gold standard” for infection and from the heterogeneous nature of intra-amniotic inflammation. For the last decade it became increasingly evident that the process of intra-amniotic inflammation is not necessarily linked to an exogenous microbial attack. For instance, abruption and decidual hemorrhage fuel into the same downstream inflammatory and oxidative

pathways as intra-amniotic infection through release of thrombin, heme and free iron and globin-centered free radicals [35].

There is a priority in identifying clinically relevant biomarkers that can predict pregnancies complicated by intra-amniotic infection which are at a high risk for fetal damage. Proteomics provides a unique opportunity given that in the short run such markers may aid with medical decision making including timing of delivery and steroid administration. Conversely, ruling out intra-amniotic inflammation or a vulnerable fetus may allow for safe expectant management and unnecessary treatment.

2.2. Role of proteomics in diagnosis of intra-amniotic infection and inflammation

It has become increasingly recognized that in most cases intrauterine infection in pregnancy is not clinically apparent. [18,36]. Thus, clinical chorioamnionitis is an insensitive measure of the presence of inflammation, infection or fetal sepsis [37,38]. Direct analysis of the amniotic fluid with the use of amniocentesis remains the most accurate modality to diagnose intra-uterine infection and to determine the extent of intra-amniotic inflammation [39]. One of the problems in establishing clinical utility of amniocentesis in preterm birth rests with the lack of sensitive and specific diagnostic “gold standards” for infection and inflammation. In addition, the turn-around time when test results become available to clinicians is too long for clinical decision-making. For example, the median time when amniotic fluid culture results are available is 7 days [range: 2–24 days] after the amniocentesis procedure [18]. Meanwhile, decision of either expectant management or indicated delivery has already occurred as guided by clinical manifestations and results of rapid tests such as amniotic fluid Gram stain, white blood cell (WBC) count, glucose concentration and lactate dehydrogenase (LDH) activity. In one of our studies, the results of this battery were concordant in excluding intra-amniotic inflammation in the setting of preterm birth in only 50% of cases and in confirming inflammation in 14% which often is not sufficient to remove uncertainty required for medical decision making [18]. Although portrayed by some as a simple, rapid and accurate test of infection the results of the amniotic fluid WBC count alone could be misleading especially in clinical scenarios such as contamination of the amniotic fluid with blood or bacterial-induced lysis of leukocytes [18].

Historical data using amniotic fluid cultures detects presence of intra-amniotic infection in 10% of patients with preterm labor and intact membranes and in 38% of patients with PPROM [40,41]. However, molecular biology techniques which are more sensitive than cultures detect bacteria in up to 60% of pregnancies complicated with preterm labor [42]. A variety of microbial pathogens have been implicated as etiologic agents of intra-amniotic infection [29, 43,44]. The most frequently found isolates are thought to originate primarily from the genital flora such as *Gardnerella vaginalis*, *Mycoplasma hominis*, *Ureaplasma*, *Peptostreptococcus* and *Bacteroides* spp. [44]. Regrettably, this assumption is significantly biased by the limited number of laboratory techniques for pathogen cultivation which normally target for identification only a handful of microbes [45]. Thus, “uncultivated” or “difficult-to-cultivate” bacteria cannot be found when relying on culture conditions alone [21,46,47]. In contrast, culture-independent methods, such as PCR, can detect bacterial DNA in up to 35% to 60% of pregnancies complicated by preterm birth [42,48]. Yet, their use alone as diagnostics cannot discriminate between *in vivo* infection and *ex vivo* contamination and thus may result in unnecessary early deliveries. Therefore, there is a real need for better tools to diagnose intra-amniotic infection and inflammation.

In recent years proteomics has been extensively used by us and others to search for biologically relevant biomarkers and to generate protein profiles characteristic of intra-amniotic inflammation and preterm birth [15,49,50]. As choice of sample for proteomics experimentation, amniotic fluid offers clear advantages over maternal blood or plasma. Given

that almost 99% of neutrophils extravasated in amniotic fluid are of fetal origin, evaluation of this compartment reflects much closer the nuances of the fetal inflammatory response to infection [51]. Other biological samples such as maternal urine and cervico-vaginal secretions are not significantly modified in the proteome over biological noise despite fetal sepsis (Buhimschi IA, unpublished observation). Several groups have investigated the cervico-vaginal proteome in search for biomarkers predictive of preterm birth [52,53,54]. Although the preliminary results are encouraging, a **challenge phase** to confirm the clinical utility for the proposed identities has not yet been published. Their superiority over the best performing cervico-vaginal biomarker to date (fetal fibronectin) in predicting the risk for preterm birth remains to be tested in a prospective blinded fashion.

