[Frontiers in Bioscience, Landmark, 21, 165-177, January 1, 2016]

Retinopathy of prematurity: an oxidative stress neonatal disease

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1. ABSTRACT

Proteomics is the global study of proteins in an organism or a tissue/fluid and is clinically relevant since most disease states are accompanied by specific alterations in an organism's proteome. This review focuses on the application of proteomics to neonatology with particular emphasis on retinopathy of prematurity (ROP), which is a disease in which oxidative stress plays a key pathophysiological role. Oxidative stress is a physiologically relevant redox imbalance caused by an excess of reactive oxygen (ROS) or reactive nitrogen oxide species (RNOS). A major conclusion of this review is that proteomics may be the optimal technology for studying neonatal diseases such as ROP, particularly in the setting of a neonatal intensive care unit (NICU). Proteomics has already identified a number of ROP serum biomarkers. This review will also suggest novel therapeutic approaches to ROP and other neonatal oxidative stress diseases (NOSDs) based on a systems medicine approach.

2. INTRODUCTION

2.1. The power of a systems biology approach

Systems biology/medicine is a non-reductionist approach to biological problems in which an organism is considered a time-dependent complex set of interconnecting protein, genetic and metabolic systems with environmental inputs. This approach relies on the rapidly evolving technology of genomics, proteomics and metabolomics. The genome is all the DNA sequence information found in a cell and is the least dynamic of the "omics". Recent thinking makes clear, however, that the genome cannot be considered static since it responds

to environmental stressors and chemical exposure, i.e., genomic plasticity. Nevertheless, the transcriptome, which is all the RNA transcripts present in a given cell, organs or organism, is more dynamic than the genome. The proteome, which is all the proteins expressed by a genome in a cell, tissue or organism at a given time, is also very dynamic. The proteome is particularly relevant to disease processes since it is proteins, by-and-large, that perform most cellular functions, and most disease states are accompanied by alterations in cellular functions. Metabolomics is the most dynamic of the "omics" and it is the large-scale study of all the metabolites in a biological cell, tissue, organ or organism. While the "omics" technologies provide very large and complex "parts lists" for cells and organisms, it is the dynamic integration of these technologies into the holistic framework of systems biology (via bioinformatics) that provides a path to "P4 medicine" which is characterized by the potential for being predictive, preventive, personalized and participatory (1,2).

Systems biology is extremely useful because it alone frames complex medical problems in a sufficiently broad context so as to capture significant etiological factors and thereby suggest evidence-based disease interventions (3). Recently, a ground breaking issue of *Pediatric Research* was totally devoted to the application systems biology to pediatric research (4). The application of systems biology to the problems of the newborn is an emerging area and the number of publications in this area will certainly increase in the near future. As reviewed by Buonocore *et al.* (5), urinary metabolomics has enormous untapped potential in both pediatrics and neonatology.

2.2. A systems biology approach to premature birth

In 2004 the USA National Institutes of Health/ National Institute of Child Health and Human Development (NIH/NICHD) issued a solicitation to initiate a genomic and proteomic network for premature birth (less than 37 weeks of pregnancy) research. The expressed purpose of the NIH/NICHD solicitation was to accelerate the pace of research on premature birth research by creating genomic and proteomic databases that could be used to provide a more molecular based understanding of premature pathophysiologies (e.g., ROP) and thereby pave the way for more effective therapeutic interventions. A key goal of this research initiative is to also discover potential biomarkers with the ability to predict whether a woman will spontaneously deliver a preterm infant and therefore provide therapeutic interventions with the potential of preventing premature birth as well as the diseases associated with prematurity. Results from this initiative are just now beginning to make their way into the primary literature and are likely to revolutionize the care and treatment of premature infants and provide insights into possible therapeutic strategies. In addition to the USA NIH/NICHD initiative there is a multinational Preterm Birth International Collaborative (PREBIC) organization whose aim is to improve pregnancy and birth outcomes as well as to optimize infant health and long-term development. Gracie *et al.* (6) have reviewed the most recent discussions of the PREBIC-"Omics" Research group with an emphasis on the need to integrate the results of genomics, transcriptomics, proteomics and metabolomics using a systems biology approach.

2.3. The systems biology of neonatal oxidative stress diseases (NOSDs)

This review article is focused on the application of system biology to ROP, which is a neonatal disease with an oxidative stress component. The rationale for this narrow focus is based on the notion that clinical progress is likely to be more rapid when presented with a clear and coherent overall hypothesis, i.e., oxidative stress is at the core of ROP and interventions in redox signaling cascades may lead to useful therapies. Moreover, redox proteomics has made major advances in the last few years and is a "mature" branch of systems biology and is well poised to make clinical contributions (7-10).

Newborn infants are immediately challenged with an abrupt increase in oxygen levels since alveolar pO_2 levels after birth are at least ten times higher than during intrauterine development (11,12). This abrupt increase in oxygen levels and the accompanying increase in reactive oxