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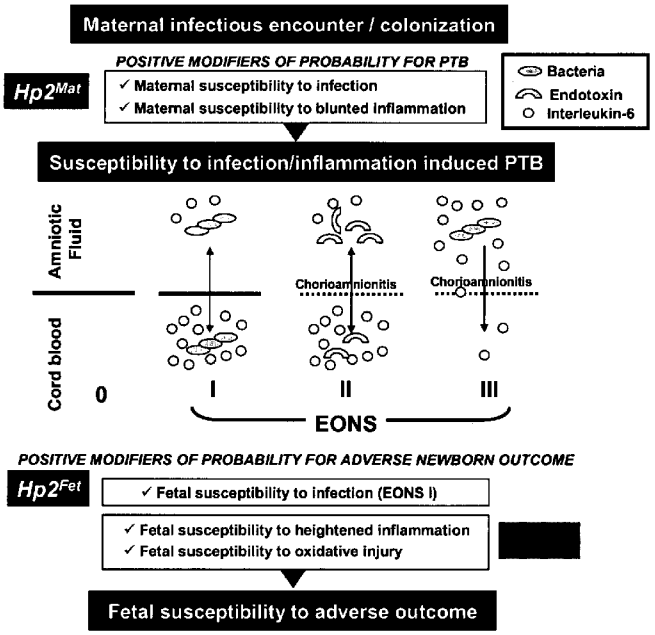

Figure 15.

(57) Abstract: Described herein are biomarkers, such as protein biomarkers, which are diagnostic of and predictive for complications that result from an in utero encounter, such as an infection by the fetus, that can lead to premature birth (PTB). The biomarkers can be used to identify fetuses and newborns at risk for complications of PTB, such as (Early Onset Neonatal Sepsis) EONS, intra-ventricular hemorrhage (IVH) and other poor outcomes.

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NOVEL MARKERS FOR DETECTION OF COMPLICATIONS RESULTING FROM IN UTERO ENCOUNTERS

GOVERNMENT FUNDING

This invention was made with government support under Grant Number R01 HD047321, awarded by the National Institutes of Health. The government has certain rights in the invention.

RELATED APPLICATION

This application claims the benefit of the filing date of U.S. Provisional application 61/206,125, entitled "Novel biomarkers for detection of early onset neonatal sepsis (EONS) and other complications of prematurity" and filed January 28, 2009. The entire teachings of the referenced application are incorporated herein by reference.

BACKGROUND OF THE INVENTION

Premature birth (PTB) is a significant public health problem. The technological advances in newborn intensive care of the past decades have increased survival of preterm infants and the awareness of the need to improve outcomes. Infection-induced PTB represents a unique environment, given that attempts to prolong pregnancy raise the risk for early onset neonatal sepsis (EONS). In such cases, there is an increased risk of poor neonatal outcomes, including intra-ventricular hemorrhage (IVH), which is a significant cause of brain injury, cerebral palsy and developmental disability. A key problem is that most hemorrhages occur in the first 24 hours and therapies aimed to prevent IVH must address the complexity of this condition. In 2001 the World Health Organization established the external Child Health Epidemiology Reference Group (CHERG) to develop epidemiological estimates for the various etiologies of death in young children.[1] In 2003, building on the work of CHERG, it was established that prematurity accounts for 75% of infant mortality and 10% of the 10.6 million yearly deaths in children younger than five years.[1] The latest U.S. vital statistics (2007) reports a 12.7% rate of PTB.[2] Compared to 1990, the percentage of infants delivered <37 completed weeks of gestation has climbed 20% resulting in ~550,000 premature infants born annually, 60,000 of which have a birthweight <1,500 grams. As support in the

NBSCU has improved, more low- and very-low-birth weight (VLBW) infants survive. It has thus become clear that improving neonatal outcomes associated with prematurity is vital.[3,4] About 5% of the nearly 55,000 preemies who survive the newborn period exhibit cerebral palsy and up to 25-50% have sensorial, cognitive and behavioral deficits which include mental retardation, visual and hearing impairments, learning and language disabilities, attention deficit-hyperactivity disorder, motor coordination defects, behavioral, emotional and social difficulties. The immediate impact of PTB for society is underscored by the rising costs of caring for premature infants which in 2005 was estimated in the US in excess of $26 billion/year. This figure does not include rehabilitation or long-term care costs.[5] Even more concerning have been the recent childhood outcome results of studies aimed at preventing PTB by universally extending pregnancy. Released in 2008, the results of ORACLE I & II clinical trials show that antibiotics given to women in preterm labor and PPROM to increase the duration of gestation also increased the risk of cerebral palsy.[6,7] The underlying mechanisms remain unclear but this data highlights the need for a paradigm shift in prematurity research from the unilateral goal of extending the duration of gestation to concurrently improving neonatal outcomes. Concerned by these issues and the alarming increase in the rate of PTB, the March of Dimes Scientific Advisory Committee on Prematurity[8] and the most recent report issued by the Institute of Medicine in 2008: Preterm Birth: Causes, Consequences and Prevention suggested that studies to identify biomarkers that may predict adverse outcomes for infants born preterm to allow for early intervention should become a priority.[9] Clearly, additional approaches to assessing neonatal and early postnatal risk independent of gestational age (GA), and particularly approaches that can provide an early diagnosis, are needed.

SUMMARY OF THE INVENTION

Described herein are biomarkers, such as protein biomarkers, which are diagnostic of and predictive for complications that result from an *in utero* encounter, such as an infection (e.g., bacterial, viral, parasitic or fungal), by the fetus. The biomarkers described herein are useful in methods of predicting fetal and neonatal outcome and can be assessed, for example, in cord blood (CB), in blood or other body fluids obtained from the neonate/newborn. In some embodiments, the biomarkers can be assessed in fetal

blood from the umbilical cord, for example, by cordocentesis a procedure sometimes called Percutaneous Umbilical Cord Blood Sampling (PUBS).

A specific embodiment described herein relates to protein biomarkers, originally identified in cord blood, that are diagnostic of and predictive for Early Onset Neonatal Sepsis (EONS), the risk of which increases when a pregnant woman is treated in an attempt to prolong pregnancy (to avoid PTB), such as in cases in which intrauterine infection occurs. As described herein, levels of biomarkers in cord blood samples from newborns who subsequently developed EONS were increased or decreased by 3-fold or more (at least three-fold), compared to controls. Newborns matched for gestational age (GA), low cord blood (CB) CB IL-6 (about 9 pg/mL) and delivered in the setting of idiopathic PTB or cord blood from a group of term babies with uncomplicated pregnancy and delivery may serve as negative controls. These groups may serve to establish the methodological baseline or reference point. Alternatively, a control or reference can be a premature neonate who progressed to EONS and/or at least one related complication of prematurity (preterm birth).

Also described herein is a rapid and relevant screening test that can be used at birth to identify neonates (newborns) at risk for intra ventricular hemorrhage (IVH) and other poor outcomes associated with premature birth. The screening test may also be performed before birth, e.g., by cordocentesis, or at any time after birth, preferably within the first 24 hours, 48 hours or 72 hours (within 1 day, 2 days or 3 days) after birth. The biomarkers described herein can also be used as markers for adverse outcomes associated with premature birth, regardless of the cause of PTB.

There are many complications that can occur as a result of PTB, only one of which is EONS. Conventionally, EONS is defined as a positive microbial culture in the first 72 hours of life. However, due to widespread use of antibiotics, culture results are frequently unreliable and diagnosis of EONS relies on clinical signs, combined with hematological and serological markers, which are non-specific. Culture results can be unreliable e.g., due to "difficult-to-cultivate" bacteria or because of the use of antibiotics to treat the mother, which may lead to false-negative diagnoses. Contamination, e.g. by personnel handling the samples, may lead to false positive results. Discovery of biomarkers that make it possible to diagnose complication(s) of PTB or predict that complication(s) of PTB will develop in a newborn or infant are critically needed. The

method of screening described herein has made it possible to diagnose complication(s) of PTB and/or predict whether (predict the future development of, predict increased risk of) complication(s) of PTB will develop; it can be used at birth and in newborns to identify those at risk for complications of PTB, such as EONS, IVH and other poor outcomes. Prompt initiation of postnatal pharmacologic intervention strategies for IVH could result in health care savings of over 3 billion dollars/year.

As described herein, Applicant has shown that expression of haptoglobin (Hp) and/or haptoglobin-related protein (HpRP) switches on precociously in newborns who develop EONS. The quantitative and qualitative changes in Hp and/or HpRP expression provide the basis for predicting newborns at risk for EONS, IVH, other complications and/or death and for providing targeted interventions at birth.

Hp is an immunomodulatory protein linked to human susceptibility, as opposed to resistance, to infection. Two allelic variants (Hp1 and Hp2) in humans code proteins with different α-chains. As a result of developmental regulation of Hp transcription, Hp is essentially absent at birth and the adult phenotype (Hp1-1; Hp2-1 or Hp2-2) emerges within the first year of life. The Hp0-0 phenotype lacks both alleles. HpRP shares greater than 90% homology with Hp. As described herein, Applicant has carried out characterization of the cord blood proteome and shown that Hp is a biomarker of EONS. As also described herein, Hp phenotype variations and levels are critical determinants of susceptibility to adverse neonatal outcomes.

Applicant, without wishing to be bound by any particular theory, considers EONS to be a heterogeneous syndrome, rather than a single condition, and describes here four possible EONS variants, each of which would benefit from a different therapeutic ("theranostics") approach. These variants are as follows:

EONS I: Vertical transmission of live bacteria to the fetus, which would require prompt identification and targeted antibiotic treatment.

EONS II: Translocation of bacterial footprints (e.g., endotoxin) and damage-associated molecular pattern proteins (DAMPs) from the mother and damaged placenta to the fetus. This would require general cardio-vascular support, 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. Treatment would include circulatory support and/or anti-inflammatory treatment.

EONS 0: None of the above, in the context of prematurity often leading to over-treatment.

Also described herein is a method of distinguishing among the variants of EONS and a method of determining which variant a neonate exhibits (determining which class or variant type a neonate falls into) and, further, determining whether therapy is needed and, if therapy is needed, the type(s) of appropriate therapy for the neonate. Such a method will result in more targeted therapy for newborns who exhibit EONS I, II, or III and avoid or reduce the use of unnecessary treatments for newborns who exhibit EONS 0.

In one embodiment, such a method for early diagnosis and pathogenic classification of EONS comprises at least one (a, one or more), at least two (two or more), at least three (three or more), at least four (four or more), or five of the following elements:

(a) Cord blood Hp and HpRP switching (antenatal switching), represented qualitatively as positive or negative and derived from an assessment of Hp and HpRP in cord blood, as assessed for example, by Hp and/or HpRP immunoreactivity above an established cut-off;

(b) Cord blood Hp and HpRP level represented quantitatively as the level of Hp and HpRP immunoreactivity;

(c) Cord blood Hp and HpRP phenotype, which is Hp 0-0, Hp 1-1, 1-2 or 2-2;

(d) Relationship of cord blood Hp and HpRP level with cord blood IL-6;

(e) Relationship of cord blood Hp and HpRP level and cord blood IL-6 with bacterial fingerprints.

In certain embodiments, methods are provided for early diagnosis and pathogenic classifications of EONS based on (a) Hp and HpRP switching represented qualitatively as positive or negative and (b) optionally further assessing IL-6 levels. The methods described herein may be carried out using body fluids other than cord blood. For example, fluids obtained from a subject such as neonatal blood, cerebral spinal (cerebrospinal) fluid, urine, saliva, tear fluid, meconium and feces, may also be used.

Analysis of e.g. Hp and HpRP switching and/or Hp and/or HpRP level can be carried out by immunological, as well as non-immunological, methods known in the art.

Specific embodiments of the method are as follows:

(1) A method, such as an ex vivo method, of diagnosing, or predicting the future development of, early onset neonatal sepsis or a (one, one or more) related complication(s) of premature birth (PTB) in a subject, comprising (the step of) detecting an increase in the level of (a) haptoglobulin and/or a fragment thereof; (b) haptoglobulin and/or a fragment thereof and haptoglobulin-related protein and/or a fragment thereof; or (c) haptoglobulin-related protein and/or a fragment thereof in a sample of blood, such as cord blood or neonatal blood from the subject. In this method, (the step of) detecting comprises detecting/determining (a) presence or absence of haptoglobin and/or a fragment thereof; (b) haptoglobin and/or a fragment thereof and/or haptoglobin-related protein and/or a fragment thereof; or (c) haptoglobin-related protein and/or a fragment thereof in a sample obtained from the subject.

(2) A method, such as an ex vivo method, for diagnosing, or predicting the future development of (risk of developing) , early onset neonatal sepsis or a (one, at least one, one or more) related complication(s) of premature birth in a subject, comprising (the step of) detecting, in a sample of blood, such as cord blood or neonatal blood from said subject, a change in the level of at least one (a, one, one or more) protein and/or a fragment thereof selected from:

Table 1

IPI ID#	Abbreviation/Name
IPI00021842	APOE apoliprotein E precursor
IPI00022434	ALB uncharacterized protein ALB
IPI00022443	AFP alpha-fetoprotein precursor
IPI00216773	ALB ALB protein
IPI00298828	APOH Beta-2-glycoprotein 1 precursor
IPI00304273	APOA4 Apolipoprotein A-IV precursor
IPI00384697	ALB isoform 2 of serum albumin procursor
IPI00431645	HP HP protein
IPI00477597	HPR isoform 1 of Haptoglobin-related protein precursor
IPI00478003	A2M Alpha-2-macroglobulin precursor
IPI00478493	HP Haptoglobin isoform 2 preprotein
IPI00555812	GC Vitamin D-binding protein precursor
IPI00607707	HPR Isoform 2 of Haptoglobin-related protein precursor
IPI00641737	HP Haptoglobin precursor
IPI00742696	GC Vitamin D-binding protein precursor
IPI00745872	ALB Isoform 1 of serum albumin precursor
IPI00847179	APOA4 apolipoprotein A-IV precursor
IPI00878517	ALB 56kDa protein
IPI00878953	APOE MRNA for apolipoprotein E
IPI00879456	APOE 25kDa protein

and fragments of each of the above-listed proteins.

(3) A method, such as an ex vivo method, of diagnosing, or predicting the future development of (risk of developing), early onset neonatal sepsis or a (one, at least one, one or more) related complication(s) of premature birth (PTB) in a subject, wherein the complication is intraventricular haemorrhage. The method can be a method of either of the preceding claims.

(4) A method, such as an ex vivo method, of any one of the preceding claims, wherein the sample of cord blood is collected at birth.

(5) A method, such as an ex vivo method, of any one of the preceding claims, wherein detecting (the detecting step) comprises detecting a decrease in the level(s) of apolipoprotein H (and/or a fragment thereof) and/or apolipoprotein E (and/or a fragment thereof) and/or vitamin D-binding protein (and/or a fragment thereof). In specific embodiments, detecting (the detecting step) comprises detecting a decrease in the level(s) of apolipoprotein H and/or a fragment thereof; apolipoprotein E and/or a fragment thereof; vitamin D-binding protein and/or a fragment thereof; apolipoprotein H and/or a fragment thereof and apolipoprotein E and/or a fragment thereof; apolipoprotein H and/or a fragment thereof and vitamin D-binding protein and/or a fragment thereof; apolipoprotein E and/of a fragment thereof and vitamin D-binding protein and/or a fragment thereof; or apolipoprotein H and/or a fragment thereof, apolipoprotein E and/or

a fragment thereof and vitamin D-binding protein and/or a fragment thereof. In further embodiments, the method comprises detecting a decrease in the level(s) of at least one (a, one, one or more) of the listed biomarkers.

(6) A method, such as an ex vivo method, of any one of claims 2 to 4, wherein detecting (the detecting step) comprises detecting an increase in the level(s) of (a) haptoglobulin and/or a fragment thereof; (b) haptoglobulin and/or a fragment thereof and haptoglobulin-related protein and/or a fragment thereof; or (c) haptoglobulin-related protein and/or a fragment thereof.

(7) A method, such as an ex vivo method, of any one of the preceding claims, wherein detecting (the detecting step) comprises detecting (a) a decrease in the level(s) of apolipoprotein H and/or apolipoprotein E and/or vitamin D-binding protein and (b) an increase in the level(s) of haptoglobulin and/or haptoglobulin-related protein. In specific embodiments, detecting (the detecting step) comprises detecting (a) a decrease in the level(s) of (i) apolipoprotein H and/or a fragment thereof; (ii) apolipoprotein E and/or a fragment thereof; (iii) vitamin D-binding protein and/or a fragment thereof; (iv) apolipoprotein H and/or a fragment thereof and apolipoprotein E and/or a fragment thereof; (v) apolipoprotein H and/or a fragment thereof and vitamin D-binding protein or a fragment thereof; (vi) apolipoprotein E and/or a fragment thereof and vitamin D-binding protein and/or a fragment thereof; or (vii) apolipoprotein H and/or a fragment thereof, apolipoprotein E and/or a fragment thereof and vitamin D-binding protein and/or a fragment thereof and (b) detecting an increase in the level(s) of (i) haptoglobulin and/or a fragment thereof; (ii) haptoglobulin and/or a fragment thereof and haptoglobulin-related protein and/or a fragment thereof; or (iii) haptoglobulin-related protein and/or a fragment thereof.

(8) A method, such as an *ex vivo* method, of any one of the preceding claims, wherein the subject is a premature neonate.

(9) The method of any one of the preceding claims wherein the blood is cord blood.

(10) A method (such as an ex vivo method) of diagnosing, or predicting the future development of, early onset neonatal sepsis and/or at least one related complication of prematurity (preterm birth) in a subject, comprising determining if antenatal Hp switching on has occurred in the subject by analyzing a sample obtained

from the subject for Hp; HpRP; or both Hp and HpRP , wherein if Hp; HpRp; or both Hp and HpRP is/are detected in the sample, antenatal HP switching on has occurred and the subject has or is at increased risk of developing EONS and/or at least one related complication of prematurity (preterm birth).

(11) The method of claim 10, wherein the subject is a premature neonate.

(12) The method of claim 10 or claim 11, wherein the sample is a cord blood sample, a neonatal blood sample, a cerebrospinal fluid sample, a urine sample, a tear fluid sample, a meconium fluid or a fecal sample.

(13) The method of any one of claims 10 to 12, further comprising determining the level of Hp; HpRP; or both Hp and HpRP in the sample and comparing the level with the corresponding level in an appropriate control.

(14) The method of any one of claims 10 to 13, wherein the appropriate control is (a) a newborn (i) matched for gestational age with the subject; (ii) with low cord blood IL-6 (such as less than 9 pg/ml); and (iii) delivered in the setting of idiopathic preterm birth or (b) a newborn delivered from an uncomplicated pregnancy and delivery.

(15) A method (such as an ex vivo method) of diagnosing, or predicting the future development of, early onset neonatal sepsis and/or at least one related complication(s) of prematurity (preterm birth) in a subject, comprising determining the level of Hp; HpRP; or Hp and HpRP in a sample of blood obtained from the subject, wherein if Hp; HpRP; or Hp and HpRP is/are present in the sample at a level equal to or greater than the level of Hp; HpRP; or Hp and HpRP in an appropriate control, the subject has or is at increased risk of future development of EONS and/or at least one related complication of prematurity (preterm birth).

(16) The method of claim 10, wherein the subject is a premature neonate.

(17) The method of claim 15 or claim 16, wherein the blood sample is a cord blood sample or a neonatal blood sample.

(18) The method of any one of claims 15 to 17, wherein the level of Hp; HpRP; or Hp and HpRP is equal to or greater than the level in an appropriate control and the appropriate control is a premature neonate who progressed to EONS and/or at least one related complication of prematurity (preterm birth).

(19) The method of any one of claims 15 to 17, wherein the level of Hp; HpRP; or Hp and HpRP is greater than the level in an appropriate control and the

appropriate control is (a) a newborn (i) matched for gestational age with the subject; (ii) with low cord blood IL-6 (such as less than 9 pg/ml); and (iii) delivered in the setting of idiopathic preterm birth or (b) a newborn delivered from an uncomplicated pregnancy and delivery.

(20) A method (such as an ex vivo method) of diagnosing, or predicting the future development of, early onset neonatal sepsis and/or at least one related complication of prematurity (preterm birth) in a subject, comprising determining if (a) cord blood IL-6 is positive, relative to an appropriate control, and (b) Hp and/or HpRP switching has occurred, wherein if cord blood IL-6 is positive and Hp and/or HpRP switching has ccurred, the subject has or is at increased risk of future development of EONS and/or at least one related complication of PTB.

(21) The method of claim 20, wherein the subject is a premature neonate.

(22) The method of claim 20 or claim 21, wherein the blood sample is a cord blood sample or a neonatal blood sample.

(23) The method of any one of claims 20 to 22, wherein the appropriate control for cord blood IL-6 is cord blood IL-6 in (a) a newborn (i) matched for gestational age with the subject; (ii) with low cord blood IL-6; and (iii) delivered in the setting of idiopathic preterm birth or (b) a newborn delivered from an uncomplicated pregnancy and delivery.

(24) The method of any one of claims 20 to 23, wherein the appropriate control has a cord blood IL-6 level of less than about 9 pg/mL.

(25) A method (such as an ex vivo method) of diagnosing, or predicting the future development of, early onset neonatal sepsis and/or at least one related complication of prematurity (premature birth) in a subject, comprising determining IL-6 level and the phenotype of Hp in a sample of blood obtained from the subject, wherein if the IL-6 level is positive and the phenotype of Hp is Hp 0-0, Hp1-1, Hp2-1 or Hp2-2, the subject has or is at increased risk of future development of early onset neonatal sepsis and/or at least one related complication and if the IL-6 level is negative and the phenotype of Hp is Hp0-0, the subject does not have or is at less risk of developing EONS and/or at least one related complication of prematurity (premature birth).

(26) A method (such as an ex vivo method) of diagnosing, or predicting the future development of, early onset neonatal sepsis and/or at least one related

complication of prematurity (premature birth) in a subject, comprising determining the relationship of Hp with the presence of bacterial DNA in a sample of blood obtained from the subject, wherein if Hp and/or HpRP and bacterial DNA are present in the sample of blood, the subject has or is at risk of developing early onset neonatal sepsis and/or at least one related complication of prematurity (premature birth).

(27) A method (such as an ex vivo method) of diagnosing, or predicting the future development of, early onset neonatal sepsis and/or at least one related complication of prematurity (premature birth) in a subject, comprising assessing at least two of the following characteristics in a sample of blood obtained from the subject: (a) antenatal Hp and/or HpRP switching on; (b) phenotype of Hp; HpRp; or Hp and HpRp; (c) level of Hp; HpRP; or Hp and HpRP; (d) cord blood IL-6 or the relationship of the level of Hp; HpRP; or Hp and HpRP to interleukin-6 level (referred to as Hp/IL-6 ratio); and (e) the relationship of Hp and/or HpRP with the presence of bacterial DNA, wherein if the sample is determined to comprise a blood signature comprising at least two characteristics indicative of early onset neonatal sepsis or an increased risk of developing at least one related complication of prematurity (preterm birth), the subject is diagnosed as having early onset neonatal sepsis or an increased risk of developing at least one related complication of prematurity (preterm birth).

(28) The method of claim 27, wherein antenatal Hp and/or HpRP switching on and cord blood IL-6 are assessed and Hp and/or HpRP switching on has occurred and cord blood IL-6 is positive.

(29) The method of claim 28 or claim 28, wherein the subject is a premature neonate.

(30) The method of any one of claims 27 to 29, wherein cord blood IL-6 is greater than about 9 pg/mL.

(31) The method of any one of claims 1 to 31, wherein the complication can be IVH, bronchopulmonary dysplasia (BPD, need for oxygen at 36 weeks postmenstrual age), retinopathy of prematurity (ROP), cerebral palsy and/or death.

(32) A method (such as an ex vivo method) of diagnosing, or predicting the future development of, early onset neonatal sepsis and/or at least one related complication of prematurity (preterm birth) in a subject, comprising assessing, in a blood

sample obtained from the subject, IL-6 level; Hp and/or HpRP switching; and, optionally, Hp type, wherein:

(a) if the CB IL-6 level is negative (not elevated), relative to an appropriate control; Hp and/or HpRP switching is negative (has not occurred); and, optionally, Hp type is Hp 0-0, the conclusion is that the neonate has not been exposed to an in utero event (e.g., bacterial encounter), Hp and/or HpRP switching has not occurred, the subject does not have and is not at increased risk of developing EONS and/or at least one related complication(s), the prognosis is good and antibiotic therapy is not needed;

(b) if the IL-6 level is elevated, relative to an appropriate control; Hp and HpRP switching is negative (has not occurred); and, optionally, Hp type is Hp 0-0, the subject has or is at increased risk of developing EONS and/or at least one related complication(s), the conclusion is that the neonate has been exposed to an in utero event (e.g., bacterial encounter), Hp and HpRP switching has not occurred, the prognosis is poor and the neonate should receive treatment, such as admission to NICU and antibiotic therapy;

(c) if the IL-6 level is elevated, relative to an appropriate control; Hp and HpRP switching is positive (has occurred); and, optionally, Hp type is Hp 1-1, Hp 1-2 or Hp 2-2, the subject has or is at risk of developing EONS and/or at least one related complication(s), the conclusion is that the neonate has been exposed to an in utero event (e.g., bacterial encounter) and the outcome (prognosis) will vary, depending on the Hp phenotype and the neonate will need further assessment and should be admitted to NICU and treated, such as by antibiotic therapy.

(33) The method claim 32, wherein the blood sample is a cord blood sample or a neonatal blood sample.

(34) The method of claim 32 or claim 33, wherein the subject is a premature neonate.

(35) The method of any one of claims 1 to 34, wherein the complication can be IVH and/or bronchopulmonary dysplasia (BPD and/or need for oxygen at 36 weeks postmenstrual age) and/or retinopathy of prematurity (ROP) and/or cerebral palsy and/or death.

In each of the embodiments of the method, the biomarkers can be any of those described herein (e.g., any of the protein biomarkers listed in the Table, CB IL-6, Hp

phenotype or HpRP phenotype, or Hp or HpRP switching), alone or in combination with one or more additional biomarker described herein. A single biomarker (at least one, one or more biomarker) can be used in a method of diagnosing, or predicting the future development of (risk of developing), early onset neonatal sepsis or a (one, at least one, one or more) related complication(s) of premature birth (PTB) in a subject. Two or more (at least two) biomarkers can be used in the method, as can any number of biomarkers described herein. The methods described herein can be used in conjunction with other methods, such as presently-available methods of diagnosing, or predicting the future development of, complication of premature birth.

In specific embodiments of diagnosing or predicting the future development of EONS, the one or more biomarker(s) detected are selected from the following group: apolipoprotein H, an apolipoprotein H fragment, apoliprotein E, an apolipoprotein E fragment, Vitamin D-binding protein, a Vitamin D-binding protein fragment, haptoglobulin, a haptoglobulin fragment, haptoglobulin-related protein and a haptoglobulin-related protein fragment. In further specific embodiments, the method comprises detecting a decrease in the level(s) of a (one, one or more, at least one) biomarker selected from apolipoprotein H, apolipoprotein H fragments, apolipoprotein E, apolipoprotein E fragments, vitamin D-binding protein, and vitamin D-binding protein fragments and detecting an increase in the level(s) of a (one, one or more, at least one) biomarker selected from haptoglobulin, haptoglobulin fragments, haptoglobulin-related protein and haptoglobulin-related protein fragments.

The biomarker(s) are detected, using methods described herein, in blood obtained from a subject (neonate, newborn, infant), using known collection techniques. The blood can be cord blood, blood other than cord blood drawn from the subject, urine, cerebrospinal fluid, tear fluid, saliva, meconium or feces.

There are further embodiments of the method. In one embodiment, the method is a method, such as an ex vivo method, of diagnosing, or predicting the future development of, early onset neonatal sepsis or a (at least one, one, one or more) related complication(s) of prematurity (or PTB) in a subject (e.g., a neonate, such as a preterm or premature neonate), comprising (the step of) determining if antenatal Hp switching on has occurred in the subject by analyzing a sample of blood obtained from the subject (e.g., cord blood, neonatal blood or umbilical cord blood) for Hp; HpRP; or both Hp and

HpRP or a fragment of any of the foregoing, wherein if Hp; HpRp; or both Hp and HpRP or a fragment thereof is/are detected in the sample, antenatal HP switching on has occurred and the subject has or is at increased risk of developing EONS and/or a (one, one or more) related complication(s) of PTB. The complication(s) can be, for example, IVH, bronchopulmonary dysplasia (BPD, need for oxygen at 36 weeks postmenstrual age), retinopathy of prematurity (ROP), cerebral palsy and/or death.

In another embodiment, the method is a method, such as an ex vivo method, of diagnosing, or predicting the future development of, early onset neonatal sepsis or a (at least one, one or more) related complication(s) of prematurity (PTB) in a subject, comprising (the step of) determining the level of Hp; HpRP; or Hp and HpRP in a sample of blood obtained from the subject, wherein if Hp; HpRP; or Hp and HpRP are present in the sample at a level equal to or greater than the level of Hp; HpRP; or Hp and HpRP in an appropriate control or reference, the subject has or is at increased risk of future development of EONS and/or a (one or more, at least one) related complication(s) of PTB. The appropriate control or reference can be the level of Hp, HpRP or Hp and HpRP in blood from newborns matched for GA with low CB IL-6 (<9 pg/mL) and delivered in the setting of idiopathic PTB, the level of HpRP or Hp and HpRP in blood from term babies with uncomplicated pregnancy and delivery assessed by the same method or the level of Hp; HpRP; or Hp and HpRP in blood from adults.

In another embodiment, the method is a method, such as an ex vivo method, of diagnosing, or predicting the future development of (risk of developing), early onset neonatal sepsis or a (one, one or more) related complication(s) of premature birth (PTB) in a subject, comprising (the step of) determining the relationship of the level of Hp; HpRP; or Hp and HpRP to interleukin-6 (IL-6) level (referred to as Hp/IL-6 ratio) in a sample of blood obtained from the subject, wherein if the Hp/IL-6 ratio is greater in the sample than the Hp/IL-6 ratio in an appropriate control or reference, the subject has or is at increased risk of future development of EONS and/or a (one or more, at least one) related complication(s) of PTB. The appropriate control or reference can be the Hp/IL-6 ratio in blood from newborns matched for GA and delivered in the setting of idiopathic PTB; the level of HpRP or Hp and HpRP in blood from term babies with uncomplicated pregnancy and delivery assessed by the same method or the level of Hp; HpRP; or Hp and HpRP in blood from adults.

In another embodiment, the method is a method, such as an ex vivo method, of diagnosing, or predicting the future development of, early onset neonatal sepsis and/or at least one related complication of prematurity (premature birth) in a subject, comprising determining IL-6 level and the phenotype of Hp in a sample of blood obtained from the subject, wherein if the IL-6 level is positive and the phenotype of Hp is Hp 0-0, Hp1-1, Hp2-1 or Hp2-2, the subject has or is at increased risk of future development of early onset neonatal sepsis and/or at least one related complication and if the IL-6 level is negative and the phenotype of Hp is Hp0-0, the subject does not have or is at less risk of developing EONS and/or at least one related complication of prematurity (premature birth).

In another embodiment, the method is a method, such as an ex vivo method, of diagnosing, or predicting the future development of (risk of developing), early onset neonatal sepsis and/or at least one (a, one, one or more) related complication of prematurity (premature birth) in a subject, comprising (the step of) determining the relationship of Hp with the presence of bacterial DNA in a sample of blood obtained from the subject, wherein if Hp and/or HpRP and bacterial DNA are present in the sample of blood, the subject has or is at risk of developing early onset neonatal sepsis and/or at least one related complication of prematurity (premature birth).

In a particular embodiment, two or more (three or more, four or more or five) of the characteristics (element) described above (antenatal Hp switching; level of Hp, HpRP, or Hp and HpRP; relationship of the level of Hp, HpRP, or Hp and HpRP to (IL-6) level (referred to as Hp/IL-6 ratio); phenotype of Hp; relationship of Hp with the presence of bacterial DNA) are assessed in diagnosing, or predicting the likelihood of future development of, early onset neonatal sepsis and/or a (one, one or more) related complication(s) of PTB in a subject. The result of this embodiment is referred to as a "cord blood Hp signature," which comprises results of assessing the two or more characteristics results. For example, in one embodiment, two or three of the following are assessed in diagnosing or predicting the likelihood of future development of EONS: Cord blood (CB) IL-6 (positive or negative); CB Hp and/or HpRP switching; and CB Hp phenotype. Diagnoses and predicted outcomes and suggested therapies are shown in Table 2. In one embodiment, CB IL-6 level; CB Hp and HpRP switching; and CB Hp type are assessed, using known methods. Several possible outcomes can result,

including the following. (1) The CB IL-6 level is negative (not elevated, relative to an appropriate control); CB Hp and HpRP switching is negative (has not occurred); and CB Hp type is Hp 0-0 in the subject; the conclusion is that the neonate has not been exposed to an *in utero* event (e.g., bacterial encounter), Hp and HpRP switching has not occurred, the prognosis is good and antibiotic therapy is not needed. (2) The CB IL-6 level is elevated, relative to an appropriate control; CB Hp and HpRP switching is negative (has not occurred); and CB Hp type is Hp 0-0 in the subject and the conclusion is that the neonate has been exposed to an *in utero* event (e.g., bacterial encounter), Hp and HpRP switching has not occurred, the prognosis is poor and the neonate should receive treatment, such as admission to Neonatal Intensive Care Unit (NICU) and/or antibiotic therapy. (3) The CB IL-6 level is elevated, relative to an appropriate control; CB Hp and HpRP switching is positive (has occurred); and CB Hp type is Hp 1-1, Hp 1-2 or Hp 2-2 in the subject, the conclusion is that the neonate has been exposed to an *in utero* event (e.g., bacterial encounter), Hp and HpRP switching has occurred. In this case, the outcome (prognosis) will vary, depending on the Hp phenotype; the neonate will need further assessment and should be considered for admission to NICU and treatment, such as by antibiotic therapy. 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 of Hp expression, which further explains why in individuals with the same genotype, Hp levels vary with exposure to environmental or epigenetic stressors (physical effort, methylation status).[10] It is known for adults that haptoglobin concentration measured by ELISA is phenotype-dependent. The reference range for haptoglobin concentration is lower in individuals carrying the Hp2-2 phenotype than individuals carrying the Hp1-1 and Hp2-1 phenotype. Therefore, Hp2-2 phenotype confers a state of relative ahaptoglobinemia. Few individuals may lack both Hp alleles, giving raise to Hp0-0 phenotype. Further assessment of the neonate can comprise determining his/her Hp phenotype and include the result of that assessment in decisions regarding treatment. (4) If the CB IL-6 level is not elevated (negative), relative to an appropriate control; CB Hp and HpRP switching is positive (has occurred); and CB Hp type is Hp 1-1, Hp 2-1 or Hp 2-2, the conclusion is that the neonate has been exposed to an *in utero* event (e.g., bacterial encounter), Hp and HpRP switching has occurred, the

prognosis is poor and the neonate should be admitted to NICU and treated, such as with antibiotic therapy. This latter outcome is rare and occurs most often in neutropenic fetuses.

1) EONS (n=46) was characterized more often by antenatal Hp switching-on (P<0.001) and significantly higher Hp levels (P<0.001), independent of GA and IL-6;

2) Phenotypes impacted Hp level: Hp2-1>Hp2-2>Hp1-1 (P<0.001);

3) Newborns with EONS and Hp2-1 had the highest Hp levels, double that of Hp1-1 (P=0.003);

4) Neonates who developed IVH or died (n=36) had higher Hp levels (P<0.001) independent of GA, IL-6, cord pH, steroid and antibiotic use;

5) Newborns with switched-on Hp at birth had an increased risk of IVH and death (RR: 3.6 [1.8-7.8]); 6) No term neonate (but all adults) had switched-on Hp.

In all of the methods described herein, any of a variety of known tecnhiques/methods can be used to assess the biomarkers. For example, inflammation, such as intraamniotic inflammation or fetal/neonatal inflammatory status can be assessed by IL-6 levels, which can be determined, for example, by SELDI-TOF mass spectrometry, ELISA (IL-6 Cytokine ELISA kit, Abnova, BD BioSciences, Cell Sciences), EIA (enzyme immune assy, Cayman Chemical), and other colorimetric assay, Western blot, semiquantitative PCR, and other nucleic acid based methods. Bacterial status (e.g., bacterial status of neonates with EONS who have negative microbial cultures) can be assessed using gene amplification techniques (e.g., PCR, RT-PCR, 16s-RNA gene amplification, other hybridization techniques, or antibody-based methods. Expression patterns and Hp presence can be detected by e.g. SELDI-TOF, ELISA, Western blot and PCR-based techniques, and phenotyping can be assessed by Western blot analysis, ELISA, high pressure gel permeation chromatography, SELDI-TOF, polyacrylamide gel isoelectric focusing (PAGIF), capillary zone electrophoresis, conventional starch gel electrophoresis or other electrophoresis methods using e.g. starch, acetate, agarose and polyacrylamide gels (J. Delanghe et al. "Fast determination of haptoglobin phenotype and calculation of hemoglobin binding capacity using high pressure gel permeation chromatography" Clinica Chimica Acta Vol.291, 2000, 43-51; Fukuda et al."Haptoglobin phenotyping by polyacrylamide gel isoelectric focusing and its application to simultaneous typing of serum proteins" Int. J. Legal Med. Vol. 101,

1988, 37-40; Wuyts et al. "A new method for fast haptoglobin phenotyping and hemoglobin binding capacity calculation based on capillary zone electrophoresis" Clin. Chem. Lab. Med. Vol. 38, 2000, 715-720; Levy et al. "ELISA for Determination of the Haptoglobin Phenotype" Clinical Chemistry, Vol. 50, 2004, 2148-2150; Wassell et al. "A new method for haptoglobin phenotyping" Ann Clin Biochem., 36, 1999, 609-12). Differential expression of Hp can be validated with ELISA. Other analytical methods can be immunological methods, for example employing one or more antibodies directed against all three polypeptide bands specific to Hp and/or HpRP, Hp isoform specific or HpRP specific and non-immunological methods. Non-immunological methods include mass spectrometry, colorimetric assays, using, for example, the ability of Hp to bind hemoglobin and of the resulting Hp-Hb complex to react with certain dyes. In addition to antbodies that are specific for Hp other antibodies that are cross reactive with other proteins (bind non-specifically with Hp and/or HpRP) can be used, as described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a representation of SELDI-TOF proteomic profiling of amniotic fluid for diagnosis of intra-amniotic inflammation, infection and early onset neonatal sepsis and Applicant's proteomic profile, the MR score, which is comprised of four biomarkers: defensin-2, defensin-1, calgranulin C and calgranulin-A.

Figures 2 and 3 show the design of proteomic studies. Figure 2 outlines the discovery phase, in which the study group consisted of 3 neonates with confirmed positive *E. coli* sepsis, high cord blood IL-6, positive amniotic fluid cultures and grade 4 histological chorioamnionitis. Three fetuses matched for gestational age and delivered in the setting of idiopathic preterm birth served as control.

Figure 3 outlines the proteomic techniques: to identify biomarkers, first fluorescence 2 dimensional differential gel electrophoresis was performed after albumin and IgG depletion. This was followed by robotic tryptic digest and tandem mass spectrometry. Pathway analysis was performed using Protein Analysis Through Evolutionary Relationships (PANTHER) ontological classification. In the validation phase, immunoassays and Western blot analysis were used to assess the differential expression of specific markers in the entire cohort.

Figure 4 shows results of study-described herein. Forty neonates were diagnosed with early onset sepsis. They delivered at an earlier gestational age, had lower birth weight and Apgar scores. Their acid base status was not different compared to controls.

Figure 5 shows further results of study described herein. Neonates with early onset sepsis were more frequently delivered in the setting of intra-amniotic infection and inflammation, as determined by microbial cultures and the MR score. Their placenta showed more often evidence of severe maternal and fetal inflammation.

Figure 6 shows results of assessment of neonatal sepsis. Analysis of hematological indices showed that neonates with early onset sepsis had lower hemoglobin levels and were more frequently lymphopenic. They had a higher absolute band count and immature to total neutrophil ratio. Overall, out of the 40 neonates with early onset sepsis, 8 had a documented positive microbial culture.

Figure 7 presents 2 dimensional differential gel electrophoresis data. Three gels were created. Each display differentially expressed proteins or peptides between a neonate with proven sepsis and its matched control. For the purpose of this study we concentrated our attention on the proteins which were at least 3 fold down-regulated (marked in red) or 3 fold upregulated (marked in blue). 332 and 230 identities were matched in 2 database resources. Using the International Protein Index database Applicant determined that of the 230 identities 68 were unique and present in at least one out of 3 gels. Applicant concentrated on the 20 identities found common in 2 out of 3 gels.

Figure 8 is a representation of the ontological classification of these 20 common identities, which showed that transfer/carrier, proteases/extracellular matrix and immunity and defense were the molecular functions and biological processes most affected in neonates with early onset sepsis.

Figure 9 shows results of analysis of potential protein biomarkers, using PANTHER. Of the 20 matched identities, several converged into the same protein and a smaller number of potential down- or up-regulated protein biomarkers was identified. These included Apolipoprotein H, Apolipoprotein E, Vitamin D binding protein, Haptoglobin and Haptoglobin related protein.

Figure 10 shows results of assessment of Hp and HpRP (EONS, no EONS). These differences remained significant following correction for gestational age at delivery.

Figure 11 shows results of an assessment of relevance of haptoglobin or haptoglobin-related proteins for neonatal outcomes other than early onset sepsis. Multivariate logistic regression analysis showed that gestational age at delivery and haptoglobin are independent predictors of intra-ventricular hemorrhage or death. Neonates who were to develop IVH or die had significantly higher haptoglobin levels at birth even after correction for gestational age.

Figure 12 shows results of Applicant's study of haptoglobin in the adult and fetus. Previous studies showed that haptoglobin is absent in the normal term fetus, as shown on the left of this Western blot. Applicant demonstrated that switching of haptoglobin and haptoglobin related protein expression toward an adult phenotype occurs prior to birth in infants with early onset neonatal sepsis, as shown on the right.

Figures 13a and 13b show 2D-DIGE gels obtained with cord serum from two newborns with EONS I and two with EONS 0, matched for GA at birth (28±1 wk). Nine proteins were differentially expressed > 3-fold in at least 2 of 3 of the gels. They were either up-regulated or down-regulated. Different spots matching to the same precursor could appear both up and down-regulated, as shown for ApoA4. TTR (transthyretin) exceeded the cut-off on a single gel (A) and thus was not classified as biomarker by Applicant's algorithm. Circled spots were picked for identification.

Figures 14A and 14B show, respectively: Hp and HpRP immunoreactivity in cord blood (CB) of 155 preterm newborns born at YNHH. Of these, 40 had a diagnosis of EONS, either confirmed (by positive cultures, n=8) or suspected (by clinical and hematological criteria, n=36). FIG. 14B: CB Hp&HpRP in the subgroup of 92 newborns that were evaluated for IVH (head ultrasound and/or MRI) or that died postnatally. Note the y-axis units in micrograms/mL. Standard curve ranged from 4-250 ng/mL. Serum was diluted 150-fold. Hp and HpRP are >90% homologous and are not differentiated in this ELISA.

Figure 15 shows a working model of how interactions of environmental stressors (bacteria) with genetically determined Hp phenotypes may impact on susceptibility to PTB and adverse neonatal outcomes. Positive modifiers facilitate disease progression.

For each, Applicant postulated how maternal (Mat) or fetal (Fet) carriage of Hp2 allele will impact on disease susceptibility (red) vs. resistance (blue). The proposed pathogenic variants of EONS are differentiated as EONS 0 (lack thereof), EONS I (passage of a live bacterial inoculum to the fetus), EONS II (passage of endotoxin, other bacterial products or DAMPs through a damaged maternal-fetal interface) and EONS III (spillage of inflammatory cytokines only).

Figure 16 shows Hp and HpRP signatures revealed by Western blot using an antibody that reacts with both Hp chains and with HpRP. The lanes to the left contain serum of Hp1-1 or Hp 2-2 adults (Sigma). Lanes a-n were loaded with cord serum from preterm newborns who were admitted to Yale NBSCU and had a sepsis workup. EONS was diagnosed in 4 newborns who received i.v. antibiotics (marked with E) but only in one cultures were positive (baby "e"; S. aureus). Red squares note babies with elevated cord IL-6. Adverse short-term outcomes are specified above each lane as death, intra-ventricular hemorrhage (IVH grade 2 or more), bronchopulmonary dysplasia (BPD, need for oxygen at 36 weeks postmenstrual age) and retinopathy of prematurity (ROP grade 2 or more). All newborns have known short-term outcomes and some are enrolled in the long-term follow-up program where they are followed with neuro-developmental assessments part of the NICHD Research Network. Babies "h" and "j" have severe forms of cerebral palsy.

DETAILED DESCRIPTION OF THE INVENTION

Using DNA-based technologies corroborated with proteomic analysis of amniotic fluid (AF), Applicant determined that microbial triggers involve a myriad of uncultivated or difficult-to-culture bacterial species capable of inducing potent fetal immune responses and cellular damage. Applicant found that such etiological agents are present in the cord blood (CB) of newborns who have early onset neonatal sepsis (EONS), yet have negative blood cultures. Using proteomics (2D-DIGE) Applicant further discovered that CB haptoglobin (Hp) and haptoglobin-related protein (HpRP) are biomarkers of EONS and IVH, independent of GA at birth or birth weight. Specifically, Applicant has shown that Hp, an abundant antimicrobial, anti-oxidant and immunomodulatory protein normally absent at birth, precociously switches on expression in fetuses who had an antenatal encounter with an infectious trigger. From a

diagnostic standpoint, this marked quantitative and qualitative change creates a signature for use to predict newborns at high risk for adverse outcomes, thus enabling targeted immediate interventions in this select subgroup. Hp is a highly abundant and stable protein and, thus, it can be reliably and rapidly detected in minute amounts of blood, using technologies easily adaptable for point-of-care (immunoassay, biosensors, mass spectrometry).

Proteomic analysis of cord blood samples (using the methodology described in Buhimschi et al. PLoS ONE 3 (4): e2049) resulted in identification of 20 proteins and protein fragments (listed in the Table 1) in cord blood samples from babies who subsequently developed EONS. The levels of the 20 proteins and protein fragments were either increased or decreased by 3-fold or greater, compared to controls. Thus, proteomic analysis resulted in identification of biomarkers (proteins and protein fragments) and functional protein networks characteristic of EONS. Analysis of the levels of one or more (a, at least one) of these proteins or protein fragments is useful in diagnosing EONS in a newborn and predicting the likelihood that a newborn will develop EONS and/or related complications of prematurity. Based on these data, it is clear that detecting changes in the level of one or more of these proteins and/or protein fragments is diagnostic of EONS and predictive for the development of EONS and related complications of prematurity, such as intraventricular hemorrhage (IVH). Decreases in the levels of Apolipoprotein H and/or Apolipoprotein E and/or Vitamin D-binding protein and/or increases in Haptogobin and/or Haptoglobin-related protein are markers that can be used for assessing complications of prematurity or preterm birth (PTB), such as EONS and related complications of prematurity.

Use of such markers as described herein provides a significant advance. The ease of detection of such proteins and protein fragments makes it possible to provide diagnostic and prognostic information about a subject more rapidly than presently-available methods, with the result that situations where therapeutic intervention is necessary can be better identified and appropriate treatment can be initiated sooner.

As discussed herein, Applicant has assessed the hypothesis that interactions of environmental stressors with genetically determined variations in Hp phenotypes and/or Hp levels are important determinants of susceptibility to adverse neonatal outcomes. The environmental stressors can be an infectious agent, such as bacteria, virus, yeast or

parasite. As further discussed, Applicant has developed a method of assessing the likelihood that a newborn or infant has (diagnosing or aiding in diagnosing) or will develop (is at risk of developing) early onset neonatal sepsis (EONS) or related complications of prematurity. The method is generally carried out up to and including three days after birth, but it can be used in older newborns, such as up to 4, 5, 6, 7 or more days after birth. The method is a method of diagnosing (the presence of) EONS in a subject (e.g., a human newborn or a human infant human, or predicting the future development of, early onset neonatal sepsis or related complications of prematurity). In one embodiment, the method of diagnosing, or predicting the future development of (risk of developing), early onset neonatal sepsis or related complications of prematurity in a subject (newborn), comprises detecting an increase in the level of haptoglobulin and/or haptoglobulin-related protein (detecting an increase in the level of at least one, one or more of haptoglobulin and/or haptoglobulin-related protein) in a sample of cord blood from the subject. In a specific embodiment, the method comprises the step of detecting an increase in the levels of haptoglobulin and/or haptoglobulin-related protein in a sample of cord blood from the subject. In other embodiments haptoglobulin and/or haptoglobulin-related protein levels are detected in a body fluid that is not cord blood, such as neonatal blood, cerebral spinal fluid, urine, tear fluid, meconium or feces. As also described herein, the biomarker is abundant in blood samples and is known to be stable, which supports its reliable detection in dried blood spots, such as archived Guthrie cards, dried fingerstick and dried CB spots. One embodiment described herein is a method of assessing the likelihood that a newborn or infant has or will develop early onset neonatal sepsis (EONS) or related complications of prematurity by detecting an increase in Hp and/or HpRP in a dried sample obtained from the subject, dried finger stick and dried CB, such as dried CB spots.

Also described herein is a method in which Hp and/or HpRP signature assessment at birth (in CB) and longitudinally in NICU thereafter (in neonatal blood) serves as (is used prospectively as) a biomarker of risk stratification for IVH and other adverse short-term outcomes in preterm newborns. The method is based on the relationship of the postnatal temporal change in Hp and/or HpRP signature and to outcome of preterm newborns. In the method, Hp, HpRP or Hp and HpRP signature is assessed in cord blood or other blood obtained from a preterm newborn and subsequently at intervals in neonatal

blood and compared with an appropriate control or reference to assess the risk of IVH in the subject. These and other methods described herein can be carried out using one or a combination of a variety of known techniques, such as western blot analysis, ELISA, EIA, mass spectrometry, SELDI-TOF, OCR or AMMP biosensor technology. For example, assessment of CB Hp and HpRP signature can be carried out using one or a combination of western blot analysis, ELISA, EIA, mass spectrometry, SELDI-TOF, PCR and AMMP biosensor technology. Antibodies raised against Hp are known in the art and are commercially available, e.g., from DAKO, Abnova, Abcam, ABR/Thermo-Fisher-Pierce. See also Kuhajda, FP et al., Proc. Natl. Acad. Sci. USA 1989 86(4): 1188-92; Kuhajda FP et al. Proc Natl. Acad. Sci. USA 1994 91(14):6379-83; Epalbaum R. et al., Pathol. Oncol. Res. 1998 4(4):271-6. These antibodies are derived from sheep, rabbit, goat, chicken (polyclonal) and mice (monoclonal) and can be used for example in ELISA, Western blot, immuno histochemistry (IHC) and radio immune assay (RIA). The antibodies can be specific for Hp, HpRP or both Hp and HpRP or cross react with proteins other than Hp or HpRP (nonspecific). Methods described herein can be carried out in samples, such as cord blood or other blood samples obtained from a neonate at or soon after birth or longitudinally after birth. Blood samples can be fresh or dried, such as archived samples or dried blood spots.

Interactions between maternal and/or fetal Hp phenotypes or levels can be assessed for their role as determinants of susceptibility to spontaneous PTB in pregnancies with infectious etiology. Hp 1-1 has the highest hemoglobin binding capacity but appears to be linked to greater susceptibility to infections. Hp 2-2 is the weakest binder to hemoglobin, which means that Hp 2-2 does not scavenge free radicals as well as the other phenotypes. After a neonate is exposed to an *in utero* event, (e.g., bacterial encounter) the Hp switching that occurs can determine the outcome. For example, a switching to Hp 1-1 phenotype leads to less of an ability to counter the bacterial infection, but the fetus or neonate is protected from oxidative injury. A switching to Hp 2-2 phenotype is effective in countering the bacterial infection but the fetus or neonate is less well protected from oxidative injury that can lead to brain injury. The phenotypic interplay between mother and fetus can be a determinant for outcome. For example, an Hp 1-1 mother may be more susceptible to infection and if the fetus/newborn exhibits switching to Hp 2-2 phenotype upon *in utero* encounter this may lead to brain damage; although a fetus/neonate with an

Hp 2-2 phenotype can fight a bacterial infection, it is less well protected from brain injury caused by oxidative injury. Therefore, Hp phenotyping of the father and mother may be used for pre-birth counseling and determination of possible PTB-related outcomes, such as EONS, IVH and brain injury, according to genetic predispositions.

In another method, the Bioscale platform that is based on Acoustic Membrane Microparticle (AMMP™) resonance, a variant of surface plasmon resonance spectroscopy is used for detection of biomarkers. The AMMP technology was first developed at Massachusetts Institute of Technology (Cambridge, MA) and uses baits (antibodies, DNA strings) coupled to super-paramagnetic polymer particles that can be adsorbed onto a membrane using a magnetic field and quantified by the resulting change in frequency of a vibrating membrane as detected by a sensor. Because the detection relies on change in frequency of sound waves, it enables quantitative measurements of analytes at picogram levels in whole blood without need of centrifugation or dilution that are otherwise required for proper standard curve interpolation. A similar change in technology has led to virtually universal replacement of conventional end-point PCR with real-time PCR. Results with AMMP technology are available in 10-15 min. A significant advancement is that the sensor surfaces can be regenerated, reducing the cost per assay compared to other technologies (ELISA or mass spectrometry). The instrumentation varies from a hand held reader (ultra-rapid mode) to a tabletop model for enhanced sensitivity (ultra-sensitive mode).

Applicant has shown that PTB is a heterogeneous syndrome and that not all PTBs are equivalent, in terms of neonatal outcome. A major obstacle to a universal therapeutic intervention to prevent prematurity is the heterogeneous nature of PTB and the difficulties with defining the populations of women benefiting most from each available intervention versus no intervention. While several interventions (progesterone, cerclage, antibiotics) have been quoted as effective in preventing spontaneous PTB in subgroups of women at risk, not all have proven beneficial when neonatal outcomes are taken into consideration, as shown by the ORACLE trials.[11,12] Alternatively, given that long-term outcomes are only available for analyses after extended periods of time, it is difficult to evaluate in the short run the benefit versus detriment attributable to each particular intervention.

While multiple pathogenic mechanisms have been implicated in triggering PTB, several have received increased attention, specifically, genetic predisposition, stress, excessive stretching, decidual hemorrhage and infection inflammation.[13] The implicit paradigm that has governed the concept of the PTB syndrome is that independent activation of each of these pathways activates a final converging cascade of events leading to premature onset of myometrial contractions, preterm premature rupture of the membranes (PPROM) or both. Evidence suggests, however, that the poor outcome of many premature children is not entirely dependent on GA at birth or birthweight, but rather results from adverse processes that damage the fetus while *in utero*. Unfortunately, this process antedates the onset of PTB symptoms.[14] In particular, of all pathogenic pathways, intra-uterine infection and subsequent inflammation is the cause that contributes disproportionately to neonatal mortality and morbidity when adjusted for GA at birth.[15] The implication of this model is that in a subgroup of cases, particularities of the fetal innate immune response to infection cause pathology unique to the premature fetus including heightened oxidative and inflammatory states that act synergistically with microbial insult to induce damage. Obstetricians and their patients are faced daily with the dilemma of choosing between early delivery and the risk of iatrogenic prematurity and complications resulting from antenatal damage. The element which universally tips the balance towards early delivery in intra-uterine infection is the imminent risk for early-onset neonatal sepsis (EONS).[16] There is evidence to support the paradigm that prolonged exposure of the fetus to a noxious intra-uterine environment results in antenatal injury to vital organs, including the brain. One of the working models for infection-induced fetal damage is that some newborns have an inborn susceptibility to mount an increased state of inflammation in response to infection.[17] However, although infection-related PTBs have a disproportionately worse outcome than what could be attributed to prematurity alone, not all cases have a bad outcome. From an intervention standpoint, this model predicates the idea of "individualized medicine" aiming to use biomarkers to identify cases who may benefit from targeted rather than a "one size fits all therapy" in a modern diagnostic – therapeutic framework -"theranostics." To this end, predicting which infants are most likely to develop postnatal complications from infectious encounters *in utero*, with potential to exceed in severity those resulting from prematurity alone, remains a key problem.

The link between perinatal infection, inflammation and PTB is underestimated. The greatest etiological factor for PTB worldwide is infection, mainly due to malaria, HIV and parasites. [18] This is in contrast with most developed countries, where iatrogenic delivery is responsible for approximately half of the births between 28 and 35 weeks of gestation. One of the first evidence of intrauterine infection involvement in triggering PTB was provided by Larsen et al. at Yale more than 30 years ago.[19] Since then, data from Yale and other institutions directly implicate intrauterine infection as etiological factor for one quarter of pregnancies delivered before 34 weeks of gestation.[20,21] Adverse pregnancy outcomes related to infection are due to a direct microbial attack on the fetus and/or premature activation of the myometrial contractile machinery, dissolution of collagen architecture leading to cervical ripening or Preterm Premature Rupture of Membranes PPROM.[22,23]

Evidence of the causal role of infection in PTB is supported by a body of work demonstrating that microbial invasion of the amniotic cavity, as identified by positive amniotic fluid (AF) cultures, occurs in 10% of patients with preterm labor and intact membranes and in as many as 38% of patients with PPROM.[24] A variety of microbial pathogens have been implicated as etiologic agents of intra-amniotic infection.[25,26,27] The most frequently isolated pathogens are thought to originate primarily from the genital flora (*Gardnerella vaginalis, Mycoplasma hominis, Ureaplasma, Peptostreptococcus & Bacteroides spp.*).[27] 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. [28] Thus, "uncultivated" or "difficult-to-cultivate" bacteria cannot be found when relying on culture conditions alone.[29,30] In contrast, culture-independent methods, such as PCR, can detect bacterial DNA in up to 35% to 60% of pregnancies complicated by PTB.[31,32] Yet, their use alone as diagnostics cannot discriminate between *in vivo* infection and *ex vivo* contamination and thus may result in unnecessary early deliveries. Irrespective of this potential bias, identification of intra-amniotic inflammation in the absence of a positive microbial culture result is a frequent finding in the clinical setting.[20] Using a proteomic fingerprint (the "MR score") as an indicator, Applicant observed that many women presenting with signs of PTB showed AF evidence of "severe inflammation," yet had negative cultures.[20] All samples that tested positive using Applicant's proteomics algorithm ultimately contained bacterial

footprints.[33,34] Furthermore, most samples of AF with positive cultures contained additional bacteria compared to those found by cultures. In fact, 60% of species detected by culture-independent methods were missed by general laboratory cultures.[34] 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.*[34] This suggests that in pregnancies complicated by PTB, the prevalence of AF infection and microbial diversity is underestimated. Moreover, it brings into perspective that the fetus may encounter pathogenic bacteria more often than previously thought. Interestingly, the majority of identified bacterial species were normal flora constituents with relatively low virulence, suggesting that their interaction with host's susceptibility may play a role.[35,36]

Early onset neonatal sepsis (EONS) is a major cause of neonatal morbidity and mortality. Sepsis in hospitalized neonates is a global problem and a significant contributor to morbidity and death. Both early- and late-onset sepsis occur with increased frequency in neonates born prematurely.[37,38,39] Mortality of culture positive (proven) neonatal sepsis ranges from 15% to 50%. In statistics published by Yale NBSCU, which holds the longest running, single-center database of neonatal sepsis started in 1928, mortality attributable to sepsis remains at 11% despite advances in neonatal care.[39] It is extremely important to make an early diagnosis of sepsis, because prompt institution of antimicrobial therapy improves outcomes. This is true for all newborns irrespective of GA at birth and more so for premature newborns because of their immature immune system. Isolation of bacteria from a central body fluid (usually blood) is the standard method to diagnose neonatal sepsis. However, the time required for the clinical laboratory to report results of cultures varies from 2-7 days, a timeframe which does not allow selection of cases for antibiotic therapy. Thus, the guidelines for clinical practice are that all newborns receive "empiric" broad spectrum antibiotics based on clinical suspicion of sepsis. In most circumstances, including at our institution, if blood culture results are not reported as positive by 48 to 72 hours, then antibiotics are discontinued.

There are significant downsides to overuse of this "initial empirical antibiotic therapy" for EONS. Aminoglycosides may be associated with important adverse effects and they require frequent monitoring of blood levels because of renal and oto-toxicity.

Preterm infants have immature organs and therefore may not tolerate some antibiotics as well as term infants. In addition, the use of broad spectrum antibiotics in neonates may alter gut flora and increase the risk of developing necrotizing enterocolitis (NEC). A recent analysis conducted by the Neonatal NICHD Network on outcomes of over 3,000 premature newborns, concluded that each empiric antibiotic treatment day was associated with increased odds of death and/or NEC.[40] The current clinical practice dictates that antibiotics are mandatory in the antepartum period to decrease the risk of neonatal group B streptococcal (GBS) infections and to prolong pregnancy in women with PPROM. This antibiotic overuse in perinatal settings has and continues to create an environment for emerging bacterial resistance with potential for additional poor outcomes in the premature newborn population.[41] Moreover, the recent emergence of resistant Gram negative strains such as the ampicillin-resistant *E. coli*[24,25] is of concern and of major public health relevance. Not surprisingly, there is concern that continuing this practice will further change the diversity of microbes in NBSCUs, and will pose in the future a significant therapeutic challenge. The continuous increase in prevalence of antibiotic-resistant and ampicillin-resistant *E. coli* infections in preterm infants is a challenge and suggests that antibiotic prophylaxis in this group needs scientifically based restrictive guidelines.

There are several reasons why it is difficult to accurately diagnose EONS and why newborns are treated with empiric antibiotics, even when it is not necessary. Bloodstream infections fluctuate widely from 8% to 73% in the diagnosis of "suspected" EONS.[42,43] Another level of complexity is added by the observation that there are many newborns who have nonspecific clinical manifestations of EONS (e.g., lethargy, apnea, respiratory distress, hypoperfusion and shock). The majority of these newborns have negative cultures.[44] A different obstacle is technical and relates to the narrow spectrum of pathogens sought in microbiology laboratories. For example, searching for *Ureaplasma* and *Mycoplasma* spp. is not part of routine sepsis work-up in neonates. A study that evaluated the frequency of umbilical CB infections with these species found that that 23% of newborns born <32 weeks tested positive for these pathogens.[45] It is also plausible that analogous to intra-amniotic inflammation, the fetal and newborn insult is induced by additional uncultivated and difficult-to-cultivate species. Data supporting

this premise has shown that 16S rDNA PCR technology improves the accuracy of culture-based methods for diagnosis of neonatal sepsis.[46]

In the context of the narrow spectrum of currently identifiable bacteria, attempts have been made to use physiologic parameters, such as hematologic indices and cytokine profiles, to identify neonates with sepsis and guide decisions related to initiation and duration of antibiotic treatment. Although the majority of the proposed cytokine markers (such as IL-6) have high negative predictive values (for ruling out sepsis), they have not been adopted for general use. This relates to the large volume of sample required (relative to the blood volume of a premature neonate), long interval to obtain the results (especially if ELISA techniques are used), high cost of tests and need for trained personnel. Hematologic indices on the other hand are readily achievable from a complete blood count and leukocyte differential assays. At Yale NBSCU results are available in 1 hour. Their disadvantages are the poor specificity for diagnosing culture proven sepsis and need for subjective interpretation. Applicant has developed a newborn hematological scoring system for EONS which in Applicant's clinical setting is used consistently to guide with antibiotic treatment. The Bhandari criteria are: absolute neutrophil count (ANC) <7,500/mL or >14,500/mL, absolute band count (ABC) >1,500/mL, immature/total neutrophil ratio (I:T) ratio>0.16, platelet count<150.000 cells/mm^3. Provided herein are methods to determine or to suspect the occurrence of sepsis in a newborn using one, two, three or four of the Bhandari criteria. In some embodiments, at least two criteria are used to determine or to suspect the presence of EONS, optionally further corroborated with one or more clinical manifestations. Per clinical protocol, at Yale NBSCU all infants with proven and suspected sepsis (by clinical and/or laboratory criteria) receive empiric antibiotherapy for at least 48 to 72 hours after which the indication is reevaluated. Therefore, there is an urgent need for improved diagnostic modalities of neonatal sepsis and of enabling technologies that can use either CB or minimal amounts of neonatal blood.

Another challenge posed in the diagnosis of EONS is its heterogeneous nature. This may thus explain why some neonates can manifest clinical symptoms or have hematological indices suggestive of EONS in the absence of a positive microbial culture results.[42,47,48] For instance, it is tempting to propose that there may not necessarily be a requirement for bacteria to passage live into fetal circulation to induce EONS

manifestations. It would suffice for endotoxin, other bacterial products, lipophylic damage associated molecular pattern proteins (DAMPs) or just cytokines to "spill" from AF into the fetal circulation and cause manifestations consistent to septic shock. Evidence described herein supports this premise.

As described herein, Applicant has developed diagnostic proteomic profiles in AF and vaginal fluid characteristic of intra-amniotic inflammation and PTB.[49,50,51,52,53,54] Applicant used surface enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI) and devised a novel, stepwise strategy based on mathematical filter preferences applied sequentially. This strategy was named mass restricted (MR) scoring. Four peaks (and thus the proteins they represent) were used to devise the MR score. The MR score ranges from 0 to 4, depending on the presence or absence of each of these 4 biomarkers. Proteomics identification techniques established that the component SELDI peaks corresponded to neutrophil defensin-2 (3.3 kDa), neutrophil defensin-1 (3.4 kDa), S100A12 (10.4 kDa) and S100A8 (10.8 kDa), all members of the innate immunity arm of antimicrobial defenses (3 or 4 peaks present of 4 possible) was highly successful in detecting intra-amniotic inflammation (90.1%). MR score has a unique ability to predict funisitis and EONS. In a prospective follow-up study Applicant observed a sequential appearance of biomarkers, as the process of intra-amniotic inflammation progresses from acute to chronic, with the peaks corresponding to the DAMPs S100A12 and S100A8 appearing last. This finding enabled stratification, based on severity of AF inflammation from MR 0 (absent) to MR 1-2 (mild) to MR 3-4 (severe) characterized by irreversible cellular injury.

Recent data from a prospective cohort of 132 consecutive mothers who had an amniocentesis to rule-out infection indicated that neonates of women with "severe" intra-amniotic inflammation by the MR score had higher CB IL-6 levels and a higher frequency of proven and suspected EONS. Of all newborns, 34 (26%) were clinically diagnosed with EONS, although only 6 (5%) of these had a positive blood culture. Overall, newborns with EONS had significantly elevated CB IL-6 levels, compared to those without sepsis when correcting for GA at birth. Yet, there was a wide margin of variation among CB IL-6 levels between individuals (newborns) from normal levels (despite positive AF culture and severe intra-amniotic inflammation by MR score), to extremely elevated concentrations (despite normal AF analysis). Wide variations in

human cytokine levels data are frequently encountered in other studies and thus Applicant investigated in greater depth the reasons for this marked individual variation in newborns. The CB-to-AF IL-6 ratio (CB/AF IL-6; an indicator of the differential inflammatory response in the fetal versus the AF 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 (funistis). This finding suggests that inflammation-induced damage to the maternal-fetal interface may play a permissive role in cytokine trafficking between the AF and CB. In most EONS cases with evidence of severe intra-amniotic inflammation (88% of all EONS cases in the study), the absolute AF IL-6 concentration was significantly higher than that measured in CB. 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 CB/AF IL-6 reversed. This finding provided support for the conclusion that an outpouring of cytokines may occur in the fetal compartment independent of AF space. In fetuses with a reversed CB/AF IL-6 ratio, EONS confirmed by positive blood cultures reached 50%. It is possible that in the remaining 50% sepsis was induced by "uncultivated" bacteria or that these fetuses had a disproportionate activation of their innate immune response to bacterial products leaked into fetal circulation. Data in support of this premise is coming from the evidence that maternal antibiotic treatment, which results in killing of maternal bacteria, may induce excess release of endotoxin. 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.[55] This may explain why attempts to prevent PTB with antibiotic treatment in patients with bacterial vaginosis, Trichomonas or preterm labor either had no effect or paradoxically increased the rate of PTB.

For the last 3 decades obstetricians, neonatologists and developmental neurobiologists had debates regarding the appropriate time to deliver a fetus exposed to a hostile intra-uterine environment. In the absence of either a preventive or curative therapy for intra-amniotic infection and inflammation the answers to 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 steroid when infection/inflammation is diagnosed remain

rhetorical. A key to providing answers to all these questions is discovery of biomarkers highly predictive of fetal and neonatal outcome. The work and method described herein offer a new opportunity to provide evidence-based answers to several of the above queries.

There will be a clinical and economic impact of a rapid method that makes it possible to improve identification of EONS and other complications of PTB. Early initiation of antibiotic treatment in the neonatal period has been shown to reduce mortality due to sepsis.[56] However, as described herein, antibiotic treatment remains contingent on early identification of signs of infection, which is a difficult task. Given that even a 4-hour delay may increase mortality rate and the fact that it takes at least 48 to 72 hours until bacterial growth can be confirmed, NICUs around the world initiate "empirical antibiotic therapy" in all cases of suspected EONS immediately after microbial cultures have been obtained.[57] This treatment is intended to cover both Gram-negative and Gram-positive bacteria, generally through a combination of an aminoglycoside (i.e., gentamicin) and a beta-lactam antibiotic (i.e., penicillin or ampicillin).[58] A study that evaluated the duration of therapy for suspected, but not proven, sepsis found that antibiotic administration in NICUs across the U.S. is highly inconsistent, lasting approximately 2-3 days in most cases, but extending to 10 days for some neonates.[59] This non-discriminatory practice adds significant cost from use of NICUs, specialized nursing, physician and pharmacy resources, in addition to sometimes prohibitive drug costs.

For the late preterm and term infants admitted in NICUs for sepsis evaluation and/or antibiotic treatment the benefits of a tool to 'rule-out EONS' and to discriminate among pathophysiological EONS variants is cost beneficial and can be incorporated into the hospital setting without delay. In addition to decreasing the length of stay reducing hospital costs, such tool will facilitate infant-parental attachments and successful establishment of breast-feeding, as well as lower the risks of exposure to resistant microorganisms frequently encountered in the setting of prolonged hospitalization.

Hp and HpRP

Hp is an acute phase glycoprotein with a great variety of important biological functions, of which the most recognized is hemoglobin binding.[60] Hp is a tetrameric protein of two α and two β chains derived from a single mRNA that encodes a precursor that is cleaved post-translationally to generate the two chains. The liver is the main site of Hp synthesis, although Hp synthesis has been demonstrated many extrahepatic sites such as adipose tissue, lung, ovary, testis, arteries and placenta. However, beyond its role as a hemoglobin scavenger, Hp is also a stimulator of angiogenesis and an inhibitor of prostaglandin synthesis.[61] Importantly, Hp plays a vital role in regulating innate host defense mechanisms, which is consistent with a global immunosuppressive and antioxidant function.[62] Hp blocks neutrophil responses by binding directly to neutrophils and inhibiting their oxidative burst. Hp also inhibits lymphocyte transformation and decreases antibody production.[63] Hp has a direct bacteriostatic activity by limiting utilization of iron by adventitious bacteria. [64] There is evidence to support a key role of Hp in modulating immune mechanisms at the materno-fetal interface by regulating NK-decidual cell interaction. [65] However, the extent to which Hp satisfies these functions is subjected to significant phenotypic and developmental variation.

In humans, but not in other mammals, Hp has been shown to occur in two allelic forms, Hp1 and Hp2, which differ in the length of the α-chain. The longer α-chain (in Hp2) seems to have arisen by an internal duplication of a gene segment coding for almost the entire α-chain of Hp1.[66] The human population has 3 major haptoglobin phenotypes (Hp1-1, Hp2-2 and the heterozygous type 2-1) derived from variations in the α-chain with identical β-chains.[67] Hp1 has two additional allelic variations $Hp1^{Fast}$ and $Hp1^{Slow}$ which code for α-chains of equal length but different charge and generate minor phenotypic differences among carriers.

The complex evolution of the human Hp gene cluster offers the classical example of how rearrangements in parts of existing genes can generate new genes coding for new proteins which confer survival advantages in certain stressing environments. Highly conserved homologs to Hp1 allele are found in all mammals. The Hp2 allele is found only in humans and is believed to have arisen ~100,000 years ago early in human evolution. Today Hp2 allele frequency is higher that Hp1 allele frequency in nearly every ethnic group and geographic area and continues to increase, suggesting that the driving force for

this positive selection continues to be present. It has been hypothesized that the ability of Hp2 allele to spread so rapidly in humans was due to its ability to provide a selective advantage against life-threatening infectious diseases which was the dominant environmental pressure early in human evolution. It is postulated that the severe environmental challenge of Plasmodium falciparum infection has resulted in emergence of Hp 2-2 phenotype in malaria endemic environments to boost Th1 cytokine and oxidative responses to which the parasite is vulnerable in a somewhat similar manner to sickle cell trait.[68]

Hp posses a phenotype-dependent antioxidant activity that exceeds by far that of vitamin C. [69] However, Hp2-2 has a lesser anti-oxidant capacity, compared to that of Hp1-1, due to the lower affinity of Hp2-2 for free hemoglobin. Moreover, Hp2-2, but not Hp1-1, has been shown to have direct antimicrobial activity *in vitro*. Hp2-2 binds to the streptococcus T antigen, thereby resulting in its aggregation and slowing its growth. Recently, there has been an increasing interest in relationships of Hp phenotypes and individual variation in susceptibility to infectious diseases versus susceptibility to chronic diseases with pro-inflammatory and oxidative component. Several studies demonstrate that individuals homozygous for the Hp1 allele (Hp1-1) are more susceptible to acquire infectious diseases, such as malaria, streptococcal or staphylococcal infections. This is in contrast to Hp2 allele carriers (Hp 2-1 or Hp2-2 phenotypes), who display resistance. These individuals, however, seem to have increased risk of diseases with oxidative and inflammatory mediated-tissue damage[70,71,72] of either infectious (pulmonary tuberculosis, HIV) or non-infectious etiology (diabetes, arteriosclerosis, coronary disease).

An important regulator of Hp expression is the inflammatory cytokine IL-6. 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 of Hp expression, which further explains why in individuals with the same genotype, Hp levels vary with exposure to environmental or epigenetic stressors (physical effort, methylation status).[73] It is known for adults that haptoglobin concentration measured by ELISA is phenotype-dependent. The reference range for haptoglobin concentration is lower in individuals carrying the Hp2-2 phenotype than individuals carrying the Hp1-1 and Hp2-1 phenotype. Therefore, Hp2-2 phenotype confers a state of relative ahaptoglobinemia.

Few individuals may lack both Hp alleles, giving raise to Hp0-0 phenotype. In the US adult population ahaptoglobinemia varies from 0.1% in Caucasians to much higher frequency 4% in African-Americans.

Another interesting feature is that in humans, the entire Hp gene is itself duplicated as Hp-related (HpRP) gene, which also contains elements of retroviral insertion. The transcription of the HpRP gene generates 21 kDa protein, but is silenced in the adult liver through an unknown mechanism. However, based on published reports, the human serum, breast carcinoma, decidua and placenta are four compartments where the HpRP protein product appears expressed. At amino-acid level HpRP is 90% identical with Hp, which explains cross-reactivity in Western blots and ELISA assays with most available antibodies.[74] Other than conferring innate immunity to humans against Trypanosoma brucei, the function of HpRP protein remains a unknown.[75] Recently, expression of HpRP has been implicated an oncofetal tumor marker in breast cancer.

Applicant has shown that Hp and HpRP levels in CB of newborns with EONS are elevated, which is remarkable, in view of the well established developmental regulation of Hp transcription and the evidence that in normal term newborns, Hp is absent at birth. Normal levels of Hp in newborns at term are measured at <2 mg/dL (clinical assay limit) and the switch to the adult level of 100-150 mg/dL occurs within the first year of life.[76] The mechanism responsible for Hp gene silencing in the fetal developmental period and its activation switch in the adult life remains unknown. The presence and levels of HpRP in CB, have not previously been established. Hp and HpRP are referred to herein as components of Hp. Using ELISA, Applicant confirmed that a group of 7 healthy term newborns had undetectable CB Hp and HpRP and their mothers had levels of ~100 μg/mL.

As described herein, Applicant has shown that an elevated Hp level at birth is a predictor of EONS (independent of GA) and of the composite outcome variable IVH and death (independent of EONS). Applicant's results are consistent with the current knowledge on the significance of genetically determined Hp phenotypes for susceptibility to infectious diseases (Hp0-0>Hp1-1>Hp2-1>Hp2-2) versus susceptibility to inflammation-induced oxidative stress and cellular damage (Hp2-2>Hp2-1>Hp1-1>Hp0-0), led Applicant to formulate the hypothesis that interactions of environmental stressors (bacteria) with genetically determined variations in haptoglobin phenotypes and/or

haptoglobin levels are critical determinants of susceptibility to adverse neonatal outcomes. A schematic representation of this view is shown in Figure 15. Based on assessment of Hp switching (from absent to adult level) in CB, a rapid diagnostic test for EONS is available. The methods described herein improve existing point-of-care technologies that seek to detect EONS at birth (analysis of CB Hp and/or HpRP signatures) and monitor thereafter sepsis in preterm newborns (analysis of neonatal blood). Useful here is a biosensor technology which has attributes to differentiate among pathogenic EONS variants, thus enabling a "theranostic" (diagnostics + targeted therapy) treatment, in which one "treatment" is no treatment e.g., no antibiotic therapy. This approach may result in substantial health care savings. One strategy utilizes SELDI-TOF, which has the potential to provide a rapid modality for individual Hp phenotyping. Other assays are known in the art. Together with evidence of exposure to infection (positive Hp switching), SELDI-TOF or other methods could be used at birth in each premature newborn to estimate risk of developing EONS, IVH and other major complications of premature birth. A significant advantage is that Hp subtyping is a well-established forensics method. In old blood spots, Hp forms adducts with hemoglobin which render the protein resistant to degradation. Hp may be detected in archived Guthrie cards, dried fingerstick spots and CB spots.

Platforms to evaluate Hp and HpRP signatures

In preliminary experiments Applicant confirmed the ability to detect differences among Hp and Hp signatures in CB using ELISA and western blot. Western blot results correlate strongly with densitometric analysis of westerns ($P>0.001$, suggesting that normalization for total protein may not be required). However for a rapid diagnosis other technologies may further facilitate detection of Hp and HpRP signatures for point-of-care in Labor and Birth and NICUs. An advantage of SELDI-TOF is the antibody-independent separation and the high resolution which enables detection not only of the major but also minor Hp phenotypes. An important step for separation in mass spectrometry is breaking the bonds among Hp chains and among Hp and hemoglobin through stringent reducing conditions. The results could be available within 1 hour reliant on availability of a SELDI-TOF mass spectrometer.

Furthermore, it is also possible to carry out methods described herein using analytical instruments based on AMMP technology, a hybrid variant of surface plasmon resonance and MEMS sensors. This cutting-edge technology offers the promise to provide the needed speed for simultaneous quantitative information for Hp and HpRP, IL-6 and bacterial fingerprints (endotoxin) within the same CB sample. The company has already validated the platform for bacteria (whole bacterial cells and endotoxin) and IL-6 measurement in adult whole blood. One approach that can be used is concurrent assessment of Hp and HpRP (using the same antibody validated by Applicant in ELISA and western blots) and IL-6 within the same sensitivity range.

It is likely that in most newborns with EONS, Hp and HpRP levels will exceed by several orders of magnitude those of IL-6, while in a minority of cases IL-6 will far exceed Hp and HpRP. Both these scenarios will prompt a need for intervention. This information would thus suffice for point-of-care enabling of a theranostic approach within 1 hour of birth, which can be followed by a clinical laboratory type analysis (Western blot, SELDI or other techniques) to determine the phenotype and compute the risk assessment.

"Antenatal switching" or Hp switching, as used herein, can be assessed, for example by western blot, with the appearance of ~ 40 kDa Hp and/or HpRP bands (corresponding to the beta-chain) and optionally accompanied by one or two additional bands that are Hp and/or HpRP specific. Full term newborns have essentially no Hp and normal levels of Hp and/or HpRP in newborns are considered undetectable and below clinical assay limits. Applicant showed that when employing the same polyclonal antibody which detects all three polypeptide bands specific for Hp and/or HpRP in paired ELISA and western blot assays, a determination of switched Hp could be made when a signal (or assay value) is measured that is above a pre-established cut-off. The cut-off for the signal (or assay value) upon which a determination can be made may vary with method sensitivity depending on the assay. For example, a determination of switched Hp and/or HpRP obtained from visual inspection of a western blot may, under the conditions used, correspond to an immunoreactivity level above 3,370 ng/mL in ELISA immunoreactivity (>3.37 micrograms/mL). A similar analysis in a group of 19 normal term newborns (controls) measured an immunoreactivity level (for ELISA) ranging from 1.9 to 0.9 micrograms/mL with none exhibiting switching based on visual inspection of

western blots. In contrast, the control mothers all showed a switched Hp and/or HpRP signature and the measured level in ELISA ranged from 909-63 micrograms/mL. In certain assays, minimum detection levels can be around 2 mg/dl of protein and an analyte (e.g. a protein) is considered undetectable if the concentration is below the detection limit. Normal levels of newborns at term are at undetectable levels. In newborns with uncomplicated pregnancy and delivery the switch to adult levels of about 100-150 mg/dL occurs within the first year of life.

In certain embodiments, antibodies are provided that are specific for Hp and/or HpRP or cytokines, such as IL-6 or other biomarkers described herein, for example, those listed in Table 2 and neutrophil defensin-1 and -2, S100A12 and S100A8, found in fluid samples of a fetus or newborn. Antibodies provided herein include polyclonal and monoclonal antibodies, as well as antibody fragments and derivatives that contain the relevant antigen binding domain of the antibodies. The term "antibody" as used herein refers to immunoglobulin molecules or other molecules which comprise at least one antigen-binding domain. The term "antibody" as used herein is intended to include whole antibodies (e.g. IgG, IgA, IgE, IgM, or IgD), monoclonal antibodies, polyclonal antibodies, chimeric antibodies, humanized antibodies, primatized antibodies, multi-specific antibodies, single chain antibodies, epitope-binding fragments, e.g., Fab, Fab' and $F(ab')_2$, Fd, Fvs, single-chain Fvs (scFv), disulfide-linked Fvs (sdFv), fragments comprising either a VL or VH domain, and totally synthetic and recombinant antibodies.

Immunoglobulin or antibody molecules of the invention can be of any type (e.g., IgG, IgE, IgM, IgD, IgA, and IgY), class (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule.

Monoclonal antibodies may be produced in animals such as mice and rats by immunization. B cells can be isolated from the immunized animal, for example from the spleen. The isolated B cells can be fused, for example with a myeloma cell line, to produce hybridomas, that can be maintained indefinitely in *in vitro* cultures. These hybridomas can be isolated by dilution (single cell cloning) and grown into colonies. Individual colonies can be screened for the production of antibodies of uniform affinity and specificity. Hybridoma cells may be grown in tissue culture and antibodies may be isolated from the culture medium. Hybridoma cells may also be injected into an animal, such as a mouse, to form tumors *in vivo* (such as peritoneal tumors) that produce

antibodies that can be harvested as intraperitoneal fluid (ascites). The lytic complement activity of serum may be optionally inactivated, for example by heating.

For example, specific proteins, peptides, haptens, and chemical compounds may be used to generate antibodies. One skilled in the art will recognize that the amount of polypeptides used for immunization will vary based on a number of factors, including the animal which is immunized, the antigenicity of the polypeptide selected, and the site of injection. The polypeptides used as an immunogen may be modified as appropriate or administered in an adjuvant in order to increase the peptide antigenicity. In some embodiments, polypeptides, peptides, haptens, and small compounds may be conjugated to a carrier protein to elicit an immune response or may be administered with and adjuvant, e.g. incomplete Freund's adjuvant.

Suitable methods to increase antigenicity are well known in the art, and include, for example, coupling the antigen with a heterologous protein (such as globulin or β-galactosidase) or through the inclusion of an adjuvant during immunization.

Antibody titers can be monitored e.g., by antigen-specific ELISA, western blot analysis, or radioimmunoassay. One or more animals are commonly used for antibody production. Antibodies or immunospecific fragments thereof of provided herein may be from any animal origin including rabbits, sheep, goats, chicken, mice, rats, hamsters, guinea pigs, donkey, camel, llama, or horse.

After one or more injections of the antigen, approximately 7-10 days after each boost, serum may be taken to determine the production of specific antibodies (titer). The test bleeds may be assayed against the immunogen itself, for example in an ELISA assay. Antibodies may be stored in several different buffers, for example at neutral pH, such as 0.01 M phosphate-buffered saline (PBS) at pH 7.4, optionally containing, for example 0.1% sodium azide to inhibit microbial growth. For long-term storage, antibodies may be kept at a low temperature, such as 4°C, -20^{0}C or -70^{0}C. Antibodies may be stored at > 0.5 mg/mL and/or in the presence of a carrier protein (e.g., 1% bovine serum albumin (BSA)), or if frozen, for example in 50% glycerol.

Protocols for generating antibodies, including preparing immunogens, immunization of animals, and collection of antiserum may be found in Antibodies: A Laboratory Manual, E. Harlow and D. Lane, ed., Cold Spring Harbor Laboratory (Cold Spring Harbor, NY, 1988) pp. 55-120 and A. M. Campbell, Monoclonal Antibody

Technology: Laboratory Techniques in Biochemistry and Molecular Biology, Elsevier Science Publishers, Amsterdam, The Netherlands (1984).

The term "antibody fragment" as used herein is intended to include any appropriate antibody fragment which comprises an antigen-binding domain that displays antigen binding function. Antibodies can be fragmented using conventional techniques. For example, $F(ab')_2$ fragments can be generated by treating the antibody with pepsin. The resulting $F(ab')_2$ fragment can be treated to reduce disulfide bridges to produce Fab^1 fragments. Papain digestion can lead to the formation of Fab fragments. Fab, Fab' and $F(ab')_2$, scFv, Fv, dsFv, Fd, dAbs, TandAbs, ds-scFv, dimers, minibodies, diabodies, bispecific antibody fragments and other fragments can also be synthesized by recombinant techniques or can be chemically synthesized. Techniques for producing antibody fragments are well known and described in the art. Antibody fragments, including single-chain antibodies, may comprise the variable region(s) alone or in combination with the entirety or a portion of the following: hinge region, CH1, CH2, and CH3 domains.

In some aspects, the antibody or antibody fragment comprises an antibody light chain variable region (VL) and an antibody heavy chain variable region (VH) which generally comprise the antigen binding site. In certain embodiments, the antibody or antibody fragment comprises all or a portion of a heavy chain constant region, such as an IgGl, IgG2, IgG3, IgG4, IgAl, IgA2, IgE, IgM or IgD constant region. In some aspects, the heavy chain constant region is an IgGl heavy chain constant region, or a portion thereof. Furthermore, the antibody or antibody fragment may comprise all or a portion of a kappa light chain constant region or a lambda light chain constant region, or a portion thereof. In some aspects, the light chain constant region is a lambda light chain constant region, or a portion thereof. All or part of such constant regions may be produced naturally or may be wholly or partially synthetic. Appropriate sequences for such constant regions are well known and documented in the art.

As used herein, the term "heavy chain portion" includes amino acid sequences derived from an immunoglobulin heavy chain. A polypeptide comprising a heavy chain portion comprises at least one of: a CH1 domain, a hinge (e.g., upper, middle, and/or lower hinge region) domain, a CH2 domain, a CH3 domain, or a variant or fragment thereof. For example, a binding polypeptide for use in the invention may comprise a

polypeptide chain comprising a CH1 domain; a polypeptide chain comprising a CH1 domain, at least a portion of a hinge domain, and a CH2 domain; a polypeptide chain comprising a CH1 domain and a CH3 domain; a polypeptide chain comprising a CH1 domain, at least a portion of a hinge domain, and a CH3 domain, or a polypeptide chain comprising a CH1 domain, at least a portion of a hinge domain, a CH2 domain, and a CH3 domain. In another embodiment, a polypeptide of the invention comprises a polypeptide chain comprising a CH3 domain. Further, a binding polypeptide for use in the invention may lack at least a portion of a CH2 domain (e.g., all or part of a CH2 domain). It will be understood by one of ordinary skill in the art that these domains (e.g., the heavy chain portions) may be modified such that they vary in amino acid sequence from the naturally occurring immunoglobulin molecule.

As used herein, the term "light chain portion" includes amino acid sequences derived from an immunoglobulin light chain. Preferably, the light chain portion comprises at least one of a VL or CL domain.

Recombinant techniques are preferred for generating large quantities of antibodies, antibody fragments and single chain antibodies. In general, recombinant production of antibodies, antibody fragments or derivatives thereof, uses mRNA encoding an antibody which is isolated from hybridoma cells that produce the desired antibody. This mRNA is used as a source for generating a cDNA molecule which encodes the antibody, or a fragment thereof. Once obtained, the cDNA may be amplified and expressed according to known methods in a variety of eukaryotic and prokaryotic hosts.

In certain embodiments, antibody derivatives are provided. As used herein, "antibody derivatives" contain an antibody or a fragment thereof, as well as an additional moiety. Such moieties may improve the solubility, absorption, biological half-life, etc., of the antibody, decrease the toxicity of the antibody *in vivo* or *in vitro*, eliminate or attenuate any undesirable side effect of the antibody *in vivo*, or serve as a detectable marker of the presence of the antibody. Moieties capable of mediating such effects are well known in the art. In certain embodiments, detectably labeled antibodies are provided. An antibody is referred to as "detectably labeled" if the antibody, or fragment thereof, is attached to a molecule which is capable of identification, visualization, or localization using known methods. Suitable detectable labels include radioisotopic

labels, enzyme labels, non-radioactive isotopic labels, fluorescent labels, toxin labels, affinity labels, and chemiluminescent labels.

Biomarkers of the invention can be measured in different types of biological samples, preferably biological fluid samples such as blood. Examples of biological fluid samples that may be used in methods of the invention, although not intended to be limiting, include cord blood, neonatal blood, cerebral spinal fluid, tears, saliva, urine, feces, and meconium. If desired, a sample can be prepared to enhance detectability of the biomarkers. For example, a sample from the subject can be fractionated. Any method that enriches for a biomarker polypeptide of interest can be used. Sample preparations, such as prefractionation protocols, are optional and may not be necessary to enhance detectability of biomarkers depending on the methods of detection used. For example, sample preparation may be unnecessary if an antibody that specifically binds a biomarker is used to detect the presence of the biomarker in a sample. Sample preparation may involve fractionation of a sample and collection of fractions determined to contain the biomarkers. Methods of prefractionation include, for example, size exclusion chromatography, ion exchange chromatography, heparin chromatography, affinity chromatography, sequential extraction, gel electrophoresis and liquid chromatography. Examples of methods of fractionation are described in PCT/US03/00531 (incorporated herein in its entirety).

As an example, a sample is pre-fractionated by anion exchange chromatography. Anion exchange chromatography allows pre-fractionation of the proteins in a sample roughly according to their charge characteristics. For example, a Q anion-exchange resin can be used, and a sample can be sequentially eluted with eluants having different pHs. Anion exchange chromatography allows separation of biomolecules in a sample that are more negatively charged from other types of biomolecules. Proteins that are eluted with an eluant having a high pH is likely to be weakly negatively charged, and a fraction that is eluted with an eluant having a low pH is likely to be strongly negatively charged. Thus, in addition to reducing complexity of a sample, anion exchange chromatography separates proteins according to their binding characteristics.

As another example, biomolecules in a sample can be separated by high-resolution electrophoresis, e.g., one or two-dimensional gel electrophoresis. A fraction containing a biomarker can be isolated and further analyzed by gas phase ion

spectrometry. Preferably, two-dimensional gel electrophoresis is used to generate two-dimensional array of spots of biomolecules, including one or more biomarkers. See, e.g., Jungblut and Thiede, Mass Spectr. Rev. 16:145-162 (1997). The two-dimensional gel electrophoresis can be performed using methods known in the art. See, e.g., Deutscher ed., Methods In Enzymology vol. 182. In certain cases, biomolecules in a sample are separated by, e.g., isoelectric focusing, during which biomolecules in a sample are separated in a pH gradient until they reach a spot where their net charge is zero (isoelectric point). This first separation step results in one-dimensional array of biomolecules. The biomolecules in one-dimensional array is further separated using a technique generally distinct from that used in the first separation step. Typically, two-dimensional gel electrophoresis can separate chemically different biomolecules in the molecular mass range from 1000-200,000 Da within complex mixtures. The pI range of these gels is about 3-10 (wide range gels).

As another example, high performance liquid chromatography (HPLC) can also be used to separate a mixture of biomolecules in a sample based on their different physical properties, such as polarity, charge and size. HPLC instruments typically consist of a reservoir of mobile phase, a pump, an injector, a separation column, and a detector. Biomolecules in a sample are separated by injecting an aliquot of the sample onto the column. Different biomolecules in the mixture pass through the column at different rates due to differences in their partitioning behavior between the mobile liquid phase and the stationary phase. A fraction that corresponds to the molecular weight and/or physical properties of one or more biomarkers can be collected. The fraction can then be analyzed by gas phase ion spectrometry to detect biomarkers. For example, the spots can be analyzed using either MALDI or SELDI as described herein.

Optionally, a biomarker can be modified before analysis to improve its resolution or to determine its identity. For example, the biomarkers may be subject to proteolytic digestion before analysis. Any suitable protease may be used. Proteases, such as trypsin, that are likely to cleave the biomarkers into a discrete number of fragments are particularly useful. The fragments that result from digestion may function as a fingerprint for the biomarkers, thereby enabling their detection indirectly. This is particularly useful where there are biomarkers with similar molecular masses that might be confused for the biomarker in question. Also, proteolytic fragmentation is useful for

high molecular weight biomarkers because smaller biomarkers are more easily resolved by mass spectrometry. Optionally, the identity of the biomarkers can be further determined by matching the physical and chemical characteristics of the biomarkers in a protein database (e.g., SwissProt).

Optionally samples containing biomarkers can be treated with one or more stabilizing agent and the container used for collection of the sample(s) may be pretreated with one or more stabilizing agent prior to measuring the levels of biomarkers. The term "stabilizing agent" refers to one or more molecules, such as polypeptides or nucleic acids, that can be used to prevent the degradation of the biomarkers. In one embodiment, the stabilizing agent is a protease inhibitor, including any of 4-(2-Aminoethyl) benzenesulphonyl fluoride (AEBSF) and Pefabloc SC, Antipain and Antipain-dihydrochloride, Aprotinin, Benzamidine and Benzamidine hydrochloride, Bestatin, Chymostatin, E-64 (L-trans-epoxysuccinyl-leucylamide-(4-guanido)-butane or N-[N-(L-trans-carboxyoxiran-2-carbonyl)-L-leucyl]-agmatine), Ethylenediaminetetraacetic acid and its sodium salt (EDTA-Na2), Leupeptin, Ethylmaleimide, Pepstatin and Pepstatin A, Phosphoramidon, Sodium azide, Trypsin inhibitor or ε-aminocaproic acid.

Levels of a biomarker discussed herein that is useful in a method of the present invention (e.g., Hp and/or HpRP, IL-6) may be assessed by any of a wide variety of well known methods for detecting expression of a transcribed molecule or its corresponding protein. Non-limiting examples of such methods include immunological methods for detection of secreted proteins, protein purification methods, protein function or activity assays, nucleic acid hybridization methods, nucleic acid reverse transcription methods, and nucleic acid amplification methods. In one embodiment, levels of a bio marker is assessed using an ELISA assay. Resulting values may be compared to a control or known (pre-established) standard. As used herein, the term "control" refers to the levels of the biomarker in a sample obtained from a reference subject.

Biomarkers such as Hp and/or HpRP and IL-6 are preferably captured with capture reagents immobilized to a solid support, such as any biochip described herein, a multiwell microtiter plate, a resin, or other suitable support. A preferred mass spectrometric technique for use in the invention is Surface Enhanced Laser Desorption and Ionization (SELDI), as described, for example, in U.S. Patent No. 5,719,060 and No. 6,225,047, in which the surface of a probe that presents the analyte to the energy source

plays an active role in desorption/ionization of analyte molecules. In this context, the term "probe" refers to a device adapted to engage a probe interface and to present an analyte to ionizing energy for ionization and introduction into a gas phase ion spectrometer, such as a mass spectrometer. A probe typically includes a solid substrate, either flexible or rigid, that has a sample-presenting surface, on which an analyte is presented to the source of ionizing energy.

One version of SELDI, called "Surface-Enhanced Affinity Capture" or "SEAC," involves the use of probes comprised of a chemically selective surface ("SELDI probe"). A "chemically selective surface" is one to which is bound either the adsorbent, also called a "binding moiety,"" or "capture reagent," or a reactive moiety that is capable of binding a capture reagent, e.g., through a reaction forming a covalent or coordinate covalent bond.

The phrase "reactive moiety" here denotes a chemical moiety that is capable of binding a capture reagent. Epoxide and carbodiimidizole are useful reactive moieties to covalently bind polypeptide capture reagents such as antibodies or cellular receptors. Nitriloacetic acid and iminodiacetic acid are useful reactive moieties that function as chelating agents to bind metal ions that interact noncovalently with histidine containing peptides. A "reactive surface" is a surface to which a reactive moiety is bound. An "adsorbent" or "capture reagent" can be any material capable of binding a biomarker of the invention. Suitable adsorbents for use in SELDI, according to the invention, are described in U.S. Patent No. 6,225,047.

One type of adsorbent is a "chromatographic adsorbent," which is a material typically used in chromatography. Chromatographic adsorbents include, for example, ion exchange materials, metal chelators, immobilized metal chelates, hydrophobic interaction adsorbents, hydrophilic interaction adsorbents, dyes, mixed mode adsorbents (e.g., hydrophobic attraction/electrostatic repulsion adsorbents). "Biospecific adsorbent" is another category, for adsorbents that contain a biomolecule, e.g., a nucleotide, a nucleic acid molecule, an amino acid, a polypeptide, a simple sugar, a polysaccharide, a fatty acid, a lipid, a steroid or a conjugate of these (e.g., a glycoprotein, a lipoprotein, a glycolipid). In certain instances, the biospecific adsorbent can be a macromolecular structure such as a multiprotein complex, a biological membrane or a virus. Illustrative biospecific adsorbents are antibodies, receptor proteins, and nucleic acids. A biospecific

adsorbent typically has higher specificity for a target analyte than a chromatographic adsorbent.

Another version of SELDI is Surface-Enhanced Neat Desorption (SEND), which involves the use of probes comprising energy absorbing molecules that are chemically bound to the probe surface ("SEND probe"). The phrase "Energy absorbing molecules" (EAM) denotes molecules that are capable of absorbing energy from a laser desorption ionization source and, thereafter, contributing to desorption and ionization of analyte molecules in contact therewith. The EAM category includes molecules used in MALDI, frequently referred to as and is exemplified by cinnamic acid derivatives, sinapinic acid (SPA), cyano-hydroxy-cinnamic acid (CHCA) and dihydroxybenzoic acid, ferulic acid, and hydroxyaceto-phenone derivatives. The category also includes EAMs used in SELDI, as enumerated, for example, by U.S Patent No. 5,719,060.

Another version of SELDI, called Surface-Enhanced Photolabile Attachment and Release (SEPAR), involves the use of probes having moieties attached to the surface that can covalently bind an analyte, and then release the analyte through breaking a photolabile bond in the moiety after exposure to light, e.g., to laser light. For instance, see U.S. Patent No. 5,719,060. SEPAR and other forms of SELDI are readily adapted to detecting a biomarker or biomarker profile, pursuant to the present invention.

The detection of the biomarkers according to the invention can be enhanced by using certain selectivity conditions, e.g., adsorbents or washing solutions. The phrase "wash solution" refers to an agent, typically a solution, which is used to affect or modify adsorption of an analyte to an adsorbent surface and/or to remove unbound materials from the surface. The elution characteristics of a wash solution can depend, for example, on pH, ionic strength, hydrophobicity, degree of chaotropism, detergent strength, and temperature.

In some embodiments of the invention, a sample is analyzed by means of a "biochip," a term that denotes a solid substrate having a generally planar surface, to which a capture reagent (adsorbent) is attached. Frequently, the surface of a biochip comprises a plurality of addressable locations, each of which has the capture reagent bound there. A biochip can be adapted to engage a probe interface and, hence, function as a probe, which can be inserted into a gas phase ion spectrometer, preferably a mass

spectrometer. Alternatively, a biochip of the invention can be mounted onto another substrate to form a probe that can be inserted into the spectrometer.

A variety of biochips is available for the capture of biomarkers, in accordance with the present invention, from commercial sources such as Ciphergen Biosystems (Fremont, CA), Packard BioScience Company (Meriden, CT), Zyomyx (Hayward, CA), and Phylos (Lexington, MA). Exemplary of these biochips are those described in U.S. Patents Nos. 6,225,047, 6,329,209, and in PCT Publication Nos. WO 99/51773 and WO 00/56934.

A substrate with an adsorbent is contacted with the urine sample for a period of time sufficient to allow biomarker that may be present to bind to the adsorbent. After the incubation period, the substrate is washed to remove unbound material. Any suitable washing solutions can be used; preferably, aqueous solutions are employed. An energy absorbing molecule then is applied to the substrate with the bound biomarkers. As noted, an energy absorbing molecule is a molecule that absorbs energy from an energy source in a gas phase ion spectrometer, thereby assisting in desorption of biomarkers from the substrate. Exemplary energy absorbing molecules include, as noted above, cinnamic acid derivatives, sinapinic acid and dihydroxybenzoic acid. Preferably sinapinic acid is used.

Once captured on a substrate, e.g., biochip or antibody, any suitable method can be used to measure one or more biomarkers in a sample. For example, biomarkers can be detected and/or measured by a variety of detection methods including for example, gas phase ion spectrometry methods, optical methods, electrochemical methods, atomic force microscopy and radio frequency methods. Using these methods, one or more biomarkers can be detected.

In one embodiment, methods of detection and/or measurement of the biomarkers use mass spectrometry and, in particular, SELDI. SELDI refers to a method of desorption/ ionization gas phase ion spectrometry (e.g., mass spectrometry) in which the analyte is captured on the surface of a SELDI probe that engages the probe interface. In "SELDI MS," the gas phase ion spectrometer is a mass spectrometer. SELDI technology is described in more detail above.

Another method for detection of biomarkers employs acustic membrane microparticle (AMMP) resonance, a variant of surface plasmon resonance spectroscopy

(Bioscale, Cambridge, MA) as described, for example in e.g., US2009-0148857 and US2007-0281371.

In another embodiment, an immunoassay can be used to detect and analyze biomarkers in a sample. An immunoassay is an assay that uses an antibody to specifically bind an antigen (e.g., a biomarker). An immunoassay is characterized by the use of specific binding properties of a particular antibody to isolate, target, and/or quantify the antigen. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular protein at least two times the background and do not substantially bind in a significant amount to other proteins present in the sample. Specific binding to an antibody under such conditions may require an antibody that is selected for its specificity for a particular protein. For example, polyclonal antibodies raised to a biomarker from specific species such as rat, mouse, or human can be selected to obtain only those polyclonal antibodies that are specifically reactive with that biomarker and not with other proteins, except for polymorphic variants and alleles of the biomarker. This selection may be achieved by subtracting out antibodies that cross-react with the biomarker molecules from other species.

Using purified biomarkers or their nucleic acid sequences, antibodies that specifically bind to a biomarker (e.g., Hp and/or HpRP) can be prepared using any suitable methods known in the art. See, e.g., Coligan, Current Protocols in Immunology (1991); Harlow & Lane, Antibodies: A Laboratory Manual (1988); Goding, Monoclonal antibodies: Principles and Practice (2d ed. 1986); Kohler & Milstein, Nature 256:495-497 (1975); Huse et al., Science 246:1275-1281 (1989); Ward et al., Nature 341:544-546 (1989).

Generally, a sample obtained from a subject can be contacted with the antibody that specifically binds the biomarker. Optionally, the antibody can be fixed to a solid support to facilitate washing and subsequent isolation of the complex, prior to contacting the antibody with a sample. Examples of solid supports include glass or plastic in the form of, e.g., a microtiter plate, a stick, a bead, or a microbead. Antibodies can also be attached to a probe substrate or a protein chip.

After incubating the sample with antibodies, the mixture is washed and the antibody-marker complex formed can be detected. This can be accomplished by incubating the washed mixture with a detection reagent. This detection reagent may be,

e.g., a second antibody which is labeled with a detectable label. Exemplary detectable labels include magnetic beads, fluorescent dyes, radiolabels, enzymes (e.g., horse radish peroxide, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic beads. Alternatively, the biomarker in the sample can be detected using an indirect assay, wherein, for example, a second, labeled antibody is used to detect bound biomarker-specific antibody, and/or in a competition or inhibition assay wherein, for example, a monoclonal antibody which binds to a distinct epitope of the biomarker is incubated simultaneously with the mixture.

Methods for measuring the amount or presence of an antibody-marker complex include, for example, detection of fluorescence, luminescence, chemiluminescence, absorbance, reflectance, transmittance, birefringence or refractive index (e.g., surface plasmon resonance, ellipsometry, a resonant mirror method, a gating coupler waveguide method or interferometry). Optical methods include microscopy (both confocal and non-confocal), imaging methods and non-imaging methods. Electrochemical methods include voltametry and amperometry methods. Radio frequency methods include multipolar resonance spectroscopy. Useful assays are well known in the art, including, for example, an enzyme immune assay (EIA) such as enzyme-linked immunosorbent assay (ELISA), a radioimmune assay (RIA), a Western blot assay, or a slot blot assay.

Immunoassays can be used to determine presence or absence of a biomarker in a sample as well as the quantity of a biomarker in a sample. The amount of an antibody-marker complex can be determined by comparing to a standard. A standard can be, e.g., a known compound or another protein known to be present in a sample. It is understood that the test amount of biomarker need not be measured in absolute units, as long as the unit of measurement can be compared to a control.

When the sample is measured and data is generated, e.g., by mass spectrometry, the data may then be analyzed by a computer software program. In certain cases, a biomarker bound to the substrate can be detected in a gas phase ion spectrometer. The biomarkers are ionized by an ionization source such as a laser, the generated ions are collected by an ion optic assembly, and then a mass analyzer disperses and analyzes the passing ions. The detector then translates information of the detected ions into mass-to-

charge ratios. Detection of a biomarker typically will involve detection of signal intensity. Thus, both the quantity and mass of the biomarker can be determined.

Generally, data generated by desorption and detection of biomarkers can be analyzed with the use of a programmable digital computer. The computer program analyzes the data to indicate the number of biomarkers detected, and optionally the strength of the signal and the determined molecular mass for each biomarker detected. Data analysis can include steps of determining signal strength of a biomarker and removing data deviating from a predetermined statistical distribution. For example, the observed peaks can be normalized, by calculating the height of each peak relative to some reference. The reference can be background noise generated by the instrument and chemicals such as the energy absorbing molecule which is set as zero in the scale.

A computer can transform the resulting data into various formats for display. The standard spectrum can be displayed, but in one useful format only the peak height and mass information are retained from the spectrum view, yielding a cleaner image and enabling biomarkers with nearly identical molecular weights to be more easily seen, in another useful format, two or more spectra are compared, conveniently highlighting unique biomarkers and biomarkers that are up- or down-regulated between samples. Using any of these formats, one can readily determine whether a particular biomarker is present in a sample.

Software used to analyze the data can include code that applies an algorithm to the analysis of the signal to determine whether the signal represents a peak in a signal that corresponds to a biomarker according to the present invention.

EXAMPLES

Example 1 Proteomic study of cord blood obtained from newborns.

This example describes proteomic assessment of cord blood and results of that assessment.

In a prospective study design, Applicant enrolled 155 premature newborns whose mothers presented with symptoms of preterm labor or PPROM and had a clinically indicated amniocentesis to rule out infection. Intra-amniotic inflammation was assessed by SELDI-TOF mass spectrometry. Neonatal hematological indices and sepsis categorization were assessed from blood specimens and cultures obtained immediately

following delivery for all neonates. Proteomic studies were conducted in 2 phases. In the discovery phase, the study group consisted of 3 neonates with confirmed positive *E. coli* sepsis, high CB IL-6 (>90 pg/mL), positive AF cultures and grade 4 histological chorioamnionitis. Three newborns matched for GA, low CB IL-6 (<9 pg/mL) delivered in the setting of idiopathic PTB served as control (reference). Applicant performed 2D-DIGE after albumin and IgG depletion. Two of the three 2D-DIGE gels analyzed for the purpose of biomarker identification are presented in Figure 13. A total of 69 spots that were differentially expressed >3-fold. These were automatically picked and subjected to robotic tryptic digest and tandem mass spectrometry. 230 identities were matched in International Protein Index (IPI) database. 68 non-redundant IPI identities were extracted, of which 20 were common to 2/3 gels. Because several different spots matched to the same protein precursor, only 9 proteins were retained as potential biomarkers. ApoH, ApoE, vitamin D binding protein and albumin appeared in average downregulated. ApoA4, α-fetoprotein, α2-macroglobulin, Hp and HpRp appeared up-regulated. The highest fold increase differences were seen for Hp (32-fold) and for HpRP (60-fold). In the validation phase immunoassays were performed to confirm or refute the differential expression of these potential biomarkers in the entire cohort. Following correction for GA, Applicant determined that significant differences between neonates without and with EONS (confirmed by culture [n=8] or suspected [n=36]) maintained only for Hp and HpRP (Figure 14A). Applicant next asked the question whether Hp and HpRP have relevance for neonatal outcomes other than EONS, which as shown earlier, has inherent clinical diagnostic challenges. In multivariate logistic regression analysis Applicant determined that after correction for GA and EONS status, neonates who developed IVH or died had higher Hp levels at birth (F ratio=11.4, $P<0.001$) (Figure 14B). Other variables excluded from the model were level of intra-amniotic inflammation (MR score), severity of histological chorioamnionitis, CB IL-6, cord pH, antenatal steroid and antibiotic use.

Example 2 Identification of protein biomarkers identified in the cord blood take and delivery that are diagnostic of and predictive for EONS.

Proteomic analysis of cord blood samples (using the methodology described in Buhimschi et al. PLoS ONE 3 (4): e2049) identified 20 proteins and protein fragments

(listed in Table 1) in cord blood samples from babies who subsequently developed EONS, whose levels were either increased or decreased by 3-fold or greater, compared to controls. The mothers had a clinically indicated amniocentesis to rule out infection. For research purposes, intra-amniotic inflammation was assessed by SELDI-TOF mass spectrometry. In this study, histological chorio-amnionitis was scored using well recognized criteria. Umbilical cord blood was used to assess fetal acid-base and inflammatory status at birth. 16s-RNA gene amplification was applied in a select group of cord blood samples to provide proof of concept that bacteria may be present in neonates who have EONS, but negative microbial cultures. Neonatal hematological indices and sepsis categorization were assessed from blood specimens and cultures obtained immediately following delivery for all neonates. Analysis of the levels of one or more (a, at least one) of these proteins or protein fragments is useful in diagnosing EONS.

Table 1

Proteins and Protein Fragments Identified in Cord Blood of Babies Who Developed EONS

IPI ID#	Abbreviation/Name
IPI00021842	APOE apoliprotein E precursor
IPI00022434	ALB uncharacterized protein ALB
IPI00022443	AFP alpha-fetoprotein precursor
IPI00216773	ALB ALB protein
IPI00298828	APOH Beta-2-glycoprotein 1 precursor
IPI00304273	APOA4 Apolipoprotein A-IV precursor
IPI00384697	ALB isoform 2 of serum albumin procursor
IPI00431645	HP HP protein
IPI00477597	HPR isoform 1 of Haptoglobin-related protein precursor
IPI00478003	A2M Alpha-2-macroglobulin precursor
IPI00478493	HP Haptoglobin isoform 2 preprotein
IPI00555812	GC Vitamin D-binding protein precursor
IPI00607707	HPR Isoform 2 of Haptoglobin-related protein precursor
IPI00641737	HP Haptoglobin precursor
IPI00742696	GC Vitamin D-binding protein precursor
IPI00745872	ALB Isoform 1 of serum albumin precursor
IPI00847179	APOA4 apolipoprotein A-IV precursor
IPI00878517	ALB 56kDa protein
IPI00878953	APOE MRNA for apolipoprotein E
IPI00879456	APOE 25kDa protein

Example 3 Assessment of Hp phenotype variations and levels as determinants of susceptibility to adverse neonatal outcomes.

OBJECTIVE: Hp is an immunomodulatory protein linked to human susceptibility versus resistance to infection. Two allelic variants (Hp1 and Hp2) code proteins with different α-chains. Developmental regulation of Hp transcription shows that Hp is near to absent at birth, with the adult phenotype (Hp1-1; Hp2-1 or Hp2-2) emerging in the first year of life. Characterization of the cord blood proteome enabled the discovery of Hp as a biomarker of EONS. Hp phenotype variations and levels are critical determinants of susceptibility to adverse neonatal outcomes.

STUDY DESIGN: Applicant analyzed cord blood from 163 preterm neonates (GA: 29 [23-34] wks). Fetal inflammatory status was assessed by IL-6 levels. Symptoms, hematological criteria and bacterial cultures were used to define EONS. Expression patterns and Hp phenotyping was performed using Western blot. Serum from adult individuals and normal term newborns (n=19) served as reference (control). Differential expression of Hp was validated using ELISA. Relationships among Hp switching-on, levels, phenotypes and outcomes including EONS, IVH and death were explored using multivariate regression and nonparametric statistics.

RESULTS showed the following:

1) EONS (n=46) was characterized more often by antenatal Hp switching-on ($P<0.001$) and significantly higher Hp levels ($P<0.001$), independent of GA and IL-6;

2) Phenotypes impacted Hp level: Hp2-1>Hp2-2>Hp1-1 ($P<0.001$);

3) Newborns with EONS and Hp2-1 had the highest Hp levels, double that of Hp1-1 ($P=0.003$);

4) Neonates who developed IVH or died (n=36) had higher Hp levels ($P<0.001$) independent of GA, IL-6, cord pH, steroid and antibiotic use;

5) Newborns with switched-on Hp at birth had an increased risk of IVH and death (RR: 3.6 [1.8-7.8]); 6) No term neonate (but all adults) had switched-on Hp.

CONCLUSION: Applicant provides the first evidence that Hp switches on precociously to the adult phenotype in newborns with EONS. The quantitative and qualitative changes in Hp expression provide the basis for predicting newborns at risk for IVH and/or death and targeted interventions at birth.

Example 4 Cord blood Hp and HpRP signature as EONS biomarker.

To validate cord blood Hp and HpRP ELISA results and provide further understanding of how Hp phenotypes impact on total immunoreactivity, Applicant performed Western blot analysis under reducing and denaturing conditions (normalized for amount of total protein to rule out hemoconcentration as a potential bias). Control sera from adult Hp1-1 and Hp2-2 individuals served as reference. As illustrated in Figure 16 for representative CB samples, Applicant made several interesting observations: 1) Each lane yields a pattern of 3 possible Hp and HpRP bands (or lack thereof) shown by the red arrows; 2) CB Hp and HpRP signature is composed of at least 5 pieces of information for each baby: antenatal switching, defined with reference to this figure as appearance of at least 2 of 3 Hp and HpRP bands (newborns b, d, g, i-k & n); Hp and HpRP level; Hp phenotype; relationship of Hp and HpRP with IL-6 levels; relationship of Hp and HpRP with bacterial footprints; 3) The amount of sample required to yield this information is minimal (<1μL cord serum).

At this time Applicant analyzed Hp and HpRP signatures for only a small portion of the available samples. Following are several aspects derived from an analysis of CB retrieved from 14 random newborns in the cohort (Figure 16). The results point out that 7 fetuses had positive antenatal Hp switching, yet only 3 of these had a diagnosis of EONS (marked as E). This suggests that the current clinical and hematological criteria for EONS still miss newborns who progress to have major adverse outcomes. In contrast, the CB of baby "e", a 28-week newborn of 1,200 grams who did not appear to have a positive antenatal switch, tested positive for *S. aureus*. This neonate received *i.v.* antibiotics and had a favorable course in NBSCU (the baby was discharged after 24 days with no evidence of short or long-term complications). Based on Applicant's assessment, it is likely that the cultures were contaminated *ex vivo*. Also notable is baby "h," who out of all 14 newborns had the highest level of CB IL-6 (>1,200 pg/mL), yet did not appear to have switched its Hp and HpRP expression antenatally. This Hp signature is consistent with a genetically determined Hp0-0 phenotype, which results in a fetus unable to up-regulate an important endogenous antioxidant resource such as Hp, despite excess transcriptional trigger (IL-6). It is thus not surprising to determine that this newborn had the worst outcome despite a birthweight of 1,380 grams (GA at delivery $31^{2/7}$ weeks). The mother was admitted with PPROM at 27 weeks and was

managed expectantly with antibiotics in accordance to ACOG guidelines. Development of non-specific signs of chorioamnionitis on day 17 of PPROM prompted an amniocentesis to rule out infection. The AF tested positive for S. viridans. By mass spectrometry SELDI-TOF Applicant found an MR score 4 consistent with "severe inflammation." Histological analysis of the placenta demonstrated stage III chorioamnionitis and grade 4 funisitis. Based on this case, Applicant questioned whether other newborns in the cohort had a similar signature (elevated cord IL-6 and absent Hp and HpRP switching). Preliminary data analysis identified two cases who matched baby "h." One neonate (600 grams at birth) died on postnatal day 2. A second neonate weighed 1,280 grams at birth (GA at delivery $30^{2/7}$ weeks). The baby was admitted to NBSCU where EONS was established based on a positive blood culture for *E. coli*. The baby developed grade 4 IVH and undergoes follow-up at Yale Child Study Center for cerebral palsy. The AF MR score showed evidence of "severe inflammation" (MR score 3) and display proteomic biomarkers characteristic for bleeding. AF in this case tested negative for infection by conventional tests. Applicant's measure of CB IL-6 was 1,533 pg/mL. The level of total Hp and HpRP by ELISA was undetectable; lack of Hp switching was observed by Western blot.

Example 5 Assessment of elements for early diagnosis and pathogenic classification of EONS.

Described herein is an algorithm for early diagnosis and pathogenic classification of EONS, based on one (a, at least one, one or more) of the following elements:

(a) Cord blood Hp and HpRP switching, represented qualitatively as positive or negative and derived from Hp and HpRP immunoreactivity above an established cut-off;

(b) Cord blood Hp and HpRP level represented quantitatively as the level of Hp and HpRP immunoreactivity;.

(c) Cord blood Hp and HpRP phenotype, which is Hp 0-0, Hp 1-1, 1-2 or 2-2;

(d) Relationship of cord blood Hp and HpRP level with cord blood IL-6; and

(e) Relationship of cord blood Hp and HpRP level and cord blood IL-6 with bacterial fingerprints.

One or more (a, at least one) of these elements can be used for early diagnosis and pathogenic classification of EONS (one or more, two, two or more, three, three or more, four, four or more or five elements can be used). In a specific embodiment, the method comprises assessing at least elements (a) – (d). In addition, element (e) can be included in the method, if it is of interest to distinguish among the 3 variants of EONS (I, II or III), in order to identify specific drug targets, such PAMPs or DAMPs. If the testing enables sub classification of EONS, antibiotics could be substituted by anti DAMP and/or anti PAMP strategies, such as: N-acetylcysteine, ethylpyruvate or quercetin (flavonoid with potent anti-oxidant properties and DAMP inhibitor)[77] or soluble RAGE (sRAGE, an extracellular truncated form of RAGE which acts as a decoy DAMP receptor) or antibodies or peptides targeted against RAGE or HMGB1 attenuate the lethal effects of endotoxin, acetaminophen and ischemia-reperfusion.[78,79, 80,81,82,83,84]

There are several reasons why it is particularly useful to determine these elements concurrently for an accurate diagnosis of EONS (and why probably other authors concluded that haptoglobin is not a good biomarker of sepsis in newborns.[85, 86]) First, the Hp and HpRP level measured in immunoassay is dependent not on only on the amount of Hp in solution, but also on the phenotype that is measured. Applicant observed that in ELISA, Hp2-2 measures consistently higher than Hp 1-1. Although the exact reasons are still to be elucidated, it is possible that the multimeric aggregates formed by Hp2-2 have a higher antibody affinity than Hp1-1. Therefore, the ELISA level is a non-linear combination of the amount and phenotype and not of the concentration alone. To substantiate this premise, Applicant tested how phenotypes impacted on Hp level and found among newborns with positive switching a significant difference in the measured level of Hp and HpRP immunoreactivity with Hp2-1>Hp2-2>Hp1-1 ($P<0.001$). Newborns with EONS and Hp2-1 had the highest Hp levels, double that of Hp1-1 ($P=0.003$). Although this could also suggest that upon the same inflammatory insult, the heterozygote phenotype produces more Hp than does the homozygous phenotype, it could also mean that the fetal Hp phenotype has relevance for complications occurring in postnatal life that could make the heterozygote more susceptible to diseases with a free radical component. Indeed, neonates who developed IVH or died ($n=36$) had higher Hp levels ($P<0.001$) independent of gestational age, cord blood IL-6, cord pH, steroid and antibiotic use. Overall, newborns with switched Hp at

birth had an increased risk of IVH & death (Relative Risk: 3.6 [1.8-7.8]). Because a minority of fetuses may have genetically determined Hp 0-0, they will not be able to respond with Hp switching when faced with an inflammatory insult. These fetuses will have highly elevated IL-6 levels in cord blood, in the absence of Hp and HPRP switching and it is likely that they will have the worst outcomes. Because of the importance of producing a tool to rule out EONS at birth, it is important to distinguish this small subgroup from the newborns who did not have a bacterial encounter *in utero*. The phenotype corrected cord blood ratio of Hp and HpRP-to-IL6 can be used to achieve this goal.

Described herein and represented in Table 2 is a simple diagnostic and treatment algorithm that can be implemented immediately in intensive neonatal care units and could serve to decrease considerably the adverse effects the costs of empiric antibiotic therapy.

Table 2

CB IL6	CB Hp& HpRP switching	CB Hp type	Conclusion	Prognosis and recommendation relative to antibiotic profilaxis in NICU
neg	neg	Hp 0-0	non-exposed and non-switched	Good. Should not receive antibiotics
pos	neg	Hp 0-0	exposed and non-switched	Poor. Should be admitted to NICU and treated
pos	pos	Hp 1-1, 1-2 or Hp 2-2	exposed and switched	Variable depending on Hp type. Should be admitted to NICU and treated
neg	pos	Hp 1-1, 1-2 or Hp 2-2	past-exposed and switched	Poor. This is a rare instance that occurs mostly in neutropenic fetuses. Should be admitted and treated

This algorithm is underscored by the observation that no healthy term neonate tested to date (n=19), but all matched mothers, had switched Hp. Moreover, this algorithm could also be extended to ruling out EONS in term newborns. To this end, Applicant tested cord blood in one case of clinical chorioamnionitis at term and found that this newborn, who was diagnosed with suspected EONS and received antibiotics, also had positive Hp and HpRP switching at birth. An advantage of this algorithm is that it can be assessed with various methodologies within 1 hour of birth. The high level of Hp and HpRP (microgram/mL) can be easily detected in various rapid methods and can even be multiplexed with IL-6, since the only subcategory that benefits from addition of IL-6 would have a high CB IL-6 immunoreactivity.

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What is claimed is:

1. An *ex vivo* method for diagnosing, or predicting the future development of, early onset neonatal sepsis (EONS) and/or at least one related complication of prematurity (preterm birth) in a subject, comprising the step of detecting an increase in the level of haptoglobulin (Hp) and/or haptoglobulin-related protein (HpRP) in a sample of blood from the subject, wherein if the level of Hp and/or HpRP is increased, the subject is diagnosed as having or is at risk of future development of EONS and/or at least one related complication.

2. An *ex vivo* method for diagnosing, or predicting the future development of, early onset neonatal sepsis and/or at least one related complication of prematurity (preterm birth) in a subject, comprising the step of detecting, in a sample of blood from the subject, a change in the level of one or more proteins selected from:

IPI ID#	Abbreviation/Name
IPI00021842	APOE apoliprotein E precursor
IPI00022434	ALB uncharacterized protein ALB
IPI00022443	AFP alpha-fetoprotein precursor
IPI00216773	ALB ALB protein
IPI00298828	APOH Beta-2-glycoprotein 1 precursor
IPI00304273	APOA4 Apolipoprotein A-IV precursor
IPI00384697	ALB isoform 2 of serum albumin procursor
IPI00431645	HP HP protein
IPI00477597	HPR isoform 1 of Haptoglobin-related protein precursor
IPI00478003	A2M Alpha-2-macroglobulin precursor
IPI00478493	HP Haptoglobin isoform 2 preprotein
IPI00555812	GC Vitamin D-binding protein precursor
IPI00607707	HPR Isoform 2 of Haptoglobin-related protein precursor
IPI00641737	HP Haptoglobin precursor
IPI00742696	GC Vitamin D-binding protein precursor
IPI00745872	ALB Isoform 1 of serum albumin precursor
IPI00847179	APOA4 apolipoprotein A-IV precursor
IPI00878517	ALB 56kDa protein
IPI00878953	APOE MRNA for apolipoprotein E
IPI00879456	APOE 25kDa protein

and fragments thereof.

3. The method of claim 1 or claim 2, wherein the sample is a cord blood sample.

4. The method of any one of claims 1 to 3 for diagnosing or predicting the future development of intraventricular haemorrhage.

5. The method of any one of the preceding claims, wherein the sample of cord blood is collected at birth.

6. The method of any one of the preceding claims, wherein the detecting step comprises detecting a decrease in the level of apolipoprotein H and/or apolipoprotein E and/or vitamin D-binding protein.

7. The method of any one of claims 2, 4, 5 and 6, wherein the detecting step comprises detecting an increase in the levels of haptoglobulin and/or haptoglobulin-related protein.

8. The method of any one of the preceding claims, wherein the detecting step comprises detecting a decrease in the level(s) of apolipoprotein H and/or apolipoprotein E and/or vitamin D-binding protein and an increase in the levels of haptoglobulin and/or haptoglobulin-related protein.

9. The method of any one of the preceding claims wherein the subject is a premature neonate.

10. A method (such as an *ex vivo* method) of diagnosing, or predicting the future development of, early onset neonatal sepsis and/or at least one related complication of prematurity (preterm birth) in a subject, comprising determining if antenatal Hp switching on has occurred in the subject by analyzing a sample obtained from the subject for Hp; HpRP; or both Hp and HpRP, wherein if Hp; HpRp; or both Hp and HpRP is/are detected in the sample, antenatal HP switching on has occurred and the

subject has or is at increased risk of developing EONS and/or at least one related complication of prematurity (preterm birth).

11. The method of claim 10, wherein the subject is a premature neonate.

12. The method of claim 10 or claim 11, wherein the sample is a cord blood sample, a neonatal blood sample, a cerebrospinal fluid sample, a urine sample, a tear fluid sample, a meconium fluid or a fecal sample.

13. The method of any one of claims 10 to 12, further comprising determining the level of Hp; HpRP; or both Hp and HpRP in the sample and comparing the level with the corresponding level in an appropriate control.

14. The method of any one of claims 10 to 13, wherein the appropriate control is (a) a newborn (i) matched for gestational age with the subject; (ii) with low cord blood IL-6; and (iii) delivered in the setting of idiopathic preterm birth or (b) a newborn delivered from an uncomplicated pregnancy and delivery.

15. A method (such as an ex vivo method) of diagnosing, or predicting the future development of, early onset neonatal sepsis and/or at least one related complication(s) of prematurity (preterm birth) in a subject, comprising determining the level of Hp; HpRP; or Hp and HpRP in a sample of blood obtained from the subject, wherein if Hp; HpRP; or Hp and HpRP is/are present in the sample at a level equal to or greater than the level of Hp; HpRP; or Hp and HpRP in an appropriate control, the subject has or is at increased risk of future development of EONS and/or at least one related complication of prematurity (preterm birth).

16. The method of claim 10, wherein the subject is a premature neonate.

17. The method of claim 15 or claim 16, wherein the blood sample is a cord blood sample or a neonatal blood sample.

18. The method of any one of claims 15 to 17, wherein the level of Hp; HpRP; or Hp and HpRP is equal to or greater than the level in an appropriate control and the appropriate control is a premature neonate who progressed to EONS and/or at least one related complication of prematurity (preterm birth).

19. The method of any one of claims 15 to 17, wherein the level of Hp; HpRP; or Hp and HpRP is greater than the level in an appropriate control and the appropriate control is (a) a newborn (i) matched for gestational age with the subject; (ii) with low cord blood IL-6 (such as less than 9 pg/ml); and (iii) delivered in the setting of idiopathic preterm birth or (b) a newborn delivered from an uncomplicated pregnancy and delivery.

20. A method (such as an ex vivo method) of diagnosing, or predicting the future development of, early onset neonatal sepsis and/or at least one related complication of prematurity (preterm birth) in a subject, comprising determining if (a) cord blood IL-6 is positive, relative to an appropriate control, and (b) Hp and/or HpRP switching has occurred, wherein if cord blood IL-6 is positive and Hp and/or HpRP switching has occurred, the subject has or is at increased risk of future development of EONS and/or at least one related complication of PTB.

21. The method of claim 20, wherein the subject is a premature neonate.

22. The method of claim 20 or claim 21, wherein the blood sample is a cord blood sample or a neonatal blood sample.

23. The method of any one of claims 20 to 22, wherein the appropriate control for cord blood IL-6 is cord blood IL-6 in (a) a newborn (i) matched for gestational age with the subject; (ii) with low cord blood IL-6; and (iii) delivered in the setting of idiopathic preterm birth or (b) a newborn delivered from an uncomplicated pregnancy and delivery.

24. The method of any one of claims 14, 20, 21, 22 and 23, wherein the appropriate control has a cord blood IL-6 level of less than about 9 pg/mL.

25. A method (such as an ex vivo method) of diagnosing, or predicting the future development of, early onset neonatal sepsis and/or at least one related complication of prematurity (premature birth) in a subject, comprising determining IL-6 level and the phenotype of Hp in a sample of blood obtained from the subject, wherein if the IL-6 level is positive and the phenotype of Hp is Hp 0-0, Hp1-1, Hp2-1 or Hp2-2, the subject has or is at increased risk of future development of early onset neonatal sepsis and/or at least one related complication and if the IL-6 level is negative and the phenotype of Hp is Hp0-0, the subject does not have or is at less risk of developing EONS and/or at least one related complication of prematurity (premature birth).

26. A method (such as an ex vivo method) of diagnosing, or predicting the future development of, early onset neonatal sepsis and/or at least one related complication of prematurity (premature birth) in a subject, comprising determining the relationship of Hp with the presence of bacterial DNA in a sample of blood obtained from the subject, wherein if Hp and/or HpRP and bacterial DNA are present in the sample of blood, the subject has or is at risk of developing early onset neonatal sepsis and/or at least one related complication of prematurity (premature birth).

27. A method (such as an ex vivo method) of diagnosing, or predicting the future development of, early onset neonatal sepsis and/or at least one related complication of prematurity (premature birth) in a subject, comprising assessing at least two of the following characteristics in a sample of blood obtained from the subject: (a) antenatal Hp and/or HpRP switching on; (b) phenotype of Hp; HpRp; or Hp and HpRp; (c) level of Hp; HpRP; or Hp and HpRP; (d) cord blood IL-6 or the relationship of the level of Hp; HpRP; or Hp and HpRP to interleukin-6 level (referred to as Hp/IL-6 ratio); and (e) the relationship of Hp and/or HpRP with the presence of bacterial DNA, wherein if the sample is determined to comprise a blood signature comprising at least two

characteristics indicative of early onset neonatal sepsis or an increased risk of developing at least one related complication of prematurity (preterm birth), the subject is diagnosed as having early onset neonatal sepsis or an increased risk of developing at least one related complication of prematurity (preterm birth).

28. The method of claim 27, wherein antenatal Hp and/or HpRP switching on and cord blood IL-6 are assessed and Hp and/or HpRP switching on has occurred and cord blood IL-6 is positive.

29. The method of claim 27 or claim 28, wherein the subject is a premature neonate.

30. The method of any one of claims 27 to 29, wherein cord blood IL-6 is greater than about 9 pg/mL.

31. The method of any one of claims 1 to 30, wherein the complication can be IVH, bronchopulmonary dysplasia (BPD, need for oxygen at 36 weeks postmenstrual age), retinopathy of prematurity (ROP), cerebral palsy and/or death.

32. A method (such as an ex vivo method) of diagnosing, or predicting the future development of, early onset neonatal sepsis and/or at least one related complication of prematurity (preterm birth) in a subject, comprising assessing, in a blood sample obtained from the subject, IL-6 level; Hp and/or HpRP switching; and, optionally, Hp type, wherein:

(a) if the CB IL-6 level is negative (not elevated), relative to an appropriate control; Hp and/or HpRP switching is negative; and, optionally, Hp type is Hp 0-0, the subject does not have and is not at increased risk of developing EONS and/or at least one related complication(s);

(b) if the IL-6 level is elevated, relative to an appropriate control; Hp and HpRP switching is negative (has not occurred); and, optionally, Hp type is Hp 0-0, the subject

has or is at increased risk of developing EONS and/or at least one related complication(s);

(c) if the IL-6 level is elevated, relative to an appropriate control; Hp and HpRP switching is positive (has occurred); and, optionally, Hp type is Hp 1-1, Hp 1-2 or Hp 2-2, the subject has or is at risk of developing EONS and/or at least one related complication(s).

33. The method claim 32, wherein the blood sample is a cord blood sample or a neonatal blood sample.

34. The method of claim 32 or claim 33, wherein the subject is a premature neonate.

35. The method of any one of claims 32 to 34, wherein the complication can be IVH and/or bronchopulmonary dysplasia (BPD and/or need for oxygen at 36 weeks postmenstrual age) and/or retinopathy of prematurity (ROP) and/or cerebral palsy and/or death.

PROTEOMICS – MR SCORE

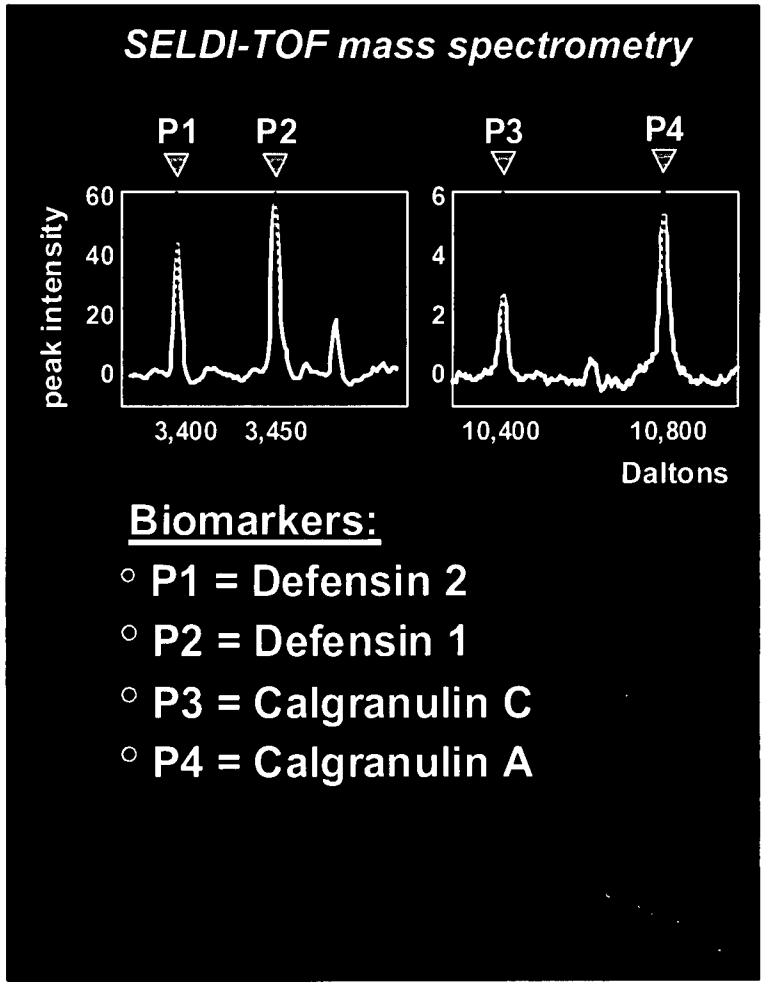


Figure 1.

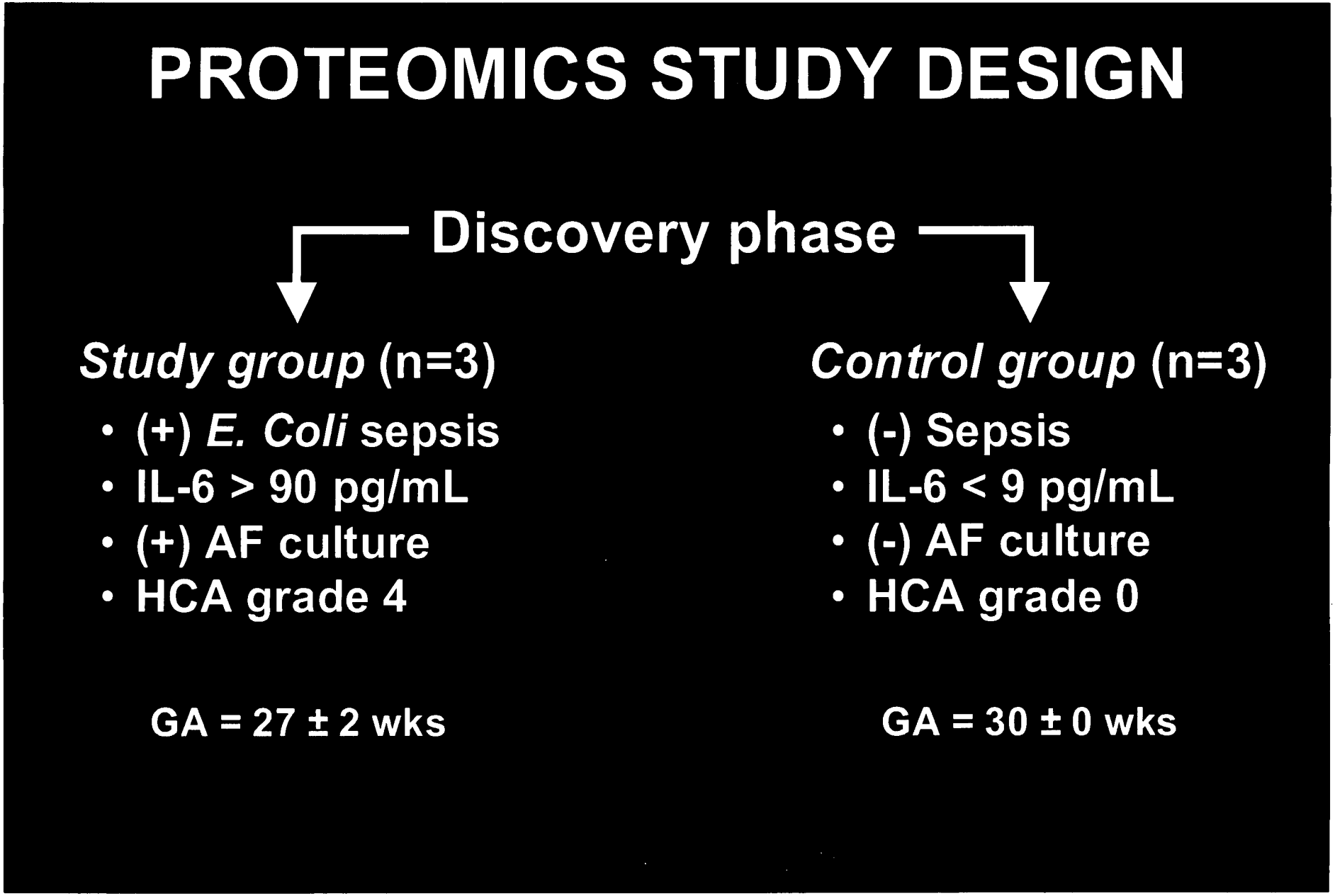

PROTEOMICS STUDY DESIGN
Discovery phase
Study group (n=3)
• (+) E. Coli sepsis
• IL-6 > 90 pg/mL
• (+) AF culture
• HCA grade 4
GA = 27 ± 2 wks
Control group (n=3)
• (-) Sepsis
• IL-6 < 9 pg/mL
• (-) AF culture
• HCA grade 0
GA = 30 ± 0 wks

Figure 2.

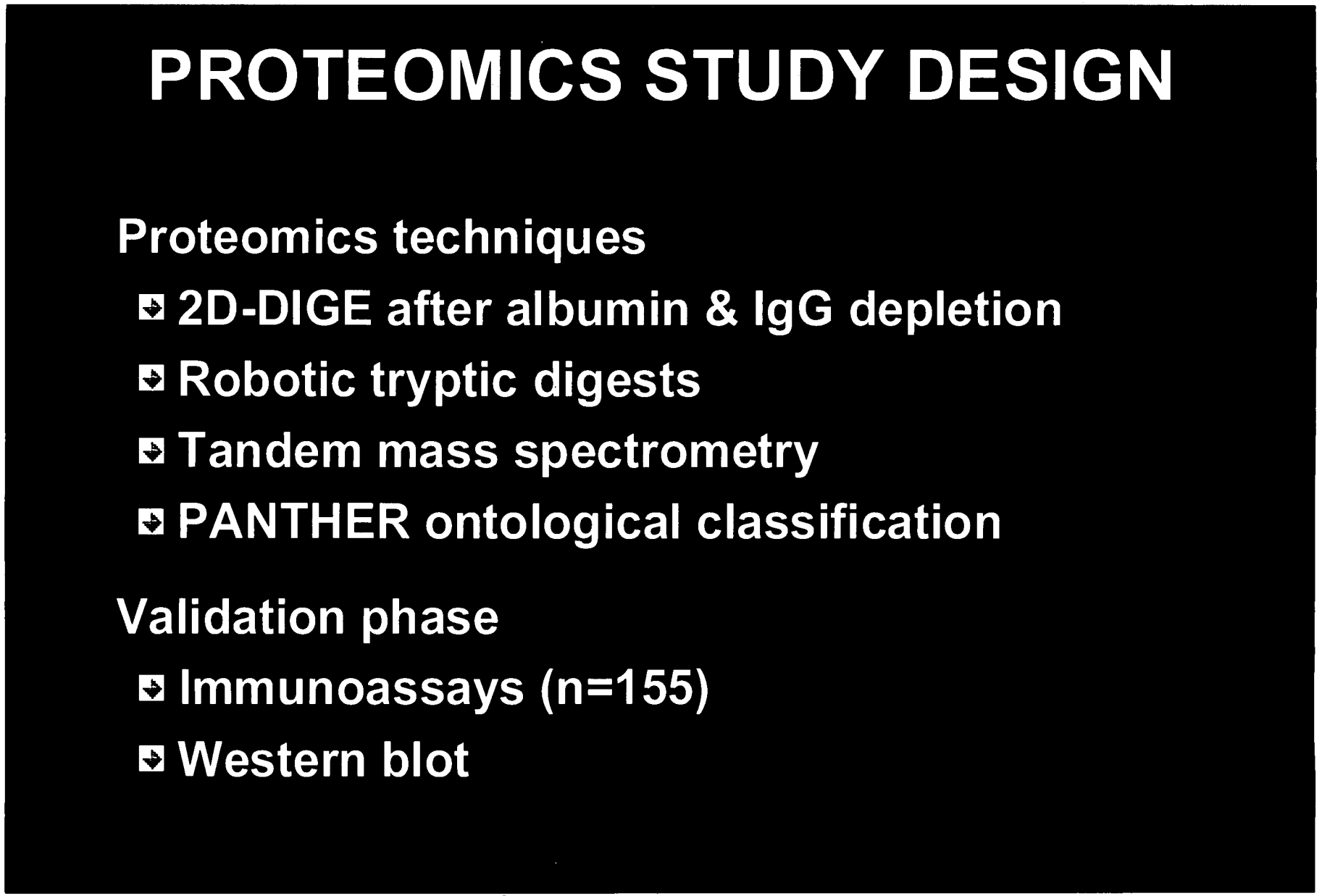

PROTEOMICS STUDY DESIGN
Proteomics techniques
2D-DIGE after albumin & IgG depletion
Robotic tryptic digests
Tandem mass spectrometry
PANTHER ontological classification
Validation phase
Immunoassays (n=155)
Western blot

Figure 3.

RESULTS

Median [IQ range]	EONS: NO (n=115)	EONS: YES (n=40)
GA at delivery *(wks)*	30 [28 - 33]	26 [25 - 30]
Birthweight *(g)*	1,450 [1,121 - 1,985]	920 [750 - 1,407]
Apgar - 1 min	8 [5 - 9]	5 [2 - 7]
Apgar - 5 min	9 [8 - 9]	8 [6 - 8]
Umbilical artery pH	7.32 [7.2 - 7.4]	7.33 [7.3 - 7.4]
Umbilical artery base deficit	4.4 [3.2 - 5.5]	5.1 [3.1 - 7.5]

Figure 4.

RESULTS

Median [IQ range]	EONS NO (n=115)	EONS YES (n=40)
Positive cultures	28 %	80 %
MR Score 3 - 4	2 [0 - 4]	4 [3 - 4]
Severe histological chorioamnionitis		
• Maternal	15 %	23 %
• Fetal	32 %	75 %

Figure 5.

NEONATAL SEPSIS

Median [IQ range]	EONS: NO (n=115)	EONS: YES (n=40)
Hematocrit (%)	47 [42 - 52]	43 [39 - 47]
Lymphocytes (%)	44 [32 - 58]	29 [23 - 44]
ABC (cells/mm3)	310 [130 - 677]	2,592 [1,557 - 3,795]
I:T ratio (%)	4 [1 - 6]	19 [14 - 25]
Positive blood cultures: n (%)	0 (0)	8 (20)

Figure 6.

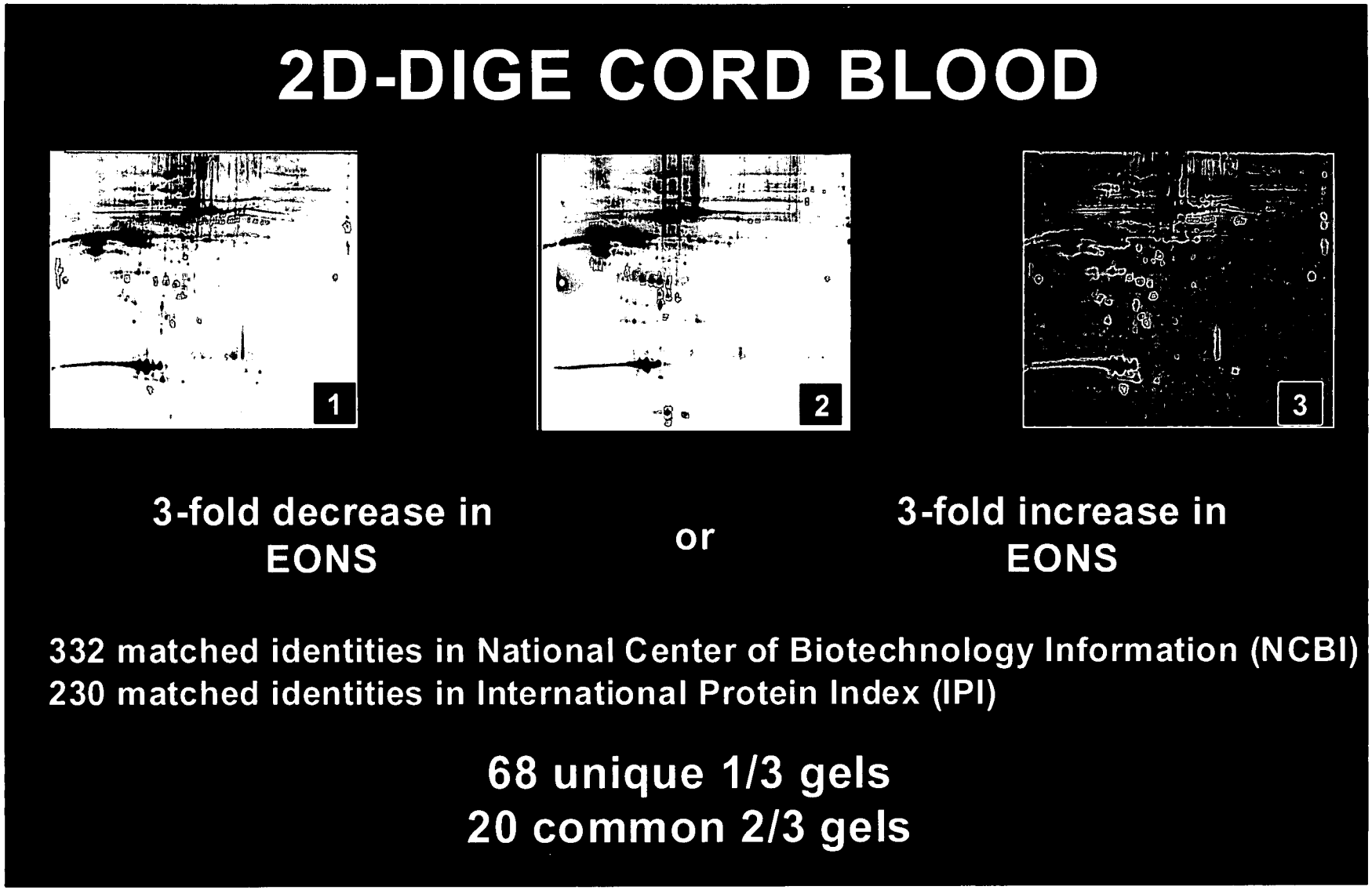
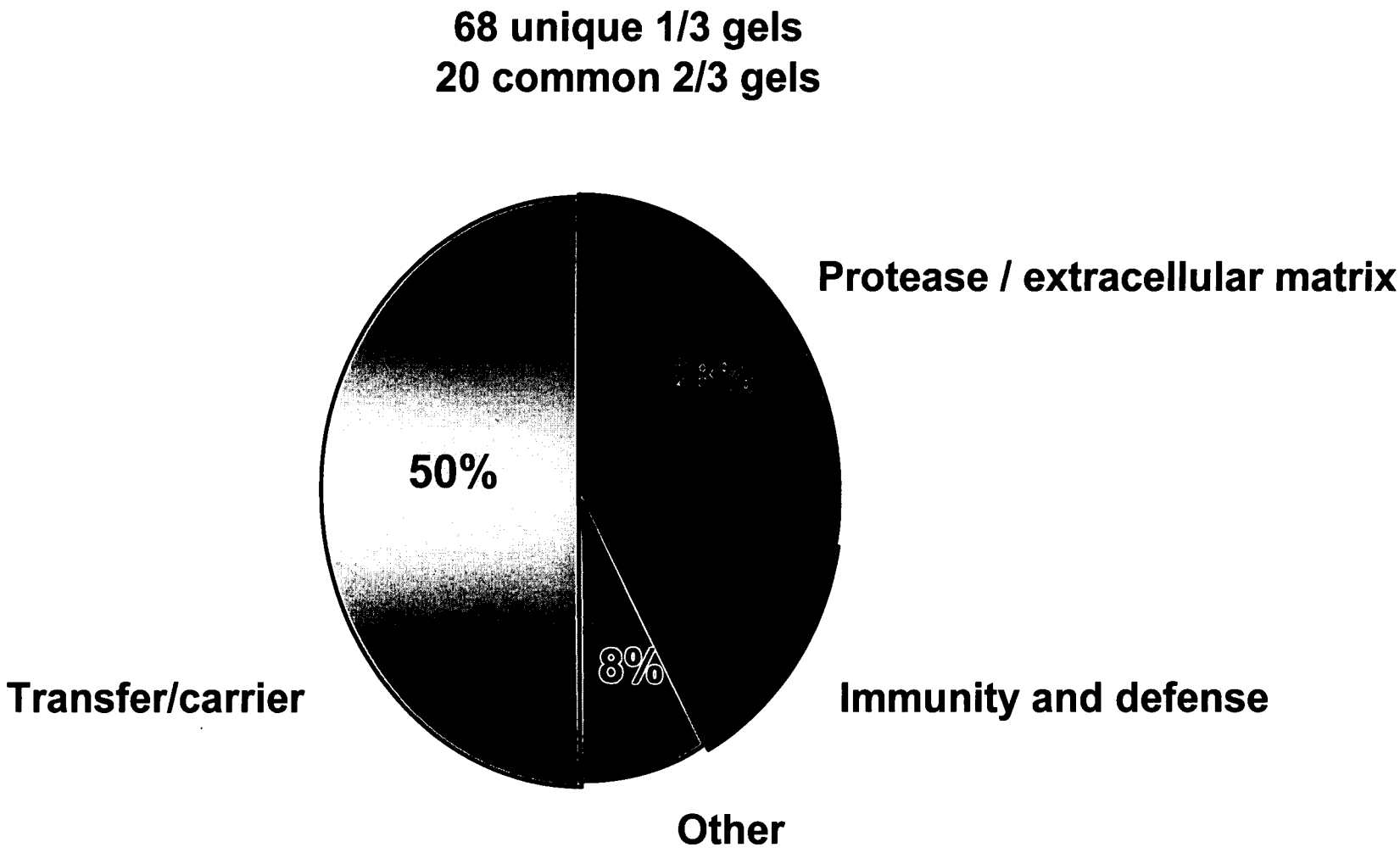

2D-DIGE CORD BLOOD
1
2
3
3-fold decrease in EONS
or
3-fold increase in EONS
332 matched identities in National Center of Biotechnology Information (NCBI)
230 matched identities in International Protein Index (IPI)
68 unique 1/3 gels
20 common 2/3 gels

Figure 7.

Figure 8.

- 332 matched identities in National Center of Biotechnology Information (NCBI)
- 230 matched identities in International Protein Index (IPI)



POTENTIAL PROTEIN BIOMARKERS

	2D- DIGE	P - value
Biomarkers	Fold change	Immunoassay
Down-regulated		
Apolipoprotein H	- 1	*ns*
Apolipoprotein E	- 3	*ns*
Vitamin D binding protein	- 3	*ns*
Up-regulated		
Haptoglobin	32	*< 0.001*
Haptoglobin-related protein	60	

Figure 9.

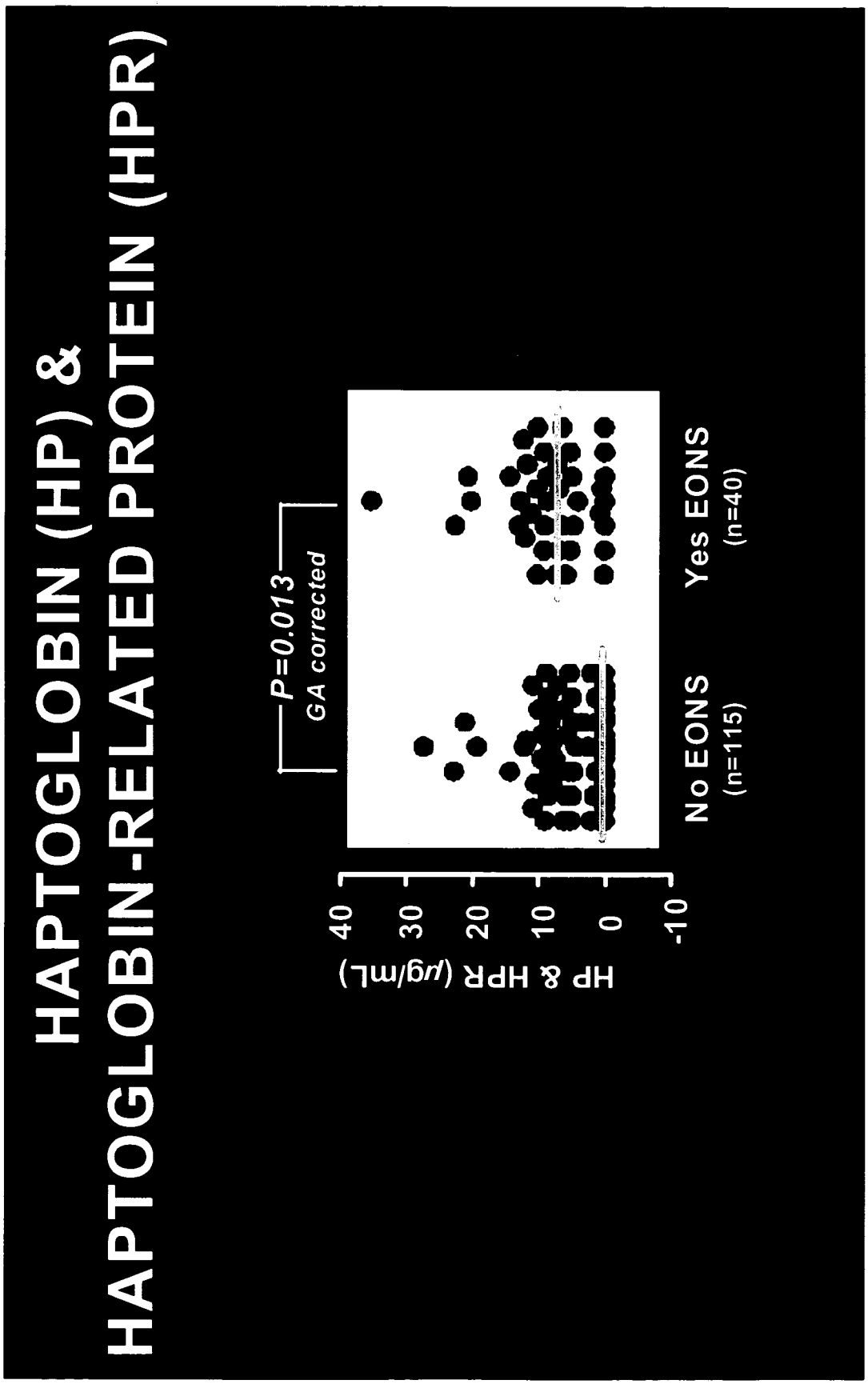

HAPTOGLOBIN (HP) &
HAPTOGLOBIN-RELATED PROTEIN (HPR)
HP & HPR (μg/mL)
40
30
20
10
0
-10
P=0.013
GA corrected
No EONS
(n=115)
Yes EONS
(n=40)

Figure 10.

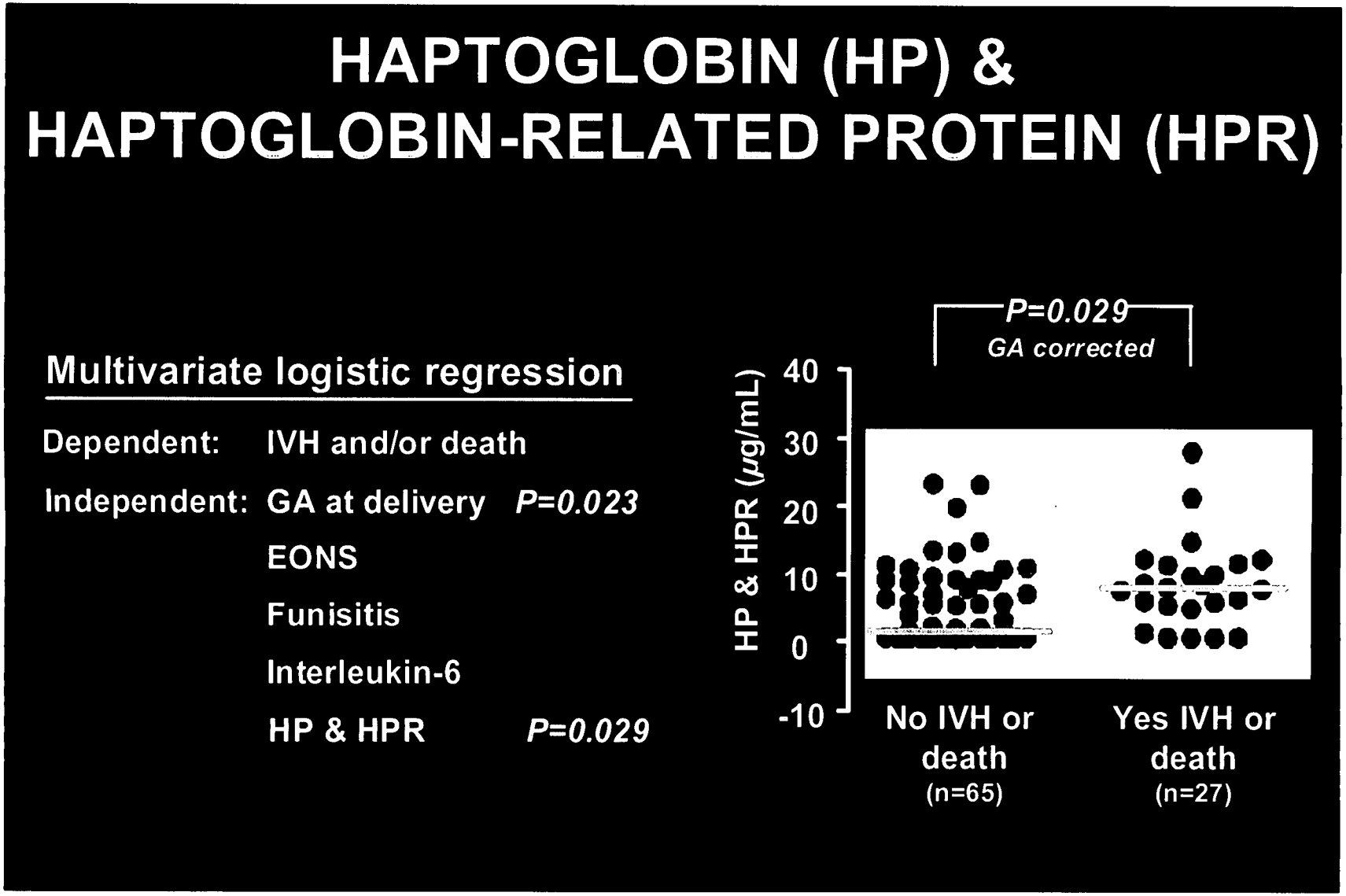

HAPTOGLOBIN (HP) &
HAPTOGLOBIN-RELATED PROTEIN (HPR)
Multivariate logistic regression
Dependent: IVH and/or death
Independent: GA at delivery P=0.023
EONS
Funisitis
Interleukin-6
HP & HPR P=0.029
P=0.029
GA corrected
HP & HPR (μg/mL)
40
30
20
10
0
-10
No IVH or death (n=65)
Yes IVH or death (n=27)

Figure 11.

HAPTOGLOBIN (HP) & HAPTOGLOBIN RELATED PROTEIN (HPR)

Haptoglobin:
- Hemoglobin scavenger
- Immuno-modulator
- Anti-microbial

Haptoglobin-related protein:
- Function unknown

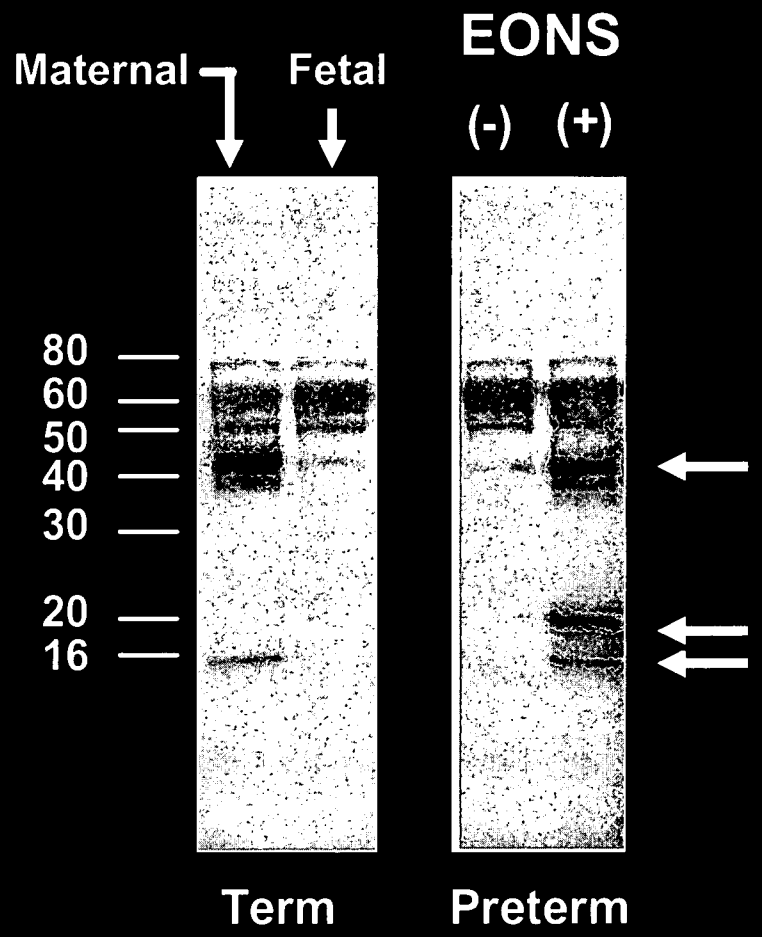
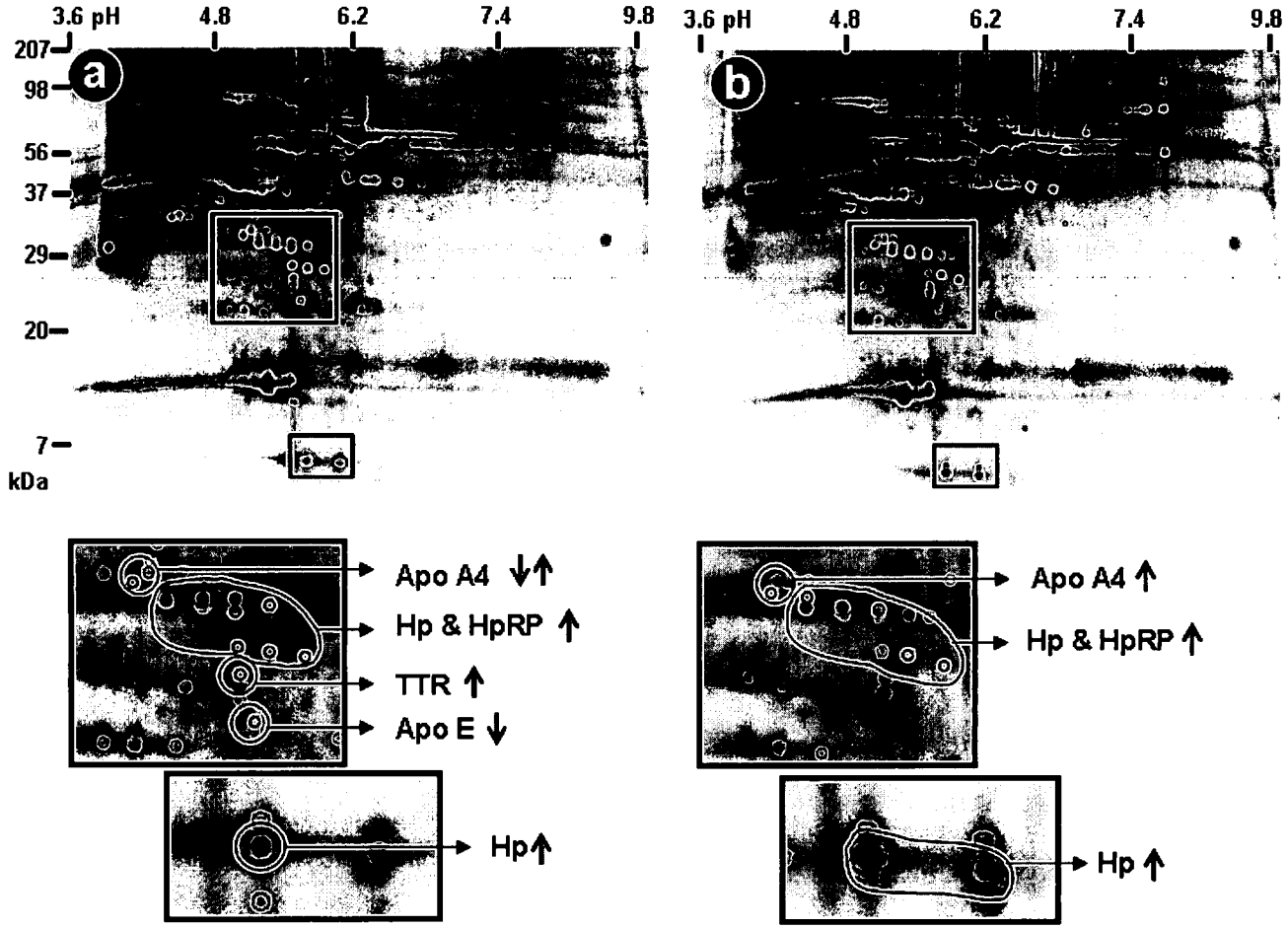


Figure 12.



➡ multidimensional analysis of the CB proteome identifies (Hp) & haptoglobin related protein

Figure 13.

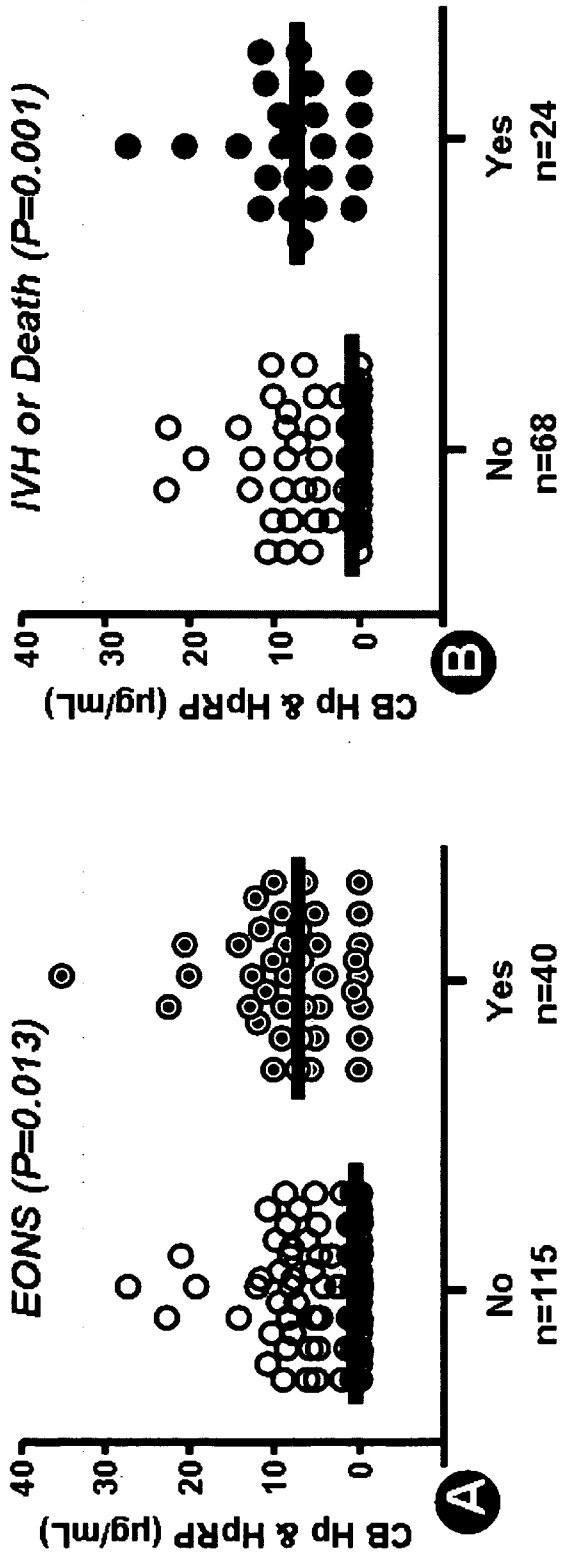

EONS (P=0.013)
CB Hp & HpRP (µg/mL)
40
30
20
10
0
No
n=115
Yes
n=40
A
IVH or Death (P=0.001)
CB Hp & HpRP (µg/mL)
40
30
20
10
0
No
n=68
Yes
n=24
B

Figure 14.

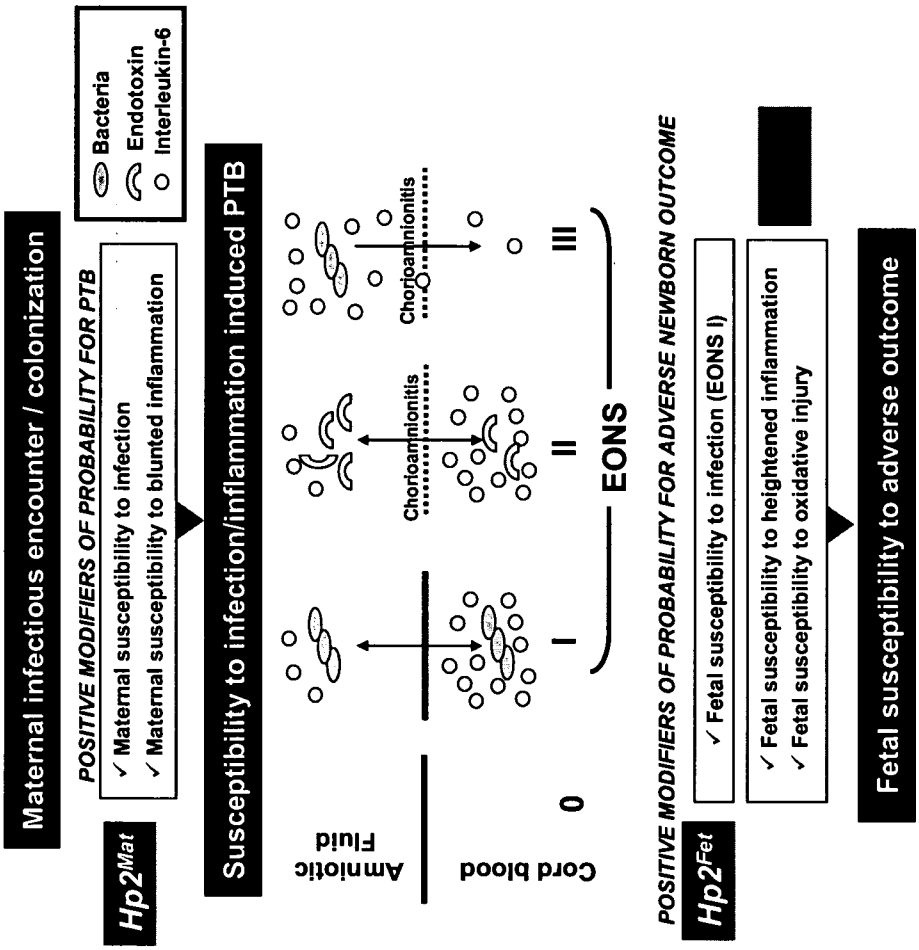

Maternal infectious encounter / colonization
POSITIVE MODIFIERS OF PROBABILITY FOR PTB
✓ Maternal susceptibility to infection
✓ Maternal susceptibility to blunted inflammation
Hp2Mat
Bacteria
Endotoxin
Interleukin-6
Susceptibility to infection/inflammation induced PTB
Amniotic Fluid
Cord blood
Chorioamnionitis
Chorioamnionitis
0
I
II
III
EONS
POSITIVE MODIFIERS OF PROBABILITY FOR ADVERSE NEWBORN OUTCOME
✓ Fetal susceptibility to infection (EONS I)
Hp2Fet
✓ Fetal susceptibility to heightened inflammation
✓ Fetal susceptibility to oxidative injury
Fetal susceptibility to adverse outcome

Figure 15.

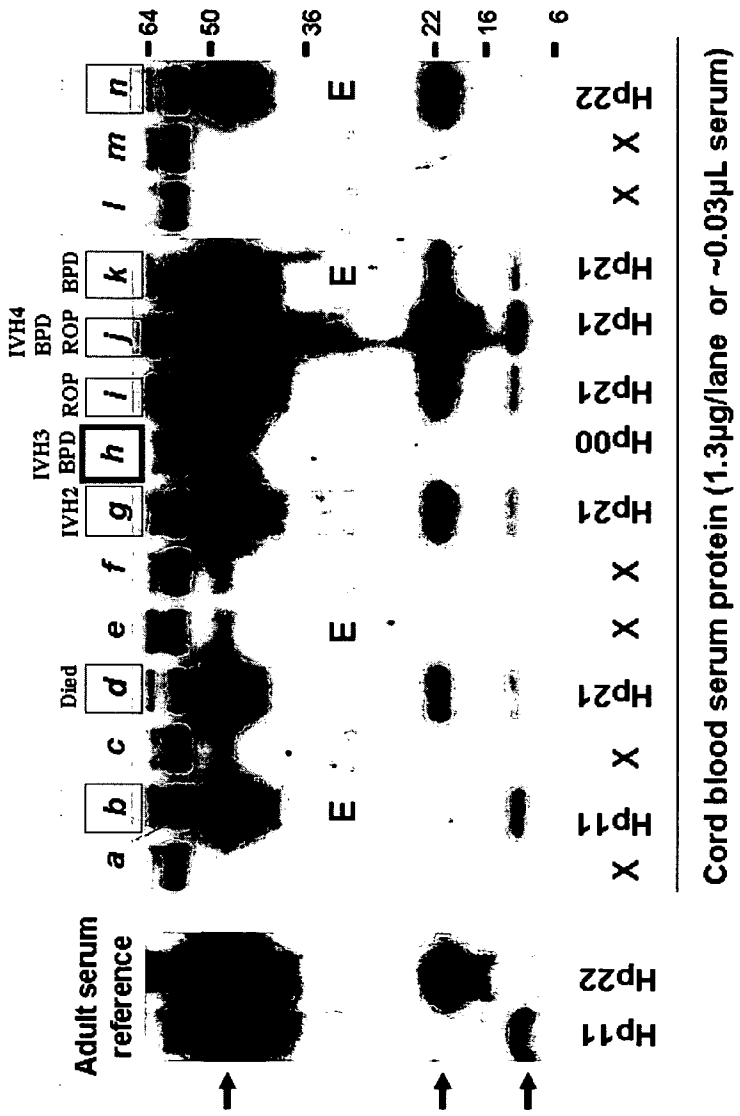

Adult serum reference
Hp11
Hp22
a
b
c
Died
d
e
f
IVH2
g
IVH3 BPD
h
IVH4 BPD ROP
i
IVH4 BPD ROP
j
IVH4 BPD
k
l
m
n
64
50
36
22
16
6
E
Hp11 X
Hp21
X
Hp21
X
X
Hp00
Hp21
Hp21
Hp21
X
X
Hp22
Cord blood serum protein (1.3µg/lane or ~0.03µL serum)

Figure 16.