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Proteomics/diagnosis of chorioamnionitis and of relationships with the fetal exposome

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Summary

Proteomics, a relatively young science, originally emerged as a complement to genomics research. By definition, the goal of proteomics is to provide a snapshot of all the proteins within an organism, tissue or biological sample at a given moment. Proteomics has the ability to single out one or more proteins (biomarkers) that change consistently in affected subjects as compared to those disease-free. From a proteomics perspective, chorioamnionitis poses both challenges and opportunities. Challenges relate to the dynamic course of the inflammatory process, and compartmentalization of the gestational sac in relation to the maternal compartment. An inability to evaluate the amniotic fluid non-invasively and repeatedly for meaningful changes in its proteome, and lack of a true gold standard for diagnosis of inflammation and/or infection, represent additional challenges. On the other hand, the unbiased and holistic nature of proteomics offers a real opportunity to improve the current diagnostic and prognostic algorithms for chorioamnionitis. Even at this current stage there are reasons to believe that proteomic biomarkers will improve the understanding of how chorioamnionitis programs or affects the fetus in utero, thus defining its exposome (sum of interactions between genetic makeup of the fetus and the intrauterine environment) of pregnancies affected by infection and/or inflammation. This review summarizes the results of proteomics studies that have aimed or reached these goals.

Keywords

Funisitis; Haptoglobin; Haptoglobin-related protein; Intra-amniotic infection; Neonatal sepsis; Proteomics

[A]Chorioamnionitis as choice of proteomics-targeted disease

By theoretical definition, proteomics provides a snapshot of all the proteins within an organism, tissue or biological sample at a given moment. In practice, however, the ability to visualize a smaller or larger part of the true proteome is highly dependent on the study design and on the three 'choices' required prior to experimentation: (i) disease; (ii)

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Conflict of interest statement

Drs Irina Buhimschi and Catalin Buhimschi are listed as inventors or co-inventors on patent applications embodying the use of proteomics biomarkers for complications of pregnancy. Neither of the authors has financial relationships with third parties related to the work described in this article.

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biological sample; and (iii) proteomics technique. The combination of these three choices will ultimately drive the chances of success in finding sensitive and specific biomarkers with biological and/or clinical relevance that will not fail during an external validation phase.^{1,2}

With respect to the choice of disease one needs to pay attention to the several interpretations to the term 'chorioamnionitis' which was originally coined to depict 'fever during labor'. Today, a clear distinction should be made between clinical and histological chorioamnionitis.³ Due to significant changes in maternal immunity and compartmentalization of intra-amniotic inflammation, only a minor fraction of women with proven histological inflammatory changes of the placenta exhibit symptoms of infection. As such, clinical chorioamnionitis occurs in 0.5–1% of all pregnancies; rates in preterm gestations are higher.⁴ With the inclusion of amniocentesis as part of the clinical management of preterm labor, it became apparent that intra-amniotic infection is a frequent encounter.⁵ In addition, although chorioamnionitis most often results from intra-amniotic infection, not all intrauterine inflammatory processes are caused by microbes and vice-versa.^{6,7} It is critical to recognize that culture techniques identify only a fraction of micro-organisms implicated as etiological agents of chorioamnionitis.⁸ Therefore, nuances in medical terminology with respect to clinical versus histological chorioamnionitis are important when choosing the 'gold standard' for either infection or inflammation. This is key given that any newly discovered biomarkers must be validated against the 'gold standard'. This article will use the term chorioamnionitis to refer to processes associated with inflammation and infection of the amniotic fluid, fetal membranes, placenta and/or fetus.

Despite its frequently encountered silent course, chorioamnionitis is a pathological process linked closely to preterm birth, early onset neonatal sepsis (EONS) and poor neurodevelopmental outcomes of both term and premature neonates.^{9–16} This paradigm is supported by the evidence that intra-amniotic inflammation superimposed on prematurity leads to devastating consequences for the neonate compared to prematurity alone.^{17,18} Exposure of the fetus to intra-amniotic inflammation increases the risk for antenatal fetal injury which postnatally results in adverse outcomes including intraventricular hemorrhage (IVH) and cerebral palsy.¹¹ Nevertheless, the risk of cerebral palsy is significantly heightened by chorioamnionitis at term gestation.¹⁹