After applying the algorithm of Mass Restricted (MR) scoring to SELDI-TOF tracings obtained from amniotic fluid of women with intra-amniotic infection and inflammation versus women of similar gestational age but normal pregnancy outcomes, we extracted a combination of 4 biomarkers that were necessary and sufficient for 100% correct classification of cases selected for the **exploratory phase** [7,8,15]. The MR score denotes the number of markers identifiable over the technical noise of the SELDI-TOF instrument. The MR score ranges from 0 (all biomarkers absent) to 4 (all biomarkers present). In the original study, an MR score of 3 or 4 (denoted MR 3–4) indicated the presence of inflammation, while an MR score of 0 or 2 (MR 0–2) was considered to exclude it [15]. On-chip immunoassays and peptide mass fingerprinting, confirmed by Western blotting, established that the 4 biomarkers of the MR score were neutrophil defensin-2 (3.3 kDa), neutrophil defensin-1 (3.4 kDa), S100A12 (10.4 kDa) and A100A8 (10.8 kDa), all members of the innate immunity arm of antimicrobial defense [15]. The relevance of these proteins as diagnostic biomarkers of intra-amniotic inflammation was subsequently confirmed independently by other group of investigators [49,50,55].

To establish the MR score's clinical value, it was critical to demonstrate that the MR score retains its diagnostic efficacy when tested in other populations at high risk for preterm birth and that it relates to relevant outcomes. In one series, we tested the ability of the MR score to predict the clinical success of rescue cerclage in women with cervical incompetence and demonstrated that an MR 3–4 in amniotic fluid at the time of surgery was highly predictive of cerclage failure [22]. We next sought to determine in a prospective and blinded fashion in a cohort of 169 consecutive women, pregnant with singletons, whether the MR score is reproducible and comparable to previously established or proposed markers of intra-amniotic inflammation or infection [18]. This approach was consistent with the **challenge phase** as described in Fig. 1. The accuracy of an MR 3–4 was the highest (> 90%) in detecting intra-amniotic inflammation followed by LDH activity. We further used stepwise logistic regression analysis to identify which clinical test or combination of tests optimally predicts inflammation and found the MR score performed better than any test or combination of tests (odds ratio: 76, $P < 0.0001$). Importantly, we observed a sequential appearance of the biomarkers as the process of intra-amniotic inflammation developed from acute to chronic, with the peaks corresponding to S100A12 and S100A8 appearing last. This finding enabled us to stratify the study population based on progression and severity of inflammation (MR 0: absent; MR 1–2: mild; MR 3–4 severe) (Fig. 2) [18]. This classification was unique and would not have been apparent without the prospective study design of the **challenge phase**. As it will be shown in the following sections we believe that such performance relates to the ability of the MR score to uniquely predict clinically relevant intra-amniotic inflammation, histological chorioamnionitis, funisitis and EONS.

We next asked the question of why the MR score was superior to all other clinical analyses of amniotic fluid in its ability to identify intra-amniotic inflammation. Using 16S rRNA sequencing and phylogenetic analysis, in collaboration with Dr. Yiping Han (Case Western Reserve University) we demonstrated that all specimens with MR score 3–4 but negative

cultures showed presence of bacterial DNA [21]. Furthermore, most samples of amniotic fluid with MR 3–4 and positive cultures contained DNA of additional bacterial species compared to those found by cultures. In fact, 60% of species detected by culture-independent methods were missed by general laboratory cultures. The key finding of this study was that the missed prokaryotes belonged to the class of uncultivated and difficult-to-cultivate species, such as *Fusobacterium nucleatum*, *Leptotrichia/Sneathia*, *Bergeyella*, *Peptostreptococcus*, *Ureaplasma parvum*, *Bacteroides* and *Clostridiales* spp. These results suggest that the prevalence of amniotic fluid infection and diversity of etiological agents linked to preterm birth is underestimated [21]. Moreover, our and other's data brings into perspective that the fetus may encounter pathogenic bacteria more often than previously thought [56].

2.3. The role of proteomics in diagnosis of histological chorioamnionitis and funisitis

The proximity of the placenta to the fetus, its common embryological origin and genotype and the availability of this reproductive tissue for research have all contributed to the significant number of studies relating various placental lesions to short and long-term neonatal outcomes including cerebral palsy [57]. The major disadvantage of placental pathological examination is that histological biomarkers are irrelevant for antenatal therapeutic choices aimed to prevent preterm birth or adverse neonatal outcome. However, valuable studies have associated short and long-term follow-up characteristics to distinct placental lesions [57,58,59,60].

Since proteomics is a young science it is intuitive that conclusions regarding the clinical utility of proteomic biomarkers in predicting the long-term outcome of the neonates is not yet possible. Until such data become available we took a step by step approach using as intermediate outcome variables results of placental examination as performed by a perinatal pathologist blinded to the MR score. We determined that the presence and severity of acute inflammation in the chorionic plate, amnion, chorio-decidua and umbilical cord (funisitis) were significantly associated with the occurrence and the degree of intra-amniotic inflammation as determined by the MR score [18,19,20]. The MR score also correlated significantly with the stages of chorioamnionitis ($p < 0.001$) and funisitis ($p < 0.001$) independent of the interval to delivery ($p = 0.160$ for MR score versus amniocentesis-to-delivery interval). We further found among the biomarkers of the MR score, the appearance of at least one of the peaks corresponding to the S100 proteins (S100A12 or S100A8) in amniotic fluid was highly indicative of biologically relevant funisitis (at least grade 2) and/or chorioamnionitis (Fig. 3). As a result we became interested to explore mechanistically the implication of these proteins for EONS and for processes that could possibly lead to antenatal fetal damage in the setting of intra-amniotic inflammation.

2.3. The role of proteomics in diagnosis of early onset sepsis (EONS)

The disconnect between EONS as a clinical entity and the responsible microorganisms may be even greater than for intra-amniotic infection. As a result, many newborns receive empiric antibiotic therapy based on nonspecific symptoms of suspected EONS and/or maternal risk factors (such as prematurity, intrapartum fever, chorioamnionitis, prolonged rupture of membranes) [61]. Several explanations are available to justify the well-recognized inability of neonatal blood culture to correctly diagnose EONS and why newborns are treated with empiric antibiotics even when unnecessary. The frequency of bloodstream infections fluctuates widely from 8% to 73% in the diagnosis of “suspected” EONS [19,62]. Moreover, microbiology laboratories only search for a narrow spectrum of pathogens. For example, culturing for *Ureaplasma* and *Mycoplasma* spp. is not part of the routine sepsis work-up in neonates. A study that evaluated the frequency of umbilical cord blood infections with these species found that 23% of newborns born at < 32 weeks tested positive for these pathogens [56]. It is also plausible that analogous to intra-amniotic inflammation, the fetal and newborn insult may be induced by additional uncultivated and difficult-to-cultivate species. Data supporting this

premise has shown that 16S rRNA PCR technology improves the accuracy of culture-based methods for diagnosis of neonatal sepsis [63].

In the context of an unreliable “gold standard” for EONS we turned our attention to clinical assessments and neonatal hematological indices at ≤ 72 hours after birth as surrogates for early sepsis [27,64,65]. In collaboration with Dr. Vineet Bhandari (Yale University) we analyzed all newborns admitted to Yale Newborn Special Care Unit included in our MR score **challenge phase** cohort (n=104) whose mothers presented with signs and symptoms of preterm birth and had an amniocentesis to rule out infection. Confirmed EONS was defined as the presence of a positive blood or any other body fluid microbial culture result. Suspected EONS was noted in the presence of clinical signs of sepsis with support from laboratory hematological results [27]. We found that neonates of women with MR 3–4 were more often lymphopenic and had significantly higher absolute band count and proportion of immature-to-total neutrophils (I:T ratio) ($P < 0.001$) compared to the other groups. Neonates of women with MR 3–4 (severe inflammation) had an increased incidence of EONS (odds ratio: 4.4, $P = 0.007$) compared to neonates from mothers with MR 0 or MR 1–2. These results remained significant after adjusting for gestational age at birth. In logistic regression, the MR 3–4 was significantly associated with EONS while both the results of amniotic fluid culture and WBC count were excluded from the equation. Of all component biomarkers of the MR score, the presence of the S100A8 had the strongest association with EONS. The association of S100A12 with chorioamnionitis and funisitis and of S100A8 with EONS suggests that these proteins may reflect a chronic process with significant relevance for antenatal fetal damage in the setting of intrauterine severe inflammation. Moreover, our data may provide first hand evidence that, similar to intra-amniotic inflammation, EONS may be a heterogeneous pathogenic entity [66]. For instance, at least some of the newborns that have nonspecific clinical manifestations of EONS (lethargy, apnea, respiratory distress, hypoperfusion and shock) may not have had true fetal infection. It is plausible to propose that EONS could be a consequence of a pathological process initiated *in utero* through chronic exposure of the fetus to a noxious pro-oxidative and pro-inflammatory intrauterine environment (see Fig.5). In this context, early delivery of the fetus may be justified.

2.4. Pathogenic basis and implications of EONS heterogeneity

Antibiotics are routinely used ante- and intrapartum in women with PPROM to extend duration of pregnancy, decrease the risk of neonatal group B streptococcal (GBS) sepsis and reduce the risk of neonatal illness after delivery [67,68,69,70]. Due to the widespread use of antepartum antibiotics, the majority of EONS cases in preterm infants are no longer caused by GBS but rather by Gram-negative bacteria with *Escherichia coli* as the most frequent pathogen [29, 71]. Antibiotics, while inducing bacterial killing, do not quell an inflammatory process already underway. A study conducted by Gravett et al. demonstrated in a primate model of GBS intra-amniotic infection that antibiotics eradicated infection while uterine activity, amniotic fluid cytokines, prostaglandins, and matrix metalloproteinases remained elevated [72]. The observation that the combination of antibiotics, anti-inflammatory and anti-prostaglandin agents suppressed inflammation and significantly prolonged gestation is extremely valuable because it suggests that multiple pathways need to be suppressed simultaneously to obtain a successful therapeutic result. Our data related to fetal inflammatory syndrome and fetal adaptation to intra-amniotic inflammation suggests that the clinical manifestations of EONS could be the result of an active trafficking process, which occurs between the amniotic fluid compartment and the fetus [19,73]. It would suffice for endotoxin, other pathogen-associated molecular pattern molecules (PAMPs), lipophilic damage associated molecular pattern proteins (DAMPs) or cytokines to “spill” from amniotic fluid into the fetal circulation to cause manifestations consistent with septic shock. In this case passage of live bacteria to the fetus may not be a requirement for clinical manifestations of EONS.

2.5. The role of DAMPs and Receptor of Advanced Glycation End-Products (RAGE) in mediating fetal injury

The “danger theory” holds that injured cells release “alarm” signals which in turn activate an immune response [74]. Consistent with this premise, DAMPs (alarmins) are a pleiotropic group of intra-cellular proteins, which when released in the extra-cellular compartment become endogenous danger signals by activating membrane receptors and amplifying the damage [75]. DAMPs have been implicated as key mediators of the host's immune response. Evidence from adults and animal models have demonstrated significantly elevated systemic concentrations of inflammatory mediators (such as monocyte chemoattractant protein-1 and interleukin-6) after traumatic crush injury or ischemia [76]. The generalized endothelial damage at a distance from the affected areas suggests that DAMPs alone are able to initiate and sustain inflammation, even in the absence of infectious triggers. Involvement of molecules such as high mobility group box protein-1 [(HMGB-1), a prototype DAMP released by somatic cells subjected to injury or necrosis] and RAGE may offer a plausible explanation as to why patients with non-infectious critical illness develop a syndrome that is indistinguishable from microbial-induced sepsis [77,78,79].

RAGE is a redox-sensitive receptor of the immunoglobulin family and acts as a pattern recognition receptor for DAMPs such as HMGB1 and S100 proteins. Engagement of RAGE converts transient cellular stimulation into sustained cellular dysfunction driven by activation of nuclear factor-kappa B (NFκB) [80,81]. A DAMP-RAGE activation axis has been implicated in the progression of an acute event to a chronic inflammatory state associated with tissue destruction. An important feature of the RAGE receptor is that its expression is low in normal tissues but increases transcriptionally in environments where RAGE ligands such as DAMPs accumulate, escalating tissue damage in a spiral positive feedback fashion [82]. In genetic animal models, deletion of RAGE and pharmacological interventions that reduce DAMP levels or block RAGE-DAMP interactions suppress inflammation and dampen tissue damage [83,84,85,86,87]. Our group was the first to provide evidence that activation of a DAMP-RAGE axis occurs in pregnancies complicated by intra-amniotic infection and inflammation [88,89]. This finding was spearheaded through the discovery of the proteomic biomarkers components of the MR score [15,90]. The identity of two of the proteomic biomarkers as S100A12 and S100A8 as proven RAGE ligands [91] led us to the thought that intrauterine infection and/or inflammation induces release of DAMPs which in turn promote fetal cellular damage via RAGE activation [92]. Evidence from our research suggests this premise is relevant for newborns exposed antenatally to a noxious intrauterine environment such as that resulting from intra-amniotic inflammation [89]. As identified by the MR score at the time of amniocentesis, many of these pregnancies had histological evidence of chorioamnionitis and funisitis and the newborns developed signs or symptoms consistent with EONS. A link between these end-points was provided by recent data suggesting that damage to the barrier between the hostile intrauterine environment and the fetus may occur *in utero* as appreciated by the degree of funisitis and chorioamnionitis [19]. In this context we demonstrated that the cord blood-to-amniotic fluid IL-6 ratio (CB/AF IL-6; an indicator of the differential inflammatory response in the fetal versus the amniotic fluid compartment), correlates with the MR score in a manner dependent on the severity of histological inflammation of the chorionic plate, chorio-decidua and umbilical cord (funisitis) [19]. Our data suggests that inflammation-induced damage of the maternal-fetal interface may play a permissive role in trafficking of cytokines DAMPs and PAMPs between the amniotic fluid and cord blood. We further observed that in most EONS cases with evidence of severe intra-amniotic inflammation (88% of all EONS cases in the study), the absolute amniotic fluid IL-6 concentration was significantly higher than that measured in cord blood. This suggests that should the maternal-fetal interface become damaged, the IL-6 gradient favors spillage into the fetal compartment. Yet, in a minority of EONS newborns, the ratio was reversed. This finding

provided support for the conclusion that progression of inflammation may occur in the fetal compartment independent of the amniotic fluid space. In fetuses with a reversed CB/AF IL-6 ratio, EONS confirmed by positive blood cultures reached 50%. It is likely that in the remaining 50% of the newborns sepsis was induced by “uncultivated” bacteria *in utero*. Equally plausible is that these fetuses had a disproportionate activation of their innate immune response to PAMPs and DAMPs leaked into fetal circulation. [19]. In Fig. 4 we illustrate our proposed mechanism leading to fetal damage. Based on the existing data we consider that bacterial PAMPs continue to engage signaling receptors such as toll-like receptors (TLRs) and participate in the release of cellular endogenous danger signal molecules (i.e DAMPs) that perpetuate the inflammatory cascade leading to fetal cellular damage via RAGE activation. This process may continue even after antibiotic treatment is initiated and thus perhaps even after birth. If such fetuses are correctly identified antenatally as enabled by our MR score, there is a window of opportunity for targeted interventions. Such therapies may in the future include anti-DAMP, anti-RAGE, or anti-cytokine strategies alone or in conjunction with antibiotics as deemed necessary for each case scenario.

Data in support of EONS heterogeneity emerges from the evidence that maternal antibiotic treatment, which results in killing of maternal bacteria, may induce excess release of PAMPs. In addition, data derived from animal models of sepsis show that antibiotic-treated rats display higher plasma endotoxin levels than untreated animals despite decreased bacteremia. Moreover, different antibiotics may induce the release of different forms of endotoxin which may be lethal for sensitized animals [93]. This may explain why attempts to prevent preterm birth with antibiotic treatment in patients with bacterial vaginosis, *Trichomonas* or preterm labor either had no effect or increased the rate of preterm birth or the risk of cerebral palsy [94,69,70]. Fig. 5 illustrates our proposed model of EONS variants.

