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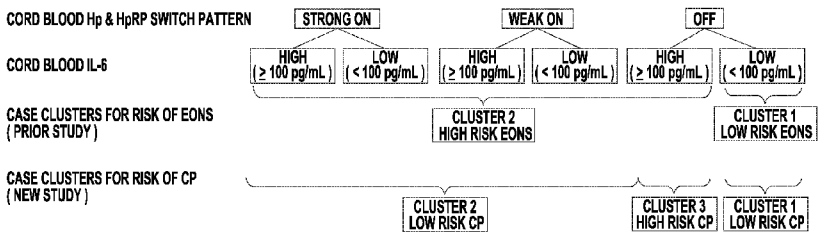


FIG. 3

(57) Abstract: A method of diagnosing a newborn as having an increased risk of developing cerebral palsy is described. The method includes obtaining a biological sample from the newborn; determining the level of Hp and/or HpRP in the biological sample and comparing it to a control value; determining the level of IL-6 in the biological sample and comparing it to a control value; and diagnosing the newborn as having an increased risk of developing cerebral palsy if the level of Hp and/or HpRP is lower than the control value, and the level of IL-6 is greater than the control value. Also described is a method of diagnosing a newborn as having an increased risk of developing retinopathy of prematurity.

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HAPTOGLOBIN AS A BIOMARKER FOR CEREBRAL PALSY IN NEWBORNS

GOVERNMENT FUNDING

[0001] This invention was made with Government support under grant numbers R01HD0620087 and U10HD027915 awarded by the National Institutes of Health and the National Cancer Institute. The Government has certain rights in the invention.

CROSS-REFERENCE TO RELATED APPLICATIONS

[0002] This application claims priority to U.S. Provisional Application Serial No. 62/086,873, filed on 12/03/2014, and U.S. Provisional Application Serial No. 62/111,278, filed on 2/03/2015, which are hereby incorporated by reference in their entirety.

BACKGROUND

[0003] Prematurity represents a key risk factor for short and long term adverse neonatal outcomes. Out of the multitude of numerous independent risk factors, infection-induced preterm birth (PTB) represents a unique clinical entity that raises considerably the risks. Acting synergistically with prematurity, intra-amniotic infection, histological chorioamnionitis and Early Onset Neonatal Sepsis (EONS) increase the risk of neonatal intra-ventricular hemorrhage (IVH), and long term consequences such as neuro-developmental delay, and cerebral palsy (CP). Allan *et al.*, Arch Pediatr Adolesc Med 1997;151:580–5 Although for many years it was assumed that EONS involves vertical transmission of genital live bacteria, particularities of the fetal response to infection indicate that prolonged exposure of a vulnerable fetus to the unfriendly intrauterine environment may lead to adverse outcomes even in the absence of a positive neonatal bacterial blood culture. Buhimschi *et al.*, BJOG. 2009;116:257-67.

[0004] With the exception of respiratory distress syndrome, which manifests immediately after birth, most neonatal complications (i.e., IVH) occur days or even weeks post-delivery. McCrea HJ, Ment LR., Clin Perinatol 35:777–92 (2008). This suggests that the decision to administer curative therapies is often taken late in the pathophysiologic process responsible for the end-organ damage. Most studies report significant associations between histological chorioamnionitis and funisitis as risk factors for EONS and IVH. Dexter *et al.* Obstet

Gynecol, 96:172–7 (2000); Moscuzza *et al.*, Gynecol Endocrinol, 27:319–23 (2011). The major disadvantage of placental histological markers of inflammation/infection is that first they are becoming available relatively late after birth. For this reason their significance is limited to late counseling and research purposes. Second, placental histologic examination, like any tests that relies on interpretation of visual inspection, has its limitations. A significant heterogeneity in assessment of tissue inflammatory responses to infection and poor to moderate intra- and inter-operator variability have been reported. Redline *et al.* Pediatr Dev Pathol 6:435–48 (2003). Third, lesions consistent with mild acute chorioamnionitis are also found in uncomplicated pregnancies and more often associated with labor at term.

[0005] Discovery of relevant biomarkers for a better understanding of the pathophysiology of early and late neonatal sepsis as well as identification of newborns at risk for increased morbidity and mortality was possible through application of proteomics. Buhimschi *et al.*, PLoS One, 6:e26111 (2011). Using an unbiased discovery algorithm the inventors demonstrated that fetal exposure to intra-amniotic infection (IAI) results in early "switch-on" of Haptoglobin (Hp) expression in the cord blood of premature neonates. This discovery came as a surprise because Hp was considered near-absent at birth switching to adult levels within the first year of life. Rausen *et al.*, Nature 191: 717 (1961). Next, the inventors asked the question if Hp switch-on pattern at birth can serve as a "biomarker" that can improve diagnosis of EONS over that achieved using clinical criteria alone. The obstacle of an absent "gold standard" diagnostic test for EONS was circumvented by using latent class analysis (LCA), a statistical method that has gained increased acceptance for situations when a perfect gold standard diagnostic test does not exist. From all the variables entered in the analysis, the Hp switch pattern had the highest discriminative power while gestational age (GA) at birth, sex, preterm premature rupture of membranes (PPROM) and Apgar scores were excluded. In our prospective cohort of 180 preterm newborns, Hp switch pattern alone was able to drive the cluster assignment (as "likely" or "unlikely exposed to inflammation") for 97% of newborns. Other significant participants to clustering the newborns were cord blood IL-6 and presumed EONS based on clinical criteria alone. Finally, the cluster algorithm was significantly better than the clinical diagnosis of EONS at predicting several neonatal morbidities including IVH. Based on the above knowledge we demonstrated that cord blood Hp level and its phenotype has the potential to improve the selection of newborns for prompt and targeted treatment at birth. Buhimschi *et al.*, PLoS One, 6:e26111 (2011).

[0006] The Maternal-Fetal Medicine Units (MFMU) Network conducted a randomized controlled trial of magnesium sulfate for prevention of CP. Rouse *et al.*, N Engl J Med, 359(9):895-905 (2008). This trial demonstrated that fetal exposure to magnesium sulfate prior to PTB did not reduce the combined risk of moderate to severe CP or death but the rate of CP was reduced among survivors. Two recent secondary analysis of the same trial showed that among offspring of women exposed to magnesium neither infusion of the drug at the time of delivery nor magnesium cord blood concentration or cytokine levels were associated with CP or death, implying that the search for identifying biomarkers predictive of short or long term outcomes must continue. Palatnik *et al.*, "Association between Cerebral Palsy or Death and Umbilical Cord Blood Magnesium Concentration" Am J Perinatol. 2015 Jun 9; Varner *et al.*, Am J Perinatol, 30(2):115-22 (2015).

[0007] The age at which cerebral palsy (CP) is diagnosed is important. The earlier CP is diagnosed correctly, the better the opportunities are to provide the child with physical and educational help, but there also might be a greater chance that CP will be confused with another problem, especially if the child is 18 months of age or younger. Infants may have temporary problems with muscle tone or control that can be confused with CP, which is permanent. Bosanquet *et al.*, Developmental medicine and child neurology 55 (5): 418–26 (2013). A metabolism disorder or tumors in the nervous system may also appear to be CP, and metabolic disorders in particular can produce brain problems that look like CP on an MRI. Disorders that deteriorate the white matter in the brain, and certain kinds of problems that cause spasms and weakness in the legs, may also be mistaken for CP when they first appear early in life. However, these disorders get worse over time, and CP does not. Accordingly, there remains a need for methods of diagnosing newborns who are at risk for developing CP.

SUMMARY OF THE INVENTION

[0008] Haptoglobin (Hp) is absent at birth in healthy term newborns. Antenatal exposure to intra-uterine inflammation results in the precocious "switch-on" of Hp with levels dependent on the major fetal phenotypes; Hp1-1, Hp1-2, Hp2-2. The inventors investigated the relationships between fetal Hp switch-on and phenotypes on immediate and late childhood outcomes independently of exposure to magnesium in-utero. The source of data for the analysis was a secondary analysis of a multicenter clinical trial of magnesium sulfate for

prevention of cerebral palsy. Rouse *et al.*, N. Eng. J. Med. 359(9), 895-905 (2008). The inventors analyzed 921 cord blood samples available from women with premature birth randomized to either placebo [n=471, gestational age (GA) median [range]: 31 [24-41] weeks or magnesium (n=450, GA 31 [24-42] weeks]. Hp levels were determined by immunoassay, while expression pattern (switch-on or off) and phenotypes were assessed by Western blot. Fetal inflammatory status was assessed by cord serum IL-6. Neonates were classified into one of 3 pre-defined clusters: Cluster 1 (non-exposed: Hp switch-off & IL-6<100 ng/mL, n=432); Cluster 2 (exposed: Hp switch-on independently of IL-6 levels, n=449); and Cluster 3 (exposed: Hp switch-off & IL-6 ≥100 ng/mL, n=40). Laboratory analyses and cluster assignments were done by investigators blinded to clinical data. Primary outcome was occurrence of intra-ventricular IVH gr3-4 and/or death.

[0009] Odd Ratios (OR) for major neonatal outcomes were adjusted for GA, birth weight and magnesium exposure. Phenotype analysis revealed that Hp1-2 or 2-2 protected against retinopathy of prematurity (OR=0.59, 95% CI 0.38-0.91). Following adjustment for magnesium treatment, GA and sex of the neonate Hp expression protected against development of cerebral palsy or death [OR=0.19, 95% CI 0.05-0.71, P =0.014). In conclusion, in the setting of in-utero inflammation, Hp switch-on protects against cerebral palsy or death, while the expression of allele 2 protects against retinopathy of prematurity.

BRIEF DESCRIPTION OF THE FIGURES

[0010] The present invention may be more readily understood by reference to the following figures, wherein:

[0011] Figures 1(A-C) provide images and a graph showing cord blood haptoglobin (Hp) and haptoglobin-related protein (HpRp) immuno-reactivity revealed by Western blot (A&B) and ELISA (C). (A) Western blot of umbilical vein (UV) serum from 4 preterm newborns of similar gestational age at birth. The newborns in Lanes 1-3 were diagnosed with suspected EONS clinical manifestations and hematological indices and all received i.v. antibiotics. The newborn in Lane 4 had a negative sepsis work-up. The presence of a conspicuous immunoreactive band corresponding to the β-chain (~42 kDa) in Lanes 1-3 is consistent with our defined switched-on Hp pattern. The absence of this band indicates for a switched-off pattern in Lane 4. The band ~9 kDa (Lanes 1&2) corresponds to the α1-chain whereas the

band at ~20 kDa (Lanes 2-3) corresponds to the α2-chain. Thus, the cord blood Hp patterns and phenotypes depicted in this gel are: switched-on pattern and Hp1-1 (Lane 1), switched-on pattern and Hp1-2 (Lane 2), switched-on pattern and Hp2-2 (Lane 3) and switched-off pattern and Hp0-0 (Lane 4). (B) Western blot of 3 representative maternal (Mat) and UV serum retrieved from women with normal deliveries at term. Note the switched-off pattern of the cord blood in contrast to the switched-on pattern of the adult blood. (C) Impact of Hp phenotypes on Hp and HpRP immunoreactivity as measured by ELISA. The red line indicates the group's median. Different letters denote statistically significant difference ($P<0.05$ Kruskal-Wallis ANOVA).

[0012] Figures 2A and 2B provide: (A) Clustering flowchart based on probability of "antenatal exposure to infection/inflammation". Posterior probabilities for inclusion in the "likely exposed" cluster were calculated for all 8 possible combinations of indicators (cord blood Hp and HpRP switch pattern, IL-6, suspected EONS) and their modal characteristics (yes/no). The number of newborns presenting each combination in the Yale cohort is included in parentheses. All newborns with cluster-2 probability levels >50% (black boxes) were considered "likely exposed". Alternatively, a diagnosis of "likely non-exposed" was assigned to newborns with probability levels <50% (grey boxes). (B) Relationships of cluster assignment, Hp phenotype and number of newborns who developed IVH or died. The percentages and the height of the grey or black portion of the bar indicate the proportion of newborns displaying the adverse composite outcome "IVH or death". Different letters denote statistical significance among newborns in the "likely exposed" cluster after correction for gestational age at birth in multivariate analysis.

[0013] Figure 3 provides a scheme representing the results of BEAM50 classification. Based on Hp and HpRP levels, whether the levels are weak or strong, and the level of IL-6, there are 6 possible combinations which can help determine the level of risk for a poor pregnancy outcome. Analysis of the data indicates that cluster III, in which IL-6 levels are high and Hp and/or HpRP levels are low includes newborns that have the highest risk of developing cerebral palsy

[0014] Figure 4 provides a set of graphs providing a model of the relationships among the timing of the infectious trigger, cord blood IL-6 and Hp levels which generate the 3 newborns clusters described herein.

DETAILED DESCRIPTION OF THE INVENTION

[0015] The present invention provides a method of diagnosing a newborn as having an increased risk of developing cerebral palsy is described. The method includes obtaining a biological sample from the newborn; determining the level of Hp and/or HpRP in the biological sample and comparing it to a control value; determining the level of IL-6 in the biological sample and comparing it to a control value; and diagnosing the newborn as having an increased risk of developing cerebral palsy if the level of Hp and/or HpRP is lower than the control value, and the level of IL-6 is greater than the control value. The present invention also provides a method of diagnosing a newborn as having an increased risk of developing retinopathy of prematurity, that includes obtaining a biological sample from the newborn; determining the level of Hp1-2 and/or Hp2-2 in the biological sample and comparing it to a control value, and diagnosing the newborn as having an increased risk of developing retinopathy of prematurity if the level of Hp1-2 and/or Hp2-2 is higher than the control value.

Definitions

[0016] As used herein, the term "diagnosis" can encompass determining the likelihood that a subject will develop a disease, or the existence or nature of disease in a subject. The term diagnosis, as used herein also encompasses determining the severity and probable outcome of disease or episode of disease or prospect of recovery, which is generally referred to as prognosis).

[0017] As used herein, the terms "treatment,""treating," and the like, refer to obtaining a desired pharmacologic or physiologic effect. The effect may be therapeutic in terms of a partial or complete cure for a disease or an adverse effect attributable to the disease. "Treatment," as used herein, covers any treatment of a disease in a mammal, particularly in a human, and can include inhibiting the disease or condition, *i.e.*, arresting its development; and relieving the disease, *i.e.*, causing regression of the disease.

[0018] The term therapy, as used herein, encompasses activity carried out to treat a disease. The specific activity carried out to conduct therapy can include use of surgery, radiotherapy, hormonal therapy, chemotherapy, or the use of one or more therapeutic agents (e.g., anticancer agents).

[0019] The terms "therapeutically effective" and "pharmacologically effective" are intended to qualify the amount of an agent which will achieve the goal of improvement in disease severity and the frequency of incidence over treatment of each agent by itself, while avoiding adverse side effects typically associated with alternative therapies. The effectiveness of treatment may be measured by evaluating a reduction in symptoms.

[0020] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

[0021] As used herein, the term "about" refers to +/-10% deviation from the basic value.

[0022] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

[0023] As used herein and in the appended claims, the singular forms "a", "and", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a sample" also includes a plurality of such samples and reference to "a biomarker" includes reference to one or more biomarkers, and so forth.

Methods of Diagnosing Cerebral Palsy in Newborns

[0024] One aspect of the invention provides a method of diagnosing a newborn as having an increased risk of developing cerebral palsy. The method includes the steps of obtaining a biological sample from the newborn or newborn umbilical cord; determining the level of Hp and/or HpRP in the biological sample and comparing it to a control value; determining the level of IL-6 in the biological sample and comparing it to a control value; and diagnosing the newborn as having an increased risk of developing cerebral palsy if the level of Hp and/or HpRP is lower than the control value, and the level of IL-6 is greater than the control value.

[0025] Hp is an acute phase glycoprotein with a great variety of important biological functions, of which the most recognized is hemoglobin binding. Dobryszycka W., Eur J Clin Chem Clin Biochem 35:647-54 (1997). 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. 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; Hp1-2 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.

[0026] 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. Bueler M R, Bersinger N A., Br J Obstet Gynaecol., 96(7), 867-9. (1989).

[0027] In some embodiments, the levels of other biomarkers for neonatal sepsis can also be determined using the methods described herein. For example, in some embodiments, the method also includes determining the level of C-reactive protein (CRP) and comparing it to a control value. Another biomarker for neonatal sepsis that can be evaluated is procalcitonin (PCT).

Cerebral Palsy

[0028] Cerebral palsy (CP) is a group of permanent movement disorders that appear in early childhood. While the central feature of CP is a disorder with movement, difficulties with thinking, learning, feeling, communication and behavior often occur along with CP. Cerebral palsy is caused by abnormal development or damage to the parts of the brain that control

movement, balance, and posture. CP is classified by the types of motor impairment of the limbs or organs, and by restrictions to the activities an affected person may perform. There are three main CP classifications by motor impairment: spastic, ataxic, and athetoid/dyskinetic CP.

[0029] Cerebral palsy is characterized by abnormal muscle tone, reflexes, or motor development and coordination. In some cases, there are joint and bone deformities and contractures (permanently fixed, tight muscles and joints). The classical symptoms are spasticity, spasms, other involuntary movements (e.g., facial gestures), unsteady gait, problems with balance, and/or soft tissue findings consisting largely of decreased muscle mass. Scissor walking (where the knees come in and cross) and toe walking (which can contribute to a gait reminiscent of a marionette) are common among people with CP who are able to walk, but taken on the whole, CP symptomatology is very diverse. The effects of cerebral palsy fall on a continuum of motor dysfunction, which may range from slight clumsiness at the mild end of the spectrum to impairments so severe that they render coordinated movement virtually impossible at the other end of the spectrum.

[0030] Babies born with severe CP often have an irregular posture; their bodies may be either very floppy or very stiff. Birth defects, such as spinal curvature, a small jawbone, or a small head sometimes occur along with CP. Symptoms may appear or change as a child gets older. Some babies born with CP do not show obvious signs right away. Classically, CP becomes evident when the baby reaches the developmental stage at 61/2 to 9 months and is starting to mobilize, where preferential use of limbs, asymmetry, or gross motor developmental delay is seen. Resulting conditions can include seizures, apraxia, dysarthria or other communication disorders, eating problems, sensory impairments, intellectual disability, learning disabilities, urinary incontinence, fecal incontinence, and/or behavioral disorders.

[0031] Cerebral palsy is typically diagnosed on the child's and mother's history and a physical examination. A general movement assessment, which involves measuring movements that occur spontaneously among those less than four months of age, appears to be most accurate. Once an initial diagnosis of CP has been made, further diagnostic tests can be carried out such as neuroimaging with computed tomography (CT) or magnetic resonance imaging (MRI), with MRI being preferred over CT due to diagnostic yield and safety.

Methods of Diagnosing Retinopathy of Prematurity in Newborns

[0032] Another aspect of the invention provides a method of diagnosing a newborn as having an increased risk of developing retinopathy of prematurity. The method includes obtaining a biological sample from the newborn; determining the level of Hp1-2 and/or Hp2-2 in the biological sample and comparing it to a control value; and diagnosing the newborn as having an increased risk of developing retinopathy of prematurity if the level of Hp1-2 and/or Hp2-2 is higher than the control value.

Retinopathy of Prematurity

[0033] The present invention also provides a method of diagnosing a newborn as having an increased risk of developing retinopathy of prematurity. Retinopathy of prematurity (ROP) is a disease of the eye affecting prematurely-born babies generally having received intensive neonatal care, in which oxygen therapy is used on them due to the premature development of their lungs. It is also known as Terry syndrome or retrolental fibroplasias. ROP involves disorganized growth of retinal blood vessels which may result in scarring and retinal detachment, and can lead to blindness in serious cases.

[0034] Risk factors for ROP include prematurity, high exposure to oxygen, low birth weight, antenatal infection, and cardiac defects. Almost all infants with ROP have a gestational age of 31 weeks or less (regardless of birth weight) or a birth weight of 1250 g (2.76 lbs) or less; these indications are generally used to decide whether a baby should be screened for ROP. The traditional screening procedure involves pupillary dilation using eye drops, after which the retina is examined using a special lighted instrument (an indirect ophthalmoscope). The peripheral portions of the retina are sometimes pushed into view using scleral depression during the examination. Examination of the retina of a premature infant is performed to determine how far the retinal blood vessels have grown, and whether or not the vessels are growing flat along the wall of the eye (the stage), with higher stages (on a scale from 1 to 5) representing increasing levels of retinal detachment.

Biological Samples

[0035] The methods involve providing or obtaining a biological sample from the subject, which can be obtained by any known means including needle stick, needle biopsy, swab, and

the like. In an exemplary method, the biological sample is a blood sample, which may be obtained for example by venipuncture. The biological sample can be obtained 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.

[0036] Biomarkers of the invention (e.g., Hp or IL-6) can be measured in different types of biological samples, preferably biological fluid samples such as blood. Examples of biological samples that may be used in methods of the invention, although not intended to be limiting, include blood, such as umbilical cord blood or neonatal blood, decidua (the thick layer of modified mucous membrane that lines the uterus during pregnancy), or placental material. 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 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.

[0037] 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.

[0038] 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 for example 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 for example 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).

[0039] 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 methods of detection described herein.

[0040] In some embodiments, 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 E-aminocaproic acid.

[0041] A biological sample may be fresh or stored. Biological samples may be or have been stored or banked under suitable tissue storage conditions. The biological sample may be a tissue sample expressly obtained for the assays of this invention or a tissue sample obtained for another purpose which can be subsampled for the assays of this invention. Preferably, biological samples are either chilled or frozen shortly after collection if they are being stored to prevent deterioration of the sample.

Newborns

[0042] The methods of the present invention are directed towards diagnosing whether a newborn has an increased risk of developing cerebral palsy or retinopathy of prematurity. A newborn having an increased risk is a newborn that is more likely to develop a condition (e.g., cerebral palsy) than an average newborn. Since about 2.1 per 1,000 live births have cerebral palsy, an increased risk for a newborn developing cerebral palsy is a probability higher than 0.21% that the newborn will develop cerebral palsy. In some embodiments, the increased risk is from 1% to 5%, from 5% to 10%, from 11% to 25%, from 26% to 50%, from 51% to 75%, from 76% to 90%, or 91% or greater.

[0043] A newborn (i.e., neonate) is a baby who has recently been delivered. Newborns are defined as human children in the period immediately succeeding birth and continuing through the first 28 days of extrauterine life. The newborn can either have been full term, or have been premature. In some embodiments of the invention, the newborn is premature. A newborn human can be considered to be premature if it is premature born before the end of the full term of gestation. In some embodiments, the newborn human is premature if it was born 1 week, 2 weeks, 3 weeks, or 4 weeks or more before the end of full term.

[0044] In some embodiments, the newborn can already have been diagnosed as having an increased risk of developing cerebral palsy (CP) or retinopathy of prematurity (ROP) as a result of other factors. In such cases, the present method is directed towards determining if the risk of developing cerebral palsy or retinopathy of prematurity is heightened relative to what it would be in view of the existing risk factors. A subject at increased risk can be at a

10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, or greater than 100% level of increased risk as compared with a typical subject. In the case of cerebral palsy, between 40% and 50% of all children who develop cerebral palsy are born prematurely. Multiple-birth infants are also more likely than single-birth infants to have CP. They are also more likely to be born with a low birth weight. In those who are born with a weight between 1 kg and 1.5 kg CP occurs in 6%. Among those born before 28 weeks of gestation it occurs in 11%. Genetic factors are believed to play an important role in prematurity and cerebral palsy generally. While in those who are born between 34 and 37 weeks the risk is 0.4%.

[0045] In babies that are born at term, risk factors for cerebral palsy include: problems with the placenta, birth defects, low birthweight, breathing meconium into the lungs, a delivery requiring either the use of instruments or an emergency Caesarean section, birth asphyxia, seizures just after birth, respiratory distress syndrome, low blood sugar, genetics, and infections in the baby. About 2% of all CP cases are inherited, with glutamate decarboxylase-1 being one of the possible enzymes involved. Most inherited cases are autosomal recessive, meaning both parents must be carriers for the disorder in order to have a child with the disorder.

[0046] In some embodiments, the newborn has an increased risk of developing cerebral palsy and/or retinopathy of prematurity as a result of being diagnosed as having been exposed to infection in utero. Infections in the mother, even infections that are not easily detected, may substantially increase the risk of a newborn developing the disorder. O'Shea, T.M., Ment Retard Dev Disabil Res Rev. 8(3):135-45 (2002). Infections of the fetal membranes known as chorioamnionitis also increases the risk.

Detection Methods

[0047] Levels of biomarkers such as Hp, allelic variants of Hp, HpRP, and IL-6 can be determined using a variety of different methods. In some embodiments, the levels of biomarkers are determined using an immunoassay. In other embodiments, the levels of the biomarker are determined using a method other than an immunoassay. For example, biomarkers such as Hp, HpRP, and IL-6 can be detected using matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF). Protein purification techniques are well known to those of skill in the art. These techniques involve, at one level,

the crude fractionation of the cellular milieu to polypeptide and non-polypeptide fractions. Having separated the polypeptide from other proteins, the polypeptide of interest may be further purified and/or quantified using chromatographic and electrophoretic techniques to achieve partial or complete purification (or purification to homogeneity). Analytical methods particularly suited to the preparation of pure proteins are immunohistochemistry, ion-exchange chromatography, exclusion chromatography; polyacrylamide gel electrophoresis; isoelectric focusing. A particularly efficient method of purifying peptides is fast protein liquid chromatography or even HPLC.

[0048] The inventors have previously confirmed the ability to detect differences among Hp and HpRP signatures in blood using ELISA and western blot. See U.S. Patent No. 8,697,367, the disclosure of which is incorporated herein by reference. 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 neonatal intensive care units (NICUs).

[0049] Hp and/or HpRP levels 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.

[0050] In certain embodiments, antibodies are provided that are specific for Hp and/or HpRP or cytokines, such as IL-6 found in fluid samples of a fetus or newborn. Antibodies provided herein include polyclonal and monoclonal antibodies, as well as antibody fragments 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, 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. The antibodies 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.

[0051] 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.

[0052] Biomarker polypeptides or effective fragments thereof 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.

[0053] 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, N.Y., 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).

[0054] 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, T and Abs, 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.

[0055] An immunoassay can be used to detect and analyze biomarkers in a biological 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.

[0056] 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.

[0057] 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.

[0058] 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 voltammetry 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.

[0059] 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.

[0060] In some embodiments, the immunoassay can include SERS sensors. Compared with immunoassays based on fluorescence, electrochemistry or ELISA, surface-enhanced Raman scattering (SERS) immunoassays have a several advantages. Li *et al.*, Analyst, 140(2):386-406 (2015). For example, SERS provides the spectral fingerprint signatures of analytes, which endows SERS sensors with better anti-interference resistance to non-specific molecules complex sample matrices (such as blood, serum and plasma). The unique attributes endow the SERS immunoassays ideal for biomarker detection in real-world biological samples.

[0061] Unfortunately, Raman scattering is extremely inefficient due to its limited scattering cross-section of ~10-30 cm^2/molecule. This drawback can be overcome using chemical enhancement (CE) and/or electromagnetic (EM) enhancement. Haynes CL, Van Duyne, R.P, J. Phys. Chem. B 107: 7426-7433 (2003); Li *et al.*, Nanotechnology, 23: 115501EM (2012). Enhancement is achieved via localized surface plasmon resonance (LSPR) to create intense local EM fields. Currently, nanoparticle (NP)-based systems are widely used in SERS detection platforms. The SERS signal is enhanced by covalently binding Raman labels on the plasmonic gold (or silver) NP surface. Furthermore, the SERS signal is enhanced by the formation of "hot spots" when the LSPR fields of two NPs are brought into close proximity. Various NPs have been explored to optimize the intensity of hot spots in solution.

[0062] In some embodiments, the immunoassay can be included in a lateral flow device. When biosensors alone are applied to whole blood samples, blood samples need to be pre-treated to remove cells prior to detection. Interference of sample matrix generally reduces the

sensitivity of electrochemical, colorimetric and visible-light fluorescent biosensors. In addition, it is difficult to realize the automation of biosensor operation. Furthermore, it is challenging to enable the multiplexed detection by a biosensor alone. One of effective solutions is to integrate a biosensor with microfluidic modules to form a microfluidic device. Compared to biosensors alone, a single microfluidic device is capable of handling sample pretreatment, concentration, transport and detection. This results in a highly automated operational workflow, allowing operation by a layperson. Also, microfluidic devices require less volume of sample and reagent; have better portability and capability of multiplexing. Hence microfluidic devices have been widely used for biomarker detection. Yetisen *et al.*, Lab Chip, 13: 2210-2251 (2013).

[0063] In a microfluidic device, the flow in microfluidic channels is driven either by an active flow with a pump or by passive control with the capillary force. The capillary force-based microfluidics such as later flow strips is very attractive because these devices are simple and inexpensive. In particular, disposable paper-based lateral flow strips utilize low-cost materials and do not require expensive fabrication equipment such as clean-room lithography. Parolo C, Merkoçi A., Chem. Soc. Rev., 42: 450-457 (2013). However, there is a technical challenge to integrate chip-based SERS substrate to a paper-based lateral flow strip, and there is constraint on tuning the flow rate and functionalization with paper-based lateral flow strip. Accordingly, in some embodiments, a plastic-base lateral flow strip can be used to conduct an immunoassay.

[0064] In other embodiments, the biomarker levels can be determined by mass spectrometry. 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. Pat. No. 5,719,060 and U.S. Pat. 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.

[0065] 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.

[0066] 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. Pat. No. 6,225,047.

Comparison of Biomarker levels to corresponding Control Values

[0067] A method of diagnosing a newborn as having an increased risk of developing cerebral palsy is disclosed. The method includes comparing the levels of a plurality of biomarkers in a biological sample obtained from a subject to corresponding control values to determine if there is an increased risk for the newborn of developing cerebral palsy. The biomarkers include Hp and/or HpRP, IL-6, and in some embodiments, C-reactive protein (CRP). Allelic subtypes of Hp (i.e., Hp1-1, Hp1-2, and Hp2-2) may also be used as biomarkers. The corresponding control values are therefore an Hp and/or HpRP control values, control values for the Hp allelic subtypes, an IL-6 control value, and in some embodiments, a CRP control value.

[0068] Control values are based upon the level of the risk predictor (*e.g.*, Hp and/or HpRP or IL-6) in comparable samples obtained from a reference cohort. In certain embodiments, the reference cohort is the general population. For example, the reference cohort can be a select population of human subjects. Control values for particular biomarkers may in some cases already be known to those skilled in the art. In certain embodiments, the reference cohort is comprised of newborns who went on to become healthy children without cerebral palsy or

retinopathy of preumaturity. The IL-6 control value is useful for identifying newborns that were exposed to infection *in utero*. Accordingly, in some embodiments, the control value for IL-6 is a value of about 100 pg/mL.

[0069] The control value can take a variety of forms. The control value can be a single cut-off value, such as a median or mean. Control values of risk predictors in biological samples obtained, such as for example, mean levels, median levels, or "cut-off" levels, are established by assaying a large sample of individuals in the general population or the select population and using a statistical model such as the predictive value method for selecting a positivity criterion or receiver operator characteristic curve that defines optimum specificity (highest true negative rate) and sensitivity (highest true positive rate) as described in Knapp, R. G., and Miller, M. C. (1992). Clinical Epidemiology and Biostatistics. William and Wilkins, Harual Publishing Co. Malvern, Pa., which is specifically incorporated herein by reference. A "cutoff" value can be determined for each biomarker that is assayed.

Methods for treating Cerebral Palsy or Retinopathy of Prematurity

[0070] In some embodiments, the method of diagnosis can be used to indicate or guide treatment of cerebral palsy. For example, the method can further comprise the step of providing treatment for the newborn having an increased risk of having or developing cerebral palsy. Suitable treatment can include use of a variety of different methods of treatment. Examples of methods of treatment include administration of therapeutic agents, surgery, and various forms of therapy intended to treat symptoms of cerebral palsy, such as physiotherapy, speech therapy, and massage therapy. Examples of therapeutic agents include Botulinum toxin, to reduce muscle tone, and magnesium sulfate.

[0071] A variety of methods of treatment are known for retinopathy of prematurity (ROP). One type of treatment is peripheral retinal ablation, which involves the destruction of the avascular retina using a solid state laser photocoagulation device. Cryotherapy, in which regional retinal destruction was done using a probe to freeze the desired areas, can also be used, but is less preferred. Scleral buckling and/or vitrectomy surgery may be considered for severe ROP where the eyes have progressed to retinal detachment. Intravitreal injection of bevacizumab (avastin) has been reported as a supportive measure in aggressive posterior

retinopathy of prematurity. Shah *et al.*, Indian journal of ophthalmology 55 (1): 75–76 (2007).

Kits

[0072] An additional aspect of the invention provides a kit for diagnosing a newborn as having an increased risk of developing cerebral palsy. The kit includes a first antibody or binding fragment thereof specific for Hp and/or HpRP, a second antibody or binding fragment thereof specific for IL-6, reagents for conducting the diagnosis, and a package for holding the antibodies and the reagents.

[0073] A further aspect of the invention provides a kit for diagnosing a newborn as having an increased risk of developing retinopathy of prematurity. This kit includes a first antibody or binding fragment thereof specific for Hp1-2, a second antibody or binding fragment thereof specific for Hp2-2, reagents for conducting the diagnosis, and a package for holding the antibodies and the reagents. In some embodiments, the kit lacks either the first or second antibody.

[0074] A kit generally includes a package with one or more containers holding the reagents, as one or more separate compositions or, optionally, as an admixture where the compatibility of the reagents will allow. The kit can also include other material(s), which may be desirable from a user standpoint, such as a buffer(s), a diluent(s), a standard(s), and/or any other material useful in sample processing, washing, or conducting any other step of the assay. In some embodiments, the kit includes a lateral flow strip which can be used to conduct an immunoassay using the antibodies included in the kit.

[0075] In some embodiments, the reagents include antibodies capable of specifically binding to the compound they are capable of detecting. For example, the kit can include an antibodies specific for Hp, HpRP, and IL-6. In some embodiments, multiple concentrations of each antibody can be included in the kit to facilitate the generation of a standard curve to which the signal detected in the test sample can be compared. Alternatively, a standard curve can be generated by preparing dilutions of a single antibody solution provided in the kit. In further embodiments, the antibodies comprise surface-enhanced Raman scattering probes.

[0076] As used herein, the terms "specific binding" or "specifically binding", refer to the interaction of an antibody, a protein, or a peptide with a second chemical species, wherein the interaction is dependent upon the presence of a particular structure (e.g., an antigenic determinant or epitope) on the chemical species; for example, an antibody recognizes and binds to a specific protein structure rather than to proteins generally.

[0077] The kit can also include instructions for using the kit to determine the levels of Hp and/or HpRP, Hp allelic subtypes, and the level of IL-6, and Hp and/or HpRP and IL-6 controls. Instructions included in kits can be affixed to packaging material or can be included as a package insert. While the instructions are typically written or printed materials they are not limited to such. Any medium capable of storing such instructions and communicating them to an end user is contemplated by this disclosure. Such media include, but are not limited to, electronic storage media (*e.g.*, magnetic discs, tapes, cartridges, chips), optical media (*e.g.*, CD ROM), and the like. As used herein, the term "instructions" can include the address of an internet site that provides the instructions.

[0078] Examples have been included to more clearly describe particular embodiments of the invention. However, there are a wide variety of other embodiments within the scope of the present invention, which should not be limited to the particular example provided herein.

EXAMPLES

Example 1: Evaluation of Hp Switch-On Patterns

[0079] Hp is a known inflammation-inducible plasma protein so the finding that EONS associates with elevated Hp and HpRP immunoreactivity would not have appeared exciting unless corroborated with the knowledge that: (a) normal newborns at term should not have detectable levels of circulating Hp; (b) the switch from the physiological neonatal ahaptoglobinemia to the adult serum level of Hp (100-150 mg/dL) occurs within the first year of life. Bishop ML, Fody EP, Schoeff LE. Clinical chemistry: principles, procedures, correlations Philadelphia: Lippincott Williams & Wilkins, 2005. The mechanism responsible for Hp gene silencing in the fetal period and its activation switch after delivery remains unknown and is the subject of our ongoing research. Using the same ELISA, we confirmed that a group of healthy term newborns had undetectable cord blood Hp&HpRP immunoreactivity while their mothers had levels >100 µg/mL, as expected. Interpreted in this

context, our data pointed us to the possibility that fetuses exposed to chorioamnionitis that were subsequently diagnosed with EONS underwent a precocious switch-on in Hp and HpRP expression which could only have occurred only in the antenatal period.

[0080] Next, we sought to confirm these findings and phenotype the premature newborns using Western blotting and the same antibody used in the immunoassay. First, we found that a significantly higher number of EONS newborns displayed a precocious umbilical cord vein (UV) switched-on Hp and HpRP at birth ($P<0.001$, Fig. 1A). To provide further evidence that this precocious switch-on pattern is EONS-specific, we then performed Western blotting and ELISA on matched cord and maternal blood from healthy term maternal-fetal dyads (Fig.1B). None of the term babies had detectable Hp and HpRP in contrast to their mothers who all had present Hp chains as expected for normal adult subjects. Third, we discovered that Hp switched-off pattern (Hp0-0) was characterized by significantly lower ELISA immunoreactivity compared to all other phenotypes ($P<0.001$, Fig. 1C). Among newborns with switch-on pattern, the Hp phenotype impacted on ELISA immunoreactivity with Hp1-1 measuring lower compared to both Hp1-2 ($P<0.001$) and Hp2-2 ($P=0.002$), independent of neonatal race, gender and gestational age at birth ($P=0.011$). This was exciting because it meant that immunoreactivity by ELISA can be biased and not necessarily representative of the actual protein amount. The most likely explanation is that multimeric aggregates characteristic of Hp2-2 phenotype measures at higher levels in ELISA compared to Hp1-1 phenotype which is a monomer and/or that the ELISA antibody has a higher affinity for Hp2-2. Therefore we concluded that a simple measurement of Hp level by ELISA would be an imperfect biomarker.

[0081] Next, we asked the question: Can Hp switch-on pattern at birth serve as a "biomarker" that can improve diagnosis of EONS over that achieved using clinical criteria alone? In clinical prematurity related research a major difficulty is absence of a "gold standard" diagnostic test for EONS. We circumvented this obstacle by using latent class analysis (LCA), a statistical method that has gained increased acceptance for situations when a perfect gold standard diagnostic test does not exist. This type of analysis assumes that a hidden (latent) variable (which in our case we named: antenatal exposure to inflammation/ infection is responsible for heterogeneity among observed variables. From all the variables entered in the analysis, the Hp switch pattern had the highest discriminative power while gestational age at birth, sex, PPROM and Apgar scores were excluded. In our prospective cohort of 180

preterm newborns, Hp switch pattern alone was able to drive the cluster assignment (as "likely" or "unlikely exposed to inflammation") for 97% of newborns. Other significant participants to clustering the newborns were cord blood IL-6 and presumed EONS. The addition of cord blood IL-6 to Hp switch pattern improved the model by driving the cluster assignment as "likely exposed" for a minority of 5 (3%) newborns whose Hp expression remained switched-off despite high levels of cord blood IL-6 (range ~100-1,500 pg/mL). The diagnosis of suspected EONS (which constitutes the current standard of care for decision to initiate broad spectrum antibiotics among others) did not change cluster assignments but it improved the level of certainty with which each case was distributed to one or the other cluster. A schematic representation of this cluster algorithm is presented in Fig. 2A along with probability levels for each possible combination.

[0082] Finally, the cluster algorithm was significantly better than the clinical diagnosis of EONS at predicting several neonatal morbidities including IVH. Equally important was that in the context of antenatal exposure to intra-amniotic inflammation (chorioamnionitis), the Hp phenotype (but not gestational age at birth) was an independent predictor of short-term neonatal morbidities and especially of IVH and ROP. Despite earlier gestational age at birth, newborns carrying the Hp2-2 exhibited significantly less incidence of major morbidities compared to those with Hp1-1 phenotype (Fig. 2B). This observation was consistent with the known function of circulating Hp and HpRP as antimicrobial, antioxidant and immunomodulatory proteins. Moreover, our analysis underlined the ability of the Hp-switch pattern to reflect the interaction between the individual genetic makeup and exposure to an environmental antenatal insult consistent with the definition of "exposome".

Example 2: Cord Blood Haptoglobin as Biomarker of Neonatal Exposure to Intra-Uterine Inflammation

[0083] Herein, we investigated the relationships between Hp switch-on and Hp phenotypes as a reflection of antenatal exposure to infection/inflammation and immediate and late childhood outcomes of fetuses exposed to magnesium in-utero. Furthermore, this trial was a unique opportunity to validate the effect of Hp phenotypes on the occurrence and severity of short/long term neonatal morbidities or death in a cohort of premature newborns exposed to magnesium sulfate that is different and significantly larger than the one used in the initial study.

METHODS

[0084] ***Study population.*** We conducted a secondary analysis of a multicenter clinical trial of magnesium sulfate for prevention of CP conducted by the Eunice Kennedy Shriver National Institute of Health and Human Development MFMU Network. Rouse *et al.*, N. Eng. J. Med. 359(9), 895-905 (2008). From the original clinical trial, out of the 2,215 women who had live births, we analyzed 921 cord blood (CB) samples available from women with PTB randomized to either placebo [n=471, GA median [range]: 31 [24-41] weeks or magnesium (n=450, GA 31 [24-42] weeks]. Samples from 27 twin pregnancies were included in this analysis [n=15 (placebo group); n=12 (magnesium group), reflecting fewer mothers due to twin gestations (Table 1).

[0085] Inclusion criteria for the original clinical trial were: singletons or twins, GA 24 - 31 weeks, high risk for spontaneous PTB due to PPROM or advanced cervical dilatation of 4 to 8 cm in the setting of intact membranes, and delivery anticipated within 2 - 24 hours. Exclusion criteria included: birth anticipated within less than 2 hours, cervical dilatation > 8 cm, PPROM <22 weeks, congenital fetal anomalies or death, hypertension or preeclampsia, contraindications to magnesium sulfate and receipt of intravenous magnesium sulfate within the previous 12 hours. Surviving infants underwent neurologic evaluation by an annually certified pediatrician or pediatric neurologist at 6, 12, and 24 months of age (corrected for GA). Infants with a normal neurologic examination at 1 year and who could walk 10 steps independently and had a bilateral pincher grasp were considered normal and did not undergo further physical examinations, although the scheduled neurodevelopmental examination was performed.

[0086] The diagnosis of CP was made by certified pediatrician or pediatric neurologist using well established criteria. Bax *et al.*, Dev Med Child Neurol 47 (8) 571-576 (2005). For severity assessment, children diagnosed with CP were further classified by the Gross Motor Function Classification System (GMFCS). Palisano *et al.*, Dev Med Child Neurol 39 (4) 214-223 (1997). Scores of ≥ 1 were considered mild CP; moderate CP ≥ 2-3; severe CP ≥ 4-5. Neurodevelopmental stages were assessed by a trained psychologist or psychometrist using the Bayley Scales of Infant Development II (BSID-II) test. Varner *et al.*, Am J Perinatol. 30(2):115-22 (2015). Components included a Mental Development Index (MDI) and a psychomotor development index (PDI). A score of < 70 indicates significant impairment (> 2

SD below the mean). The diagnoses of IVH, ROP, NEC, BPD, patent ductus arteriosus, and neonatal proven sepsis were established based on well recognized clinical criteria. Rouse *et al.*, N Engl J Med. 359(9):895-905 (2008). This secondary analysis was considered exempt by the Ohio State University Office of Responsible Research Practices.

[0087] ***Primary outcome of the analysis.*** The primary outcome was defined as death and/or IVH category 3 and 4.

[0088] ***Umbilical cord blood (CB) serum.*** CB was retrieved by sterile puncture of the umbilical vein after delivery. Samples were centrifuged for 10 min (1,000 g, 4°C), the serum aliquoted and stored at -80°C.

[0089] ***Immunoassay methods.*** Blocking buffer (5% non-fat dry milk) was used for sample dilution: 100, 1,000 or 10,000-fold. We used a mixed phenotype Hp standard (Hp1-2, Sigma) to prepare a 7-point standard curve (250-3.9 ng/ml). IL-6 (Pierce-Endogen, Rockford, IL) was quantified to assess the fetal inflammatory status. All samples were tested by laboratory investigators unaware of the clinical outcome. Bicinchoninic acid (BCA) assay (Pierce Biotechnology, Rockford, IL) was employed to quantify the CB serum total protein.

[0090] ***Hp Western blotting, determination of CB Hp switch-on pattern and Hp phenotype.*** Haptoglobin is a tetrameric protein with two α and two β-chains linked by disulfide bonds. In humans, Hp occurs in two co-dominant allelic forms, *Hp1* and *Hp2*, which differ in the length of the α-chain. Smithies et al., Nature 196: 232–236 (1962). The human population has 3 major Hp phenotypes (Hp1-1, Hp2-2 and the heterozygous Hp1-2), derived from variations in the α-chain with identical β-chains. Maeda N, Smithies O., Annu Rev Genet 20: 81–108 (1986). Absence of Hp at protein level denotes Hp0-0 phenotype (ahaptoglobinemia).

[0091] SDS-PAGE gels (10-20%, InVitrogen, Carlsbad, CA) were loaded with equal amounts of CB protein (2 μg/lane) mixed 1:2 with reducing sample buffer (Bio-Rad, La Jolla, CA) and boiled for 5 min.. After electrophoretic transfer, nitrocellulose membranes (Bio-Rad) were blocked with 5% milk and then incubated overnight at 4°C rabbit anti-Hp polyclonal antibody (1:3,000, Sigma, St Louis, MO). Detection was performed using biotinylated goat anti-rabbit secondary antibody (1:5,000, Jackson Immunoresearch, West Grove, PA) followed by streptavidin-linked horseradish peroxidase, (1:8,000, Amersham Biosystems, Piscataway, NJ), chemiluminescence (ECL-Plus, Amersham) and a timed 3 min.

exposure to film (Kodak Biomax). Optical density of the bands of interest was analyzed with Image J software, available online from the research services branch of the National Institutes of Health. The results were expressed as the optical density of each band and as total optical density calculated from the sum of α- and β-chains. Purified Hp from blood of adults with known phenotypes (Hp1-1, Hp1-2 and Hp2-2, Sigma, St. Louis, MO) was used as positive control.

[0092] In the current study, a detectable Hp β-chain (~42 kDa) on Western-blot was indicative of a "switch-on" in Hp expression. Hp phenotypes were defined by additional presence of α-chain bands at either ~9 kDa (α^1: Hp1-1), ~20 kDa (α^2: Hp2-2) or both (Hp1-2).

[0093] ***Latent-class analysis (LCA).*** We applied latent-class analysis (LCA), a statistical solution that has gained increased acceptance when a "gold standard" diagnostic test does not exist. Vermunt JK, Magidson J (2002) "Latent class cluster analysis. In Applied latent class analysis" Edited by Hagenaars J, McCutcheon A. Cambridge University Press. pp 89–106. Briefly, LCA assumes that a hidden (latent) variable is responsible for heterogeneity among observed variables. By studying the patterns of co-variation among observed variables, the nature of the latent variable can be characterized. Each model combines several indicators (variables involved in cluster formation) and covariates (other demographical or clinical variables), potentially adding to cluster characterization. A cluster model is examined first, then clusters are added until no further improvement in classification is observed. For the purpose of this study the latent variable was set as "*antenatal IAI exposure*". This variable was then investigated for its ability to predict short and long term adverse neonatal outcomes. Neonates were classified into one of 3 pre-defined clusters: Cluster 1 (non-exposed: Hp switch-off and IL6<100 ng/mL, n=432); Cluster 2 (exposed: Hp switch-on independently of IL-6 levels, n=449); Cluster 3 (exposed: Hp switch-off and IL6 ≥100 ng/mL, n=40). Laboratory analyses and cluster assignments were done by investigators blinded to clinical data.

[0094] ***Statistical analyses.*** Normality testing was performed using the Shapiro-Wilk test. Data were compared with t-test for continuous variables and Chi-square for frequencies, and adjusted for correlation between twins as appropriate. Stepwise multivariable regression analysis was used to determine concurrent relationships between variables and to correct for

possible influences of GA, birthweight and magnesium sulfate exposure. Logistic regression models were assessed using the Hosmer and Lemeshow goodness-of-fit test. Co-linearity among the variables was tested using the condition index. All models corrected for the correlation between twins. A P <0.05 was considered statistically significant. Odd Ratios (OR) for major neonatal outcomes were adjusted for GA, birth weight and magnesium exposure.

[0095] Goodness of fit of LCA models was evaluated using the Bayesian Information Criterion (BIC) and Akaike information criterion (AIC). Vermunt JK, Magidson J (2002) Latent class cluster analysis. In Applied latent class analysis. Edited by Hagenaars, J., McCutcheon A. Cambridge University Press. p. 89-106. Lower BICs indicate model improvement. Conditional bootstrap re-sampling procedures (500 random iterations) were employed to compare fit indices between models. The input of each indicator to the final model was measured by the nominal correlation coefficient Goodman-Kruskal tau-b (GK-θ). Finally, a posterior probability classifying each case to each cluster of the final model was calculated. Odds ratios (OR), positive likelihood ratio (+LR, number of times more likely that a newborn with a positive diagnosis will develop the adverse outcome compared to newborn with a negative diagnosis), negative likelihood ratio (-LR, number of times more likely that a newborn with a negative diagnosis will not develop the adverse outcome compared to newborn with a positive diagnosis) and number needed to harm (NNH, number of subjects needed to be exposed to a risk factor to cause harm in one subject that otherwise would not have been harmed) were computed to determine the risk of adverse neonatal outcomes. The lower the NNH the worse the risk factor.

RESULTS

[0096] In Table 1 we are presenting the demographics characteristics of the study population indicating no significant differences among groups except a higher number of male neonates exposed to magnesium sulfate.

[0097] Table 1: Baseline characteristics by treatment group

Characteristic	Placebo	Magnesium	*P*-value**
Mothers			
N	456	438	
Maternal age, years *	24 [15 - 44]	25 (14 - 44)	0.186$
Race/Ethnicity, n (%) African American Hispanic Other	 187 (41) 77 (17) 192 (42)	 177 (40) 75 (17) 186 (43)	0.983
High school education, n (%)	283 (62)	290 (67)	0.165
Smoking, n (%)	135 (30)	115 (26)	0.265
Alcohol, n (%)	39 (9)	43 (10)	0.513
Drug, n (%)	44 (10)	41 (9)	0.883
Steroids, n (%)	444 (97)	424 (97)	0.615
Steroids to delivery, days *	6 [0 - 87]	6 [0 - 115]	0.293$
Antibiotics, n (%)	433 (95)	418 (95)	0.739
Clinical chorioamnionitis, n (%)	46 (10)	45 (10)	0.927
PPROM, n (%)	405 (89)	384 (88)	0.595
Rupture to delivery, days	7	6	0.679
Indicated PTB, n (%)	40 (9)	46 (11)	0.380
Cesarean delivery, n (%)	147 (32)	148 (34)	0.622
Postpartum endometritis, n (%)	22 (5)	18 (4)	0.605
Neonates#			
Male sex, n (%)	231 (49)	252 (56)	0.038
Gestational age at birth, weeks	31 (24 - 41)	31 (24 - 42)	0.398
Birth weight, grams	1,575 (580 - 4,035)	1,589 (568 - 3,752)	0.450
Twins (at least one analyzed), n (%)	55 (12)	43 (10)	0.394
Twins (both analyzed), n	15	12	0.394
Major congenital anomaly, n (%)	14 (3)	21 (5)	0.182
5-Min Apgar score <7, n (%)	47 (10.0)	56 (12.4)	0.234
Need for resuscitation at birth, n (%)	383 (81.3)	358 (79.6)	0.508

* n (%) or median (min-max)
** T-test for continuous variables and Chi-square for frequencies
*** Median (25th to 75th percentile)

[$] Wilcoxon rank sums test
[#] Adjusted for correlation between twins
Preterm Premature Rupture of Membranes: PPROM; Preterm Birth: PTB

[0098] ***Hp, IL-6 and magnesium CB level.*** There was no difference between groups in CB Hp levels (placebo: mean [95% CI]: 3,297 [2,551-4,262] vs. magnesium 3,454 [2,656-4,494] ng/ml, P=0.803). Similarly, there was no difference between treatment groups in CB IL-6 (placebo: 35 (27-44) vs. magnesium 26.6 (21 - 332) pg/ml, P=0.083). Yet, while Hp phenotype did not modify the Hp and IL-6 levels among groups, there was a significant relationship among Hp status and CB IL-6 levels with higher levels of the pro-inflammatory cytokine when Hp was switch-on (P<0.001) (Table 2).

Table 2. Relationship between log IL-6 and Haptoglobin (Hp) switch patterns.

Hp Switch Pattern	Mean	SE	Min	Max
Off	2.0	0.1	1.8	2.1
On "Weak"	4.4	0.2	4.0	4.8
On "Strong"	5.1	0.1	4.9	5.5

[0099] Data presented as mean log IL-6 value ± Standard Error (SE) and range [min-max]. Each switch pattern group is statistically significantly different from each other at the Bonferroni P 0.05/3=0.0167

[00100] As expected, the magnesium CB levels was higher in the treatment group compared to placebo (placebo: 1.56 [1.52 - 1.60] vs. magnesium 2.55 [2.45 - 2.64] mg/dL, P< 0.001). Data was corrected for twins.

[00101] ***Relationship between phenotype and cluster.*** There was a statistically significant association between phenotype and cluster in both placebo and magnesium groups (P<0.001).

[00102] Relationship between cluster and neonatal outcome. The frequency of short and long term outcome is presented in Table 3. Phenotype analysis revealed that Hp1-2 or 2-2 protected against retinopathy of prematurity (OR=0.59, 95% CI 0.38-0.91) (Table 4). Following adjustment for magnesium treatment, GA and sex of the neonate Hp expression protected against development of cerebral palsy or death [OR=0.19, 95% CI 0.05-0.71, P =0.014) (Table 5).

Table 3. Frequency of the outcomes for the study population

Outcomes	**Placebo**	**Magnesium**
Samples, n	471	459
Primary outcome		
Intra-ventricular hemorrhage - Grade 1 only, n (%)	2 (0.4)	2 (0.5)
Death (fetal/neonatal death), n (%)	5 (1)	8 (2)
Secondary outcome		
Retinopathy of prematurity, n (%)	66 (14)	60 (13)
Necrotizing enterocolitis, n (%)	22 (5)	26 (6)
Patent ductus arteriosus, n (%)	44 (9)	42 (9)
Bronchopulmonary dysplasia, n (%)	54 (12)	55 (12)
Cerebral palsy, n (%)	6 (1)	7 (2)
Cerebral palsy or death, n (%)	10 (2)	14 (3)
Mental developmental delay (<70), n (%)	31 (8)	43 (11)
Psychomotor delay (<70), n (%)	33 (8)	31 (8)
Proven sepsis, n (%)	46 (10)	39 (9)
Seizures, n (%)	4 (1)	2 (1)
Fetal anomalies		
Anomalies, n (%)	2 (0.4)	6 (1)

Table 4. Relationship between short and long term neonatal outcomes and cluster.

Outcomes	Cluster 1 vs. Cluster 3		Cluster 2 vs. Cluster 3	
	OR (95% CI)	P value	OR (95% CI)	P value
Intra-ventricular hemorrhage (gr. 3-4) and/or death (n=13)	0.13 (0.03-0.68)	0.016	0.14 (0.03-0.70)	0.016
Retinopathy of prematurity (n=126)	1.84 (0.54-6.27)	0.326	1.11 (0.33-3.69)	0.866
Necrotizing enterocolitis (n=48)	0.53 (0.17-1.59)	0.255	0.42 (0.13-1.31)	0.136
Bronchopulmonary dysplasia (n=109)	2.66 (0.58-12.19)	0.207	2.19 (0.50-9.67)	0.301
Proven sepsis (n=6)	0.85 (0.24-3.00)	0.797	0.95 (0.28-3.21)	0.935
Cerebral Palsy or death (n=23)	0.27 (0.07-1.00)	0.050	0.16 (0.04-0.60)	0.006
Motor developmental index <70 (n=74)	1.55 (0.33-7.24)	0.576	1.43 (0.31-6.64)	0.649
Psychomotor index <70 (n=64)	1.46 (0.33-6.49)	0.623	0.91 (0.20-4.12)	0.900

Cluster 1 (non-exposed: Hp switch-off and IL-6<100 ng/mL, n=432)

Cluster 2 (exposed: Hp switch-on independently of IL-6 levels, n=449)

Cluster 3 (exposed: Hp switch-off and IL-6 ≥100 ng/mL, n=40)

Table 5. Relationship between Cerebral Palsy and/or Death and Cluster.

Cerebral Palsy and/or death	Adjustment for treatment group alone		Adjustment for treatment group, baby sex and GA at birth	
	OR (95% CI)	P value	OR (95% CI)	P value
Cluster 1 vs. Cluster 2	1.00 (0.41-2.43)	0.993	1.53 (0.59-3.96)	0.378
Cluster 1 vs. Cluster 3	0.21 (0.06-0.70)	0.012	0.29 (0.08-1.10)	0.068
Cluster 2 vs. Cluster 3	0.21 (0.06-0.71)	0.012	0.19 (0.05-0.71)	0.014

Cluster 1 (non-exposed: Hp switch-off and IL-6<100 ng/mL, n=432)

Cluster 2 (exposed: Hp switch-on independently of IL-6 levels, n=449)

Cluster 3 (exposed: Hp switch-off and IL-6 ≥100 ng/mL, n=40)

Haptoglobin: Hp

DISCUSSION

[00103] Sequencing of the human genome has provided an unprecedented advancement in elucidation of gene and protein expression in a variety of diseases. It also underlined the importance of the environment in the determinism of various medical conditions. That is because genetic susceptibility was unable to explain alone the non-linear relationship between presence and/or levels of various environmental etiologic agents (i.e., infectious, chemical, physical, life-style) and various individual response to the same noxious agent. Porta *et al.*, J Epidemiol Community Health 56:734–8 (2002). As a consequence the notion

of "exposome" evolved as a scientific concept aimed to explain the complex interplay between genetic susceptibility and etiologic environmental triggers. Wild CP., Cancer Epidemiol Biomarkers Prev 14:1847–50 (2005).

[00104] 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. However, although infection-induced PTB have a disproportionately worse outcome than what could be attributed to prematurity alone, not all cases have a bad outcome. To this end, predicting infants who are the most likely to develop postnatal complications from infectious encounters *in utero* remains a critical clinical and scientific issue. Stoll *et al.* Pediatrics 127:817–26 (2011).

[00105] Figure 3 shows the relationship between CP and cluster 3 was only identified as part of the new study, as the prior study investigating primarily EONS did not have CP as outcome. When we initiated the new study and requested the samples from the NICHD repository we hypothesized that the risk of CP will coincide with the risk of EONS. Interestingly, this was not the case. Thinking post factum there is a mechanistic explanation which is explained in Figure 4.

[00106] Figure 4 shows the relationships among the timing of the infectious trigger, cord blood IL-6 and Hp levels which generate the 3 newborns clusters described in this study. Newborns in Cluster 1 are identified at birth as having switch-off Hp and HpRP (identified phenotypically as haptoglobin 0-0) and low IL-6. These newborns did not have an antenatal encounter with an infectious agent. Newborns in Cluster 2 on the other hand, have had an antenatal encounter with infection yet they were able to mount an adequate inflammatory response which includes IL-6 as well as Hp and HpRP. Based amounted evidence from us and others we expect IL-6 immunoreacitity in cord blood peaks earlier than Hp and HpRP and is transitory. Since IL-6 is known to be the transcriptional stimulus for Hp and HpRP, newborns in Cluster 2 respond appropriately with elevation in Hp and HpRP immunoreactivity which measures in ELISA in accordance with their genetically-determined phenotype: Hp1-1 (slow kinetic and low plateau), Hp 2-2 (fast kinetic and high plateau) and Hp 1-2 (intermediate). Thinking post-factum, as haptoglobin is a very potent antioxidant and antimicrobial defense molecule, these newborns are the ones able to protect themselves. Interestingly in the BEAM study, we were able to identify a separate cluster which was not

apparent earlier. The newborns in Cluster 3 who have high IL-6 but are unable to increase their Hp and HpRP and it is exactly these newborns who are most likely to die or have severe morbidity (cerebral palsy). These newborns may have a genetically determined defect in either promoter region which prevents IL-6 from activating transcription or in coding region which results in silencing of the gene.

[00107] The complete disclosure of all patents, patent applications, and publications, and electronically available material cited herein are incorporated by reference. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.

CLAIMS

What is claimed is:

1. A method of diagnosing a newborn as having an increased risk of developing cerebral palsy, comprising

obtaining a biological sample from the newborn;

determining the level of Hp and/or HpRP in the biological sample and comparing it to a control value;

determining the level of IL-6 in the biological sample and comparing it to a control value; and

diagnosing the newborn as having an increased risk of developing cerebral palsy if the level of Hp and/or HpRP is lower than the control value, and the level of IL-6 is greater than the control value.

2. The method of claim 1, wherein the newborn is premature.

3. The method of claim 1, further comprising determining the level of C-reactive protein (CRP) and comparing it to a control value.

4. The method of claim 1, wherein the level of Hp and/or HpRP and the level of IL-6 are determined using an immunoassay.

5. The method of claim 4, wherein the immunoassay is a surface-enhancing Raman scattering (SERS) immunoassay.

6. The method of claim 1, wherein the biological sample is a blood sample.

7. The method of claim 6, wherein the blood sample is obtained from umbilical cord.

8. The method of claim 1, wherein the biological sample is a decidua sample or a placental sample.

9. The method of claim 1, further comprising providing treatment for the newborn having an increased risk of having or developing cerebral palsy.

10. The method of claim 1, wherein the biological sample is obtained within 72 hours after the birth of the newborn.

11. The method of claim 1, wherein the control value for IL-6 is a value of about 100 pg/mL.

12. The method of claim 1, wherein newborn was diagnosed as having been exposed to infection in utero.

13. A kit for diagnosing a newborn as having an increased risk of developing cerebral palsy, comprising a first antibody or binding fragment thereof specific for Hp and/or HpRP, a second antibody or binding fragment thereof specific for IL-6, reagents for conducting the diagnosis, and a package for holding the antibodies and the reagents.

14. The kit of claim 13, wherein the kit further comprises surface-enhanced Raman scattering probes.

15. The kit of claim 13, wherein the kit further comprises a lateral flow strip.

16. The kit of claim 13, wherein the kit further comprises instructions for using the kit to determine the levels of Hp and/or HpRP and the level of IL-6, and Hp and/or HpRP and IL-6 controls.

17. A method of diagnosing a newborn as having an increased risk of developing retinopathy of prematurity, comprising

obtaining a biological sample from the newborn;

determining the level of Hp1-2 and/or Hp2-2 in the biological sample and comparing it to a control value; and

diagnosing the newborn as having an increased risk of developing retinopathy of prematurity if the level of Hp1-2 and/or Hp2-2 is higher than the control value.

18. The method of claim 17, wherein the newborn is premature.

19. The method of claim 17, wherein the level of Hp1-2 and/or Hp2-2 are determined using an immunoassay.

20. The method of claim 17, wherein the biological sample is a blood sample.

21. The method of claim 17, wherein the blood sample is obtained from umbilical cord.

22. The method of claim 17, further comprising providing treatment for the newborn having an increased risk of having or developing retinopathy of prematurity.

23. The method of claim 17, wherein the biological sample is obtained within 72 hours after the birth of the newborn.

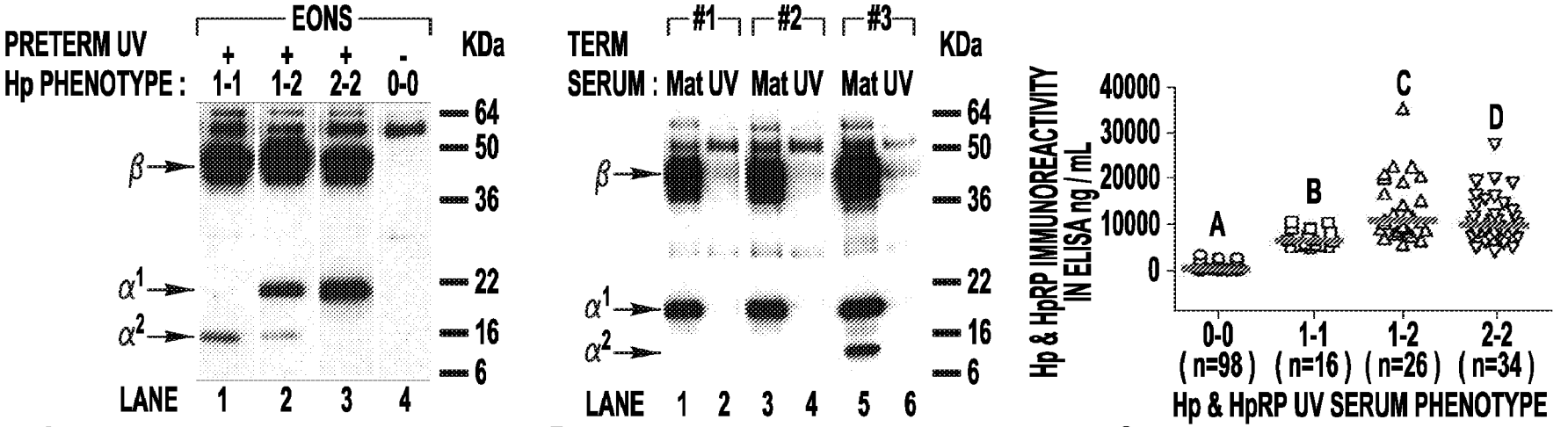


FIG. 1

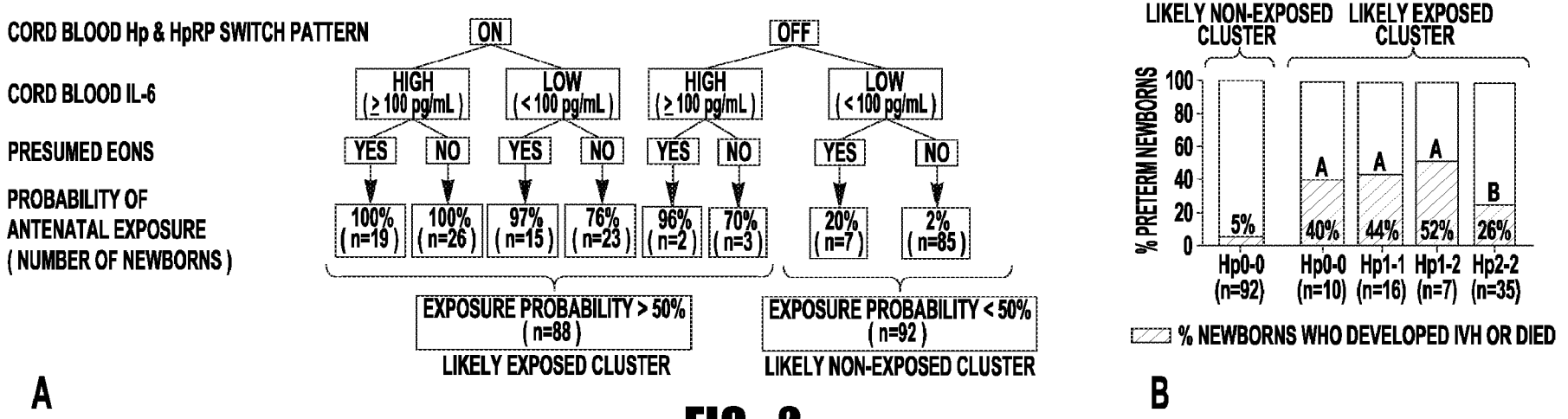


FIG. 2

SUBSTITUTE SHEET (RULE 26)

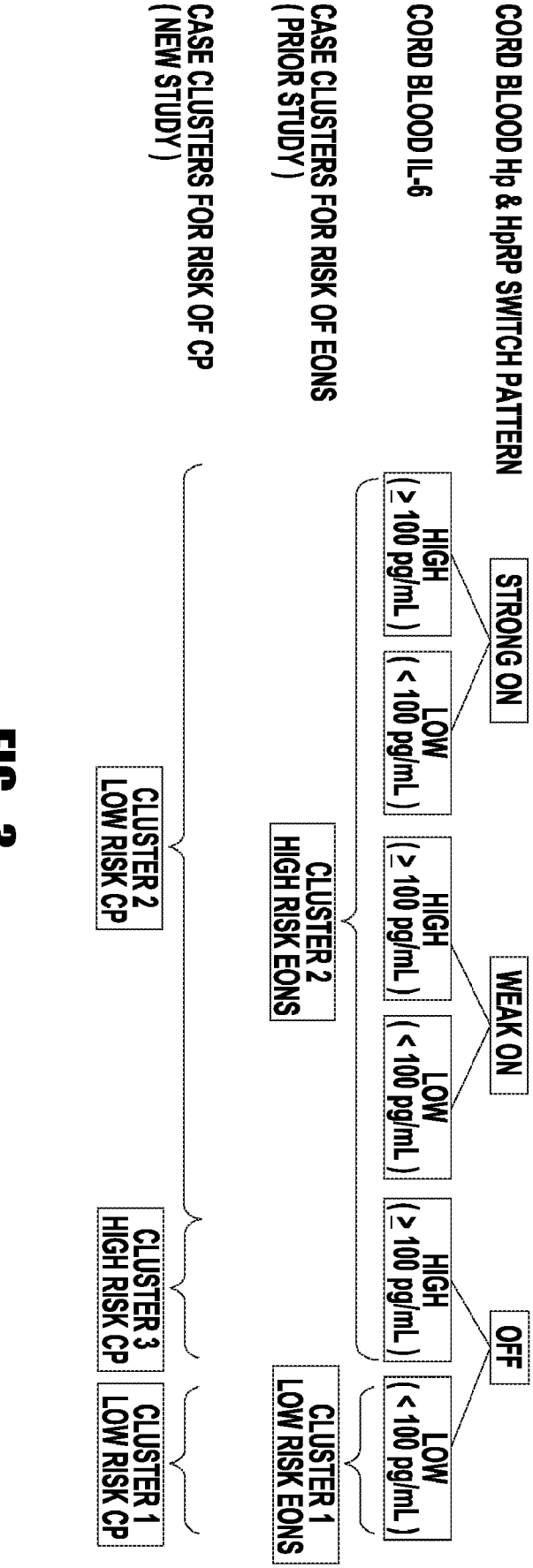

CORD BLOOD Hp & HpRP SWITCH PATTERN
CORD BLOOD IL-6
CASE CLUSTERS FOR RISK OF EONS (PRIOR STUDY)
CASE CLUSTERS FOR RISK OF CP (NEW STUDY)
STRONG ON
WEAK ON
OFF
HIGH (≥ 100 pg/mL)
LOW (< 100 pg/mL)
HIGH (≥ 100 pg/mL)
LOW (< 100 pg/mL)
HIGH (≥ 100 pg/mL)
LOW (< 100 pg/mL)
CLUSTER 2 HIGH RISK EONS
CLUSTER 1 LOW RISK EONS
CLUSTER 2 LOW RISK CP
CLUSTER 3 HIGH RISK CP
CLUSTER 1 LOW RISK CP

FIG. 3

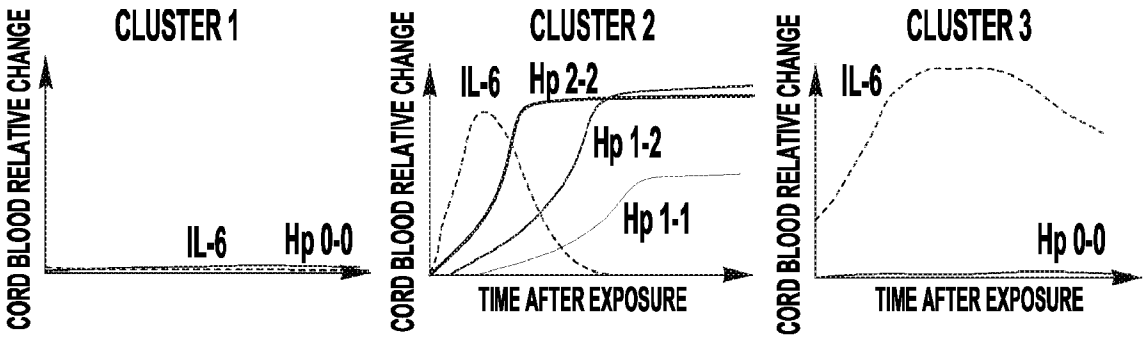

CLUSTER 1
CORD BLOOD RELATIVE CHANGE
IL-6
Hp 0-0
CLUSTER 2
CORD BLOOD RELATIVE CHANGE
IL-6
Hp 2-2
Hp 1-2
Hp 1-1
TIME AFTER EXPOSURE
CLUSTER 3
CORD BLOOD RELATIVE CHANGE
IL-6
Hp 0-0
TIME AFTER EXPOSURE

FIG. 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/063723

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - G01N 33/53 (2015.01)
CPC - G01N 33/53 (2015.12)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - G01N 33/48, 33/49, 33/50, 33/53 (2015.01)
CPC - G01N 33/48, 33/487, 33/49, 33/492, 33/53, 33/5302, 33/5306 (2015.12)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 435/7.1, 7.4, 7.72, 7.93; 506/7, 9; 702/19, 23
CPC - G01N 33/48, 33/487, 33/49, 33/492, 33/53, 33/5302, 33/5306 (2015.12) (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Orbit, Google Patents, Google Scholar, PubMed
Search terms used: haptoglobin, surface-enhanced Raman spectroscopy, neonatal, preterm birth, interleukin, rapid blood test, controls, scattering assay, lateral flow

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 2012/0021442 A1 (BUMINSCHI et al) 26 January 2012 (26.01.2012) entire document	1, 2, 4, 6-13, 16-23 ---------- 3, 5, 14, 15
Y	US 2011/0136734 A1 (BARRETT-REIS) 09 June 2011 (09.06.2011) entire document	3
Y	US 2010/0055721 A1 (LAMBERT et al) 04 March 2010 (04.03.2010) entire document	5, 14, 15
A	COSTANTINE et al. "Umbilical cord blood biomarkers of neurologic injury and the risk of cerebral palsy or infant death," Int J Dev Neurosci. 25 June 2011 (25.06.2011), Vol. 29, Pgs. 917-922. entire document	1-23
A	HELLGREN et al. "Proliferative Retinopathy Is Associated with Impaired Increase in BDNF and RANTES Expression Levels after Preterm Birth," Neonatology, 09 November 2010 (09.11.2010), Vol. 98, Pgs. 409-418. entire document	1-23

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 January 2016	02 MAR 2016
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

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