Similar to preterm birth, fetal injury can result from a wide variety of etiologies.^{20–22} Thus, in neonatology, there is a critical need for biomarkers that can discriminate between damage inflicted by antenatal exposure of the fetus to infection/inflammation or to hypoxia and ischemia. This is clinically important because each condition requires distinct postnatal interventions to prevent long term disability. Equally important is the discovery of biomarkers able to identify fetuses in pregnancies complicated by chorioamnionitis where pregnancy prolongation outweighs the risk of prematurity and vice versa. A relevant question is how can such biomarkers significantly improve the obstetrical practice with respect to clinical decision-making? The current proposal is that with the use of accurate biomarkers, clinicians can either postpone (i.e. progesterone, tocolytics, cerclage) or accelerate (i.e. induction of labor, cesarean section) delivery while optimizing the time of antenatal corticosteroid administration relative to that of delivery.

[A]Choice of proteomics tools. General principles and approaches

Proteomics tools target protein separation and/or identification of proteins in biological samples coupled to computational algorithms that allow the extraction of relevant information from the realm of data.²¹ In its early stages, proteomics relied on high resolution, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) to separate,

identify and quantitate individual proteins present in a complex sample.²² The first gel dimension allows separation according to protein charge using isoelectric focusing; the second dimension allows separation by protein size. After separation, the proteins are visualized using gel-staining procedures with dyes or fluorescent tracers. The development of 2D difference gel electrophoresis (DIGE)²³ has significantly improved the accuracy and dynamic range of gel-based proteomics. DIGE allows multiple samples to be co-separated and visualized on one 2D-PAGE gel by labeling with different fluorescent dyes.

Recent advancements in technology have made mass spectrometry (MS) an indispensable proteomics tool.²⁴ Essentially, mass spectrometers consist of three parts: an ionization source, a mass analyzer, and an ion detector.²⁵ The ionization source converts molecules into gas-phase ions, which are then separated by the mass analyzer and transferred to the ion detector. The mass analyzer uses physical properties such as electric or magnetic field, or time of flight (TOF) to separate ions by their m/z ratios. The development of electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), as 'soft ionization' techniques suitable for proteins and peptides, has revolutionized the practical application of MS for proteomics in particular and for biology in general.²⁶ Tandem-MS instruments such as triple quadrupole, ion-trap and the most recent advanced quadrupole-time-of-flight (Q-TOF) were introduced to allow protein identification by sequence database searching. The high accuracy of the Q-TOF technology makes the combination of MALDI-Q-TOF configurations the best for de-novo protein sequencing.

While accurate technology is essential for novel protein discovery, greater automation is equally important for the rapid and accurate diagnosis of human diseases. Such goals have led to the development of surface-enhanced-laser desorption/ionization (SELDI). When used in conjunction with protein chip arrays, SELDI²⁷ allows for the isolation and identification of peptides and proteins with specific properties in complex biological samples. Protein chip array assays using SELDI-TOF-MS technology provide a valuable research tool due to the multidimensional nature of protein separation, which can be optimized for complex mixtures of proteins. By varying the chip surfaces, washing conditions, incubation times, laser intensities and energy-absorbing molecules, an almost infinite number of experimental conditions can be designed for the optimal separation of the protein(s) from all the others.

Continuous development in MS platforms and of techniques related to quantitation of resolved ions has led to the development of shot-gun proteomics. In the MudPIT approach, proteins are subjected to enzymatic digestion generating an exhaustive list of protein identities. Multiple-reaction monitoring (MRM) has recently emerged as a promising antibody-independent tool to determine how much of each individual protein is contained in the original sample.²⁸

The variety of available techniques has led to two opposing views as to what end-result is important. Diagnostic-pattern proteomics uses high throughput MS approaches to generate proteomic profiles while minimizing the importance of biomarker identity. The advantages of this approach are the ability to derive the final biomarker combination from large numbers of cases, the minimal manipulation of biological sample, and the lack of bias with respect to identities. The diagnosis is thus intrinsic in the pattern and not in protein nomenclature. The disadvantages of diagnostic-pattern proteomics are that it often requires customized bioinformatics approaches for data analysis and that by itself it offers no further clues as to why the particular pattern is present or not. The contrasting method uses identification-centered proteomics, which focuses on providing the most comprehensive list of protein identities differentially expressed in the respective biological samples using an arbitrary cut-off. The advantage is that it offers a compelling indication of the identities of the proteins differentially expressed among groups. There are several disadvantages of

identification-centered proteomics. First, this method generally involves a more extensive sample manipulation, making it more difficult to establish quantitative relationships. Second, protein identities are derived from algorithms, which match peptide sequences in databases and therefore the identity depends heavily on the quality of the match. Third, biomarkers are generally fragments of proteins and the resulting database match to a protein precursor may not have any relationship with its actual biological role performed by the different fragments in vivo. Fourth, the lists of protein identities are large and need to be filtered down using a biological significance criterion, which may not be the degree of increase or decrease of the signal. However, with all the aforementioned limitations, if correctly designed, proteomic experimentation can provide invaluable tools and insight into diagnostic modalities and pathogenetic pathways for preterm birth that could not have been envisioned by any other methodology.

[A]Choice of biological sample. Relevance vs availability

Irrespective of employed technologies or approach, the design of proteomic experimentation continues to be the most important limiting factor in obtaining conclusions with biological and clinical significance. Using the basic principle that ‘no matter how powerful a microscope, one cannot find something that is not there’, the choice of cases and of the biological sample where biomarkers are first sought are critical steps to ensure that the final combination of biomarkers is indeed representative of the disease process and not a confounding event. Furthermore, only when the biomarkers perform adequately in a population different than that used for their development, can one claim their clinical utility. In our opinion, a relevant biological sample is one that changes as a consequence of the disease process under investigation and not as a consequence of unrelated pathologies. In terms of availability, the ideal biological sample would be one that is easily obtained non-invasively and repeatedly (i.e. saliva, urine).^{2,29}

In the quest of discovering novel diagnostic and prognostic markers for chorioamnionitis, we and others have explored a wide palette of biological samples. Most represented are amniotic fluid samples obtained through transabdominal amniocentesis,^{30–33} amniotic fluid from the vagina in cases of preterm premature rupture of membranes (PPROM),³⁴ cervico-vaginal secretions,^{35,36} or maternal serum.³⁷ Most recently we focused our attention on cord blood.³⁸ As a matter of relevance, amniotic fluid and cord blood offer clear advantages over samples of maternal origin. 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.³⁹ Although the preliminary results with cervico-vaginal secretions are encouraging, a proper external validation to confirm the clinical utility for the proposed identities has not yet been published. Other biological samples such as maternal urine have not been useful for proteomic analysis (I.A. Buhimschi, unpublished observation).

[A]Importance of the proper validation of potential proteomic biomarkers

A correctly designed validation phase is always required before newly discovered proteins are advocated as biomarkers. In our studies we included patients prospectively based on the ‘intent to diagnose’ and thus ‘tested’ the potential markers to a mix of symptomatic and asymptomatic patients with varying stages of chorioamnionitis and/or co-morbidities including maternal inflammatory conditions unrelated to chorioamnionitis (i.e. pyelonephritis, appendicitis, pneumonia, viral syndrome, diabetes, bacterial vaginosis, etc.). Such design is necessary to establish marker specificity as these co-morbidities are often encountered in the real-life setting where an improvement in the diagnosis of chorioamnionitis is needed most. Moreover, any new biomarker has to perform better or

improve the performance of the currently available diagnostic scheme (e.g. clinical assessment and fetal fibronectin in the case of cervico-vaginal secretions, clinical assessment and rapid tests of intra-amniotic inflammation/infection in the case of amniotic fluid, clinical assessment and neonatal hematological indices in the case of cord blood).

To date, only two studies have reported chorioamnionitis-related biomarkers that were discovered using proteomics methodology and have been validated in the above fashion.^{31,38} The following sections will expand on the biological relevance of these markers and their potential clinical utility.

[A]Amniotic fluid mass-restricted score. Relationships with intra-amniotic inflammation, true infection and histological chorioamnionitis

About 12 years ago, our laboratory embarked on discovery of biomarkers for prediction of preterm birth using proteomics on the premise that alterations at protein levels may relate closer to the disease process than alterations in genes or mRNA. Our original choice of using SELDI-TOF as a pattern-centered proteomics approach was based on the reasoning that minimal sample handling, high throughput and speed of analysis were more important in a clinical setting than mass accuracy or protein identification, especially for analysis of less complex fluids such as amniotic fluid, cerebrospinal fluid or urine.^{40,41} Figure 1 illustrates the experimental design that led to the discovery of the proteomics mass-restricted (MR) score followed by three levels of validation conducted by our group.^{29,31} Additional levels of validation have resulted from confirmatory studies conducted by other laboratories.^{42–44}

Our approach was to use basic principles of Boolean logic and translate them into a set of rules aimed at complexity reduction and applicable to proteomics data. This set of rules represents the MR method. When applied to amniotic fluid SELDI-TOF tracings from women with and without intra-amniotic infection and inflammation (Fig. 1, discovery phase), the MR method extracted four SELDI peaks (and the proteins they represent), which were then used to devise the MR score. The score ranged from 0 to 4 depending on the presence or absence of each of these four biomarkers. In the original study, an MR score of 3 or 4 indicated the presence of inflammation, whereas scores of 0, 1 or 2 were considered to exclude it. From the standpoint of diagnosis, determining the presence or absence of these four biomarkers was sufficient. However, elucidation of the pathophysiology of intra-amniotic inflammation required identification of the corresponding proteins. On-chip immunoassay and peptide mass fingerprinting confirmed by western blotting established that the four protein biomarkers were: neutrophil defensin-2 (3.3 kDa), neutrophil defensin-1 (3.4 kDa), calgranulin C (10.4 kDa, also known as S100A12 protein) and calgranulin A (10.8 kDa, also known as S100A8), all members of innate immunity arm of antimicrobial defenses.

In a different population, we tested whether the MR score relates to pregnancy outcome, pathologic evaluation of the placenta and early onset sepsis. In this separate study, the MR score was generated from fresh samples of amniotic fluid and the SELDI-TOF strips were analyzed in a blinded fashion. An important finding was the sequential appearance of the biomarkers with increasing severity of intra-amniotic inflammation. Defensin 2 and defensin 1 appeared first followed by calgranulin C (S100A12) and lastly calgranulin A (S100A8). This led us to fine-tune the original two diagnostic categories into three (MR score 0: 'absent' inflammation; MR score 1 or 2 'minimal' inflammation; MR score 3 or 4 'severe' inflammation (Fig. 2). Although women with severe inflammation had shorter amniocentesis-to-delivery intervals than women with both absent and minimal

inflammation, this latter group also delivered significantly earlier compared to those without inflammation.

Pathological examination of the placenta was the first method used to investigate the mechanisms responsible for preterm birth.¹ The proximity of the placenta to the fetus, its common embryological origin and the unconditional availability of this tissue for research after the birth of the child have all contributed to the large number of studies, which have recognized histological chorioamnionitis and funisitis as risk factors for neonatal mortality and morbidity.^{3,45–47} Given that proteomics is a relatively young science and that long term neonatal follow-up data are not yet available, we have used placental examination (as performed by a perinatal pathologist blinded to proteomic results) as an intermediate outcome variable. We found that presence and the 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.^{29,32,33} The MR score correlated significantly with the stages of chorioamnionitis ($P < 0.001$, Fig. 3) and funisitis independent of the interval to delivery ($P < 0.001$ for MR score, $P = 0.160$ for amniocentesis-to-delivery interval). The occurrence in amniotic fluid of at least one of the peaks corresponding to calgranulin C and/or calgranulin A (indicative of the transition from mild to severe intra-amniotic inflammation) was predictive of biologically relevant funisitis (at least grade 2),²⁹ and/or histological chorioamnionitis. In a concurrent study exploring the pathophysiological relevance of the biomarkers,⁴⁸ we found that calgranulin C was localized to the inflammatory cells infiltrating the umbilical cord and adhering to the endothelium (Fig. 4). The recent identification of calgranulin C as ligand for the receptor of advanced glycation end-products (RAGE) has led us to a new area of investigation on the role of RAGE and damage-associated molecular pattern molecules (DAMPs) in chorioamnionitis and antenatal fetal injury.⁴⁹ Of all component biomarkers of the MR score, the occurrence of calgranulin A in amniotic fluid (which corresponds to the MR score of 4 and to the most severe level of intra-amniotic inflammation) was significantly associated with early onset sepsis [OR: 4.8 (95% CI: 1.7–13.2), $P = 0.002$].²⁹

[A]Cord blood haptoglobin switch-on pattern as biomarker of the fetal exposome in pregnancies complicated chorioamnionitis

The sequencing of the human genome has provided advancements in elucidation of gene and protein expression in a variety of diseases. However, it also underlined the importance of the environment, as simple genetic susceptibility was unable to explain the non-linear relationships between presence and levels of etiological agents (infectious, chemical, physical, lifestyle, etc.) in the environment and individual variations in the presence and severity of the disease linked to the respective agent.⁵⁰ The notion of the ‘exposome’ as the complex interplay between genetic susceptibility and etiological triggers in the environment has recently emerged to fill this gap.⁵¹

There is evidence to support the paradigm that in pregnancies complicated by chorioamnionitis, the prolonged exposure of the fetus to the noxious intrauterine environment rich in free radicals, cytokines, bacterial products and DAMPs results in fetal injury to vital organs including the brain.⁵² One of the working models for infection-induced fetal damage is that some newborns have a genetic propensity to mount an increased state of inflammation in response to infection. Although infection-related preterm births have a disproportionately worse outcome than that attributed to prematurity alone, not all cases have a bad outcome. To this end, predicting who are the infants most likely to develop postnatal complications from infectious encounters in utero remains a pivotal issue.⁵³

With the exception of respiratory distress syndrome, which manifests immediately after birth, most of the neonatal morbidities have a delayed presentation.⁵⁴ This suggests that the decision to intervene is often taken too late in the pathophysiologic process to prevent end-organ damage.^{52,54} Many studies have reported significant associations between histological chorioamnionitis/funisitis and early onset sepsis and IVH.^{55–58} The major disadvantage of placental histological markers is that they are not available prior to birth or immediately after birth in the short time required to play a role in clinical decision-making. For this reason their significance is currently limited to counseling and research purposes. Furthermore, placental pathological examination has limitations intrinsic to pathology practice since it relates to examination of tissues rather than to biological fluids. For instance, significant heterogeneity in inflammatory responses and poor-to-moderate intra- and inter-operator variability have been reported.^{59,60} Moreover, the intricacy and redundancy of biological processes in generating cellular and tissue lesions might lead to identical pathological footprint when the initiating triggers might have been distinct. For example, lesions consistent with mild acute chorioamnionitis are also found in uncomplicated pregnancies and more often associated with labor at term.⁴⁷ Although the MR score enables antenatal identification of fetuses at risk for early onset sepsis, it suffers from the limitation that amniotic fluid is not easily obtainable and performance of transabdominal amniocentesis requires specialized healthcare settings and trained personnel. Proteomic analysis of the cord blood offers the potential to close at least some of these gaps. Our choice of proteomics technique was 2D-DIGE. The flow chart summarizing the study design is presented in Fig. 5.

In the discovery phase we used cord blood from three preterm newborns [gestational age: median, interquartile range (IQR): 28 (25–30) weeks] with confirmed early onset sepsis by positive blood cultures for *Escherichia coli*. All infants had elevated fetal inflammatory markers and their placentas had histological chorioamnionitis. Three neonates without confirmed or presumed early onset sepsis, born at similar gestational ages [26 (25–30) weeks] in the setting of idiopathic preterm birth were used as controls. One of the three 2D-DIGE gels analyzed for the purpose of biomarker discovery is presented in Fig. 6. A reductionist algorithm similar to the MR scoring method was applied to extract identities differentially expressed ≥ 1.5 fold in at least two of the three 2D-DIGE gels. This approach pointed toward haptoglobin (Hp), haptoglobin-related protein (HpRP), α -fetoprotein, vitamin-D-binding protein, apolipoprotein A4, apolipoprotein E and apolipoprotein H as potential cord blood biomarkers for early onset sepsis.

Recognizing the limitations in quantifying the exact proteomics markers using antibodies and immunoassays targeted against protein precursors and not against the differentially expressed fragments, we embarked on validating the above-mentioned proteins in the cord blood of the remaining 174 preterm newborns in the study. Forty-five infants were clinically diagnosed with early onset sepsis (presumed and/or confirmed) and received intravenous antibiotics. Newborns with the diagnosis of early onset sepsis had an increased incidence of intra-amniotic infection and inflammation. They also had increased presence and severity of histological chorioamnionitis and funisitis, a difference that remained significant after correction for gestational age at birth.

The HpRP gene product has >90% sequence homology with Hp; unfortunately, a specific antibody that discriminates HpRP from Hp does not exist. Thus a single immunoassay was employed to quantify the combined Hp&HpRP immunoreactivity. At the end of this first level validation phase, we found that cord serum Hp&HpRP immunoreactivity, but not that of the other proteomic targets, was significantly elevated in newborns with clinical early onset sepsis and this difference remained significant after correction for gestational age at birth ($P = 0.001$).

Haptoglobin is a tetrameric protein with two α - and two β -chains linked by disulfide bonds. In humans, Hp occurs in two co-dominant allelic forms, *Hp1* and *Hp2*, which differ in the length of the α -chain.⁶¹ The human population has three major Hp phenotypes (*Hp1-1*, *Hp2-2* and the heterozygous *Hp1-2*), derived from variations in the α -chain with identical β -chains. Absence of Hp at protein level denotes *Hp0-0* phenotype (ahaptoglobinemia). An important regulator of Hp expression is the inflammatory cytokine IL-6.⁶² Hepatic Hp synthesis is dependent on *cis*-acting elements localized within the first 186 bp of the 5'-flanking region of the promoter. Interaction of this promoter site with *trans*-acting elements is postulated to provide a second level of complexity in regulation in Hp expression which further explains why in individuals with the same genotype Hp levels vary with exposure to environmental or epigenetic stressors.⁶³

Haptoglobin is a known inflammation-inducible plasma protein. Therefore, the finding that early onset sepsis is associated with elevated Hp&HpRP immunoreactivity should not be surprising. However, normal newborns at term do not express detectable levels of circulating haptoglobin.^{64,65} The switch from the physiological neonatal ahaptoglobinemia to the adult level of haptoglobin of 100–150 mg/dL occurs within the first year of life.⁶⁴ The mechanism responsible for Hp gene silencing in the fetal period and its activation switch in the adult life remains unknown. Using the same enzyme-linked immunosorbent assay (ELISA), we confirmed that a group of healthy term newborns had undetectable cord blood Hp&HpRP immunoreactivity while their mothers had levels of >100 μ g/mL as expected. These data suggested that fetuses exposed to chorioamnionitis who subsequently developed early onset sepsis underwent a precocious 'switch-on' in Hp&HpRP expression, which could only have occurred in the antenatal period.

We sought to confirm these findings using western blots and the same antibody used in the immunoassay; a significantly higher number of newborns with early onset sepsis displayed switched-on Hp&HpRP at birth ($P < 0.001$, Fig. 7A). To provide further evidence that this precocious switch-on pattern was early onset sepsis-specific, we performed western blotting and ELISA on matched cord and maternal blood from healthy term maternal–fetal dyads (Fig. 7B). None of the 19 term babies had detectable Hp&HpRP in contrast to their mothers who all had Hp chains as expected for normal adult subjects. The Hp switched-off pattern (*Hp0-0*) was characterized by significantly lower ELISA immunoreactivity compared to all other phenotypes ($P < 0.001$, Fig. 7C). Among newborns with the 'switch-on' pattern, the immunoreactivity with *Hp1-1* measured lower compared to both *Hp1-2* ($P < 0.001$) and *Hp2-2* ($P = 0.002$) independent of neonatal race, gender and gestational age at birth ($P = 0.011$). Possible explanations for this observation are that the multimeric aggregates characteristic of *Hp2-2* are measured at higher levels than *Hp1-1* which is a monomer and/or that the antibody has a higher affinity for *Hp2-2*.

Our laboratory views the 'paradox of the gold standard' as a major roadblock to the quest of better diagnostic and prognostic biomarkers using different platforms including proteomics. Traditional statistical methods based on receiver operating curve (ROC) analyses cannot be applied for validation, as by definition one cannot improve accuracy over that of the 'gold standard'. On the other hand, if a 'gold standard' is perfect, there is no practical need for improvement. With respect to our study population, due to complexities related to the immune responses of the premature newborn, clinical circumstances surrounding delivery (i.e. antenatal antibiotics) and technological limitations related to the limited spectrum of bacteria sought in neonatal blood cultures, we immediately recognized that it was impossible to determine precisely how many newborns had 'true' early onset sepsis.^{8,66} We knew that with respect to funisitis, which is a more precise pathologic entity, our Hp switch-on algorithm had better accuracy (83%) than with the clinical diagnosis of early onset sepsis (73%). Therefore, we used latent class analysis (LCA),⁶⁷ a statistical method that has gained

increased acceptance for situations when a perfect gold standard diagnostic test does not exist.⁶⁸ This analysis assumes that a hidden (latent) variable (which in our case we named: antenatal exposure to intra-amniotic inflammation) is responsible for heterogeneity among observed variables. From all the variables entered in the analysis, the Hp switch pattern had the highest discriminative power, while gestational age at birth, sex, PPROM and Apgar scores were excluded. Hp switch alone was able to drive the cluster assignment (as 'likely' or 'unlikely exposed to inflammation') for 97% of newborns in our prospective cohort of 180 preterm newborns. Other significant participants to clustering the newborns were cord blood IL-6 and presumed early onset sepsis. Addition of cord blood IL-6 improved the model by driving the cluster assignment as 'likely exposed' for a minority of newborns (3%) whose Hp expression remained switched off despite high levels of cord blood IL-6 (range: ~100–1500 pg/mL). These ahaptoglobinemic outliers are the subject of current investigations in our laboratory. The diagnosis of presumed early onset sepsis (which constitutes the current standard of care for decision to initiate broad spectrum antibiotics) did not change cluster assignments but it improved the level of Bayesian certainty with which each case was distributed to one or the other cluster. A schematic representation of this cluster algorithm is presented in Fig. 8 along with probability levels for each possible combination.

The cluster algorithm was significantly better than the clinical diagnosis of early onset sepsis at predicting several neonatal morbidities including IVH.³⁸ Equally important was the finding that in the context of antenatal exposure to intra-amniotic inflammation (chorioamnionitis), the Hp phenotype (but not gestational age at birth) was an independent predictor of short-term neonatal morbidities and especially of IVH and ROP, which are known to have important antenatal pathogenic ascendants. Despite earlier gestational age at birth, newborns carrying the Hp2-2 exhibited a significantly lower incidence of major morbidities compared to those with Hp1-1 phenotype. This observation is in keeping with the known important and phenotype-dependent functions of circulating Hp and HpRP as antimicrobial, antioxidant and immunomodulatory proteins.^{69,70} Moreover, it signifies the ability of the Hp-switch pattern to reflect the interaction between the individual genetic make-up and exposure to an environmental antenatal insult consistent with the definition of the exposome.

[A]Concluding remarks

Discovery of novel biomarkers to improve pregnancy and childhood outcomes is a priority for perinatal medicine. Proteomics has the ability to untangle the complex interactions between the developing fetus and the intrauterine environment (the antenatal exposome). In pregnancies complicated by chorioamnionitis such interactions may result in both adaptive (programming) and deleterious (injury) effects that may have profound consequences on future health.

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Practice points

- Proteomic biomarkers have the potential to define the fetal exposome in the context of pregnancies complicated by chorioamnionitis.
- Identification of expressed haptoglobin in cord blood at birth is indicative of antenatal exposure to chorioamnionitis.
- In the event of antenatal exposure to chorioamnionitis, the individual disparities in fetal haptoglobin phenotypes affect postnatal outcomes.

Research directions

- Carefully designed proteomics experiments are required to discover and validate biomarkers with both biological and clinical relevance.

Figure 1.

Flow chart representation of the phases and patient groups that led to the discovery and validation of the amniotic fluid proteomics mass-restricted (MR) score. PTL, preterm labor; PTB, preterm birth; PPRM, preterm premature rupture of membranes; AF, amniotic fluid; WBC, white blood cell; EONS, early onset neonatal sepsis. Flow chart sums subjects from references 33 and 34.

Figure 2.

(A) Representative surface-enhanced-laser desorption/ionization–time of flight mass spectrometry profiles of the amniotic fluid based on the increasing severity of inflammation. P1–P4 represent the biomarker peaks of the mass-restricted (MR) score; (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). IAI, intra-amniotic infection/inflammation. Published in *PLoS Med* 2007;4:e18. Republication by authors permitted under Creative Commons Attribution License.

[Ref. 29]

Figure 3.