In light of these considerations we propose the following classification of EONS. Based on this model each newborn may require different theranostic (therapeutic/diagnostic) approaches:

EONS I—Vertical transmission of live bacteria to the fetus. This condition would require prompt pathogen identification and targeted antibiotic treatment.

EONS II—Translocation of bacterial footprints (i.e. endotoxin) and DAMPs from the mother and damaged placenta to the fetus. This condition would require general cardiovascular support and could be amenable to anti-inflammatory treatment and specific endotoxin-neutralizing strategies.

EONS III—Translocation of cytokines (such as IL-6) from the mother and damaged placenta to the fetus. This condition could require circulatory support and/or anti-inflammatory treatment.

EONS 0—None of the above, which in the context of current clinical care often is associated with over-treatment.

CONCLUDING REMARK

For the last three decades obstetricians, neonatologists and developmental neurobiologists have had powerful debates regarding the best way to diagnose intraamniotic infection and inflammation, and the appropriate time to deliver a fetus exposed to a hostile intrauterine environment. In the absence of either a preventative or curative therapy for intraamniotic infection/inflammation the answers to key questions such as how much of an inflammatory stress can each fetus withstand or whether there is time to wait for a complete course of steroids

when infection and/or inflammation is identified remain rhetorical. Proteomics offers a unique opportunity to provide answers to these questions through functional biomarkers which could represent targets for future therapeutic intervention.

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Figure 1.

Basic paradigm of proteomics work-flow in our laboratory.

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Figure 2.

A: Representative SELDI-TOF mass spectrometry profiles of the amniotic fluid based on the “severity” of inflammation (MR=0 “no” inflammation; MR=2 “mild” inflammation; MR=3–4 “severe” inflammation). P1–P4 represent the biomarkers of the Mass Restricted (MR) score which are neutrophil defensin-2 (**P1**: 3,377 Da) neutrophil defensin-1 (**P2**: 3,448 Da), S100A12 (**P3**: 10,443.85 Da) and S100A8 (**P4**: 10,834.51). The *x* axis of the tracings represents the molecular mass in Daltons; the *y* axis represents the relative peak intensity. **B:** Kaplan-Meier analysis illustrating the duration from amniocentesis-to-delivery in women with MR scores of 0 (zero, no inflammation), MR scores of 1–2 (mild inflammation), and MR scores of 3–4 (severe inflammation). Reproduced with permission from: published in PLoS Med. 2007 Jan 16; 4(1):e18. Reproduction by authors permitted under Creative Commons Attribution License

Figure 3.

Three dimensional representation of the relationship between histological markers of placental inflammation on the *x* axis (**A**: stages of chorioamnionitis, **B**: grades of choriodecidualitis, **C**: grades of amnionitis), number of analyzed cases on the *y* axis and the proteomics MR score on the *z* axis. With permission from: Obstet Gynecol 2008;111(2 Pt1):403–12. KWH permission to republish #2373861175982

Figure 4.

A: Working model for the potential roles of PAMPs (Pathogen-Associated Molecular Patterns), DAMPs (Damage-Associated Molecular Patterns, alarmins) and RAGE (Receptor of Advanced Glycation End-Products) in fueling cellular damage to the fetus exposed to acute and chronic intrauterine infection, inflammation and oxidative stress. **B:** The underlying change in the amniotic fluid MR score from an MR 0–2 to an MR 3–4 is in 93% of cases due to presence of biomarker peaks P3 and/or P4 representing the DAMPs S100A12 and S100A8. Pathogenically this would correspond to the transition of intra-amniotic inflammation from an acute perhaps reversible process to a chronic process characterized by cellular damage via RAGE activation. Reproduced with permission from: Curr Opin Infect Dis. 2009;22(3):235–43. KWH permission to republish #2373870081864

Figure 5.

Schematic representation of pathogenic variants of EONS. **EONS 0** (lack thereof), **EONS I** (passage of a live bacterial inoculum to the fetus either from the intra-amniotic compartment or via hematogenous dissemination), **EONS II** (passage of endotoxin, other bacterial products or DAMPs through a damaged maternal-fetal interface represented by the interrupted grey line) and **EONS III** (spillage of inflammatory cytokines only through the damaged interface). Ability to differentiate among these variants is important for future therapeutic purpose.