Three-dimensional representation of the relationship between the amniotic fluid mass-restricted (MR) score and histological inflammatory findings in the fetal membranes. (A) Stages of chorioamnionitis; (B) grades of chorio-decidualitis; and (C) grades of amnionitis. With permission from: *Obstet Gynecol* 2008;**111**(2 Pt 1):403–12. KWH permission to republish 2766550305749.

[Ref. 33]

Figure 4.

Morphological (Masson trichrome, A and C) and immunohistochemical (B and D) staining of umbilical cord affected by severe histological funisitis. (A and B) Neutrophils infiltrating the wall of an umbilical artery (UA) and perivascular Wharton gelatin stain positive for calgranulin C, one of the biomarkers of the MR score. (C and D) Neutrophils adhering to the endothelium of the umbilical vein (UV) intensely positive for calgranulin C. With permission from: *Am J Obstet Gynecol* 2007;**196**:181.e1–13. Elsevier permission to republish 2765421478702.

[Ref. 48]

Figure 5.

Flow chart representation of the phases and patient groups used for discovery and validation of the cord blood biomarkers of antenatal exposure to intra-amniotic infection/inflammation. PTL, preterm labor; PTB, preterm birth; PPROM, preterm premature rupture of membranes; NBSCU, NewBorn Special Care Unit; CB, cord blood; EONS, early onset neonatal sepsis; HCA, histological chorioamnionitis; IVH, intraventricular hemorrhage; ROP, retinopathy of prematurity; NEC, necrotizing enterocolitis; BPD, bronchopulmonary dysplasia; 2D-DIGE, two-dimensional differential gel electrophoresis. Published in *PLoS One* 2011;6:e18. Republication by authors permitted under Creative Commons Attribution License. [Ref. 29]

Figure 6. Merged two-dimensional differential gel electrophoresis image of one of the three gels used during the proteomics discovery phase and representative spots identified by mass spectrometry. Spots upregulated at least 1.5-fold are outlined in blue and spots downregulated at least 1.5-fold are outlined with red. The squared regions are shown with matched unambiguous protein identities and direction of change represented by blue arrows. Apo, apolipoprotein; Hp, haptoglobin; HpRP, haptoglobin-related protein. Published in *PLoS One* 2011;6:e18. Republication by authors permitted under Creative Commons Attribution License.
[Ref. 29]

Figure 7.

Cord blood haptoglobin (Hp) and haptoglobin-related protein (HpRP) immunoreactivity revealed by western blot and enzyme-linked immunosorbent assay (ELISA). (A) Western blot of umbilical vein (UV) serum from four preterm newborns of similar gestational age at birth. Newborns in lanes 1–3 were diagnosed with early onset neonatal sepsis (EONS) based on clinical manifestations and hematological indices (presumed EONS) and all received intravenous antibiotics. The newborn in lane 4 had a negative sepsis work-up. Blood cultures remained negative for all four newborns. The presence of a conspicuous immunoreactive band corresponding to the α -chain (~42 kDa) in lanes 1–3 is consistent with our defined switched-on Hp pattern. The absence of this band indicates for a switched-off pattern in lane 4. The band ~9 kDa (lanes 1 and 2) corresponds to the α^1 -chain whereas the band at ~20 kDa (lanes 2–3) corresponds to the α^2 -chain. (B) Impact of Hp phenotypes on Hp&HpRP immunoreactivity as measured by ELISA. The red line indicates the group's median. Groups assigned different letters are statistically different at $P < 0.05$ (Kruskal–Wallis analysis of variance). (C) Western blot of three representative maternal (Mat) and UV sera retrieved from women with normal term deliveries. Note the switched-off pattern of the cord blood in contrast to the switched-on pattern of the adult blood. Published in *PLoS One* 2011;**6**:e18. Reproduction by authors permitted under Creative Commons Attribution License.

[Ref. 29]

Figure 8.

Clustering algorithm based on probability of ‘antenatal exposure to intra-amniotic infection/inflammation (IAI)’. Posterior probabilities of antenatal exposure were calculated for all eight possible combinations of significant indicators [cord blood haptoglobin (Hp) switch pattern, cord blood interleukin (IL)-6, presumed early onset neonatal sepsis (EONS)] and their modal characteristics. The number of newborns presenting each combination is included in parentheses. Published in *PLoS One* 2011;6:e18. Republication by authors permitted under Creative Commons Attribution License. [Ref. 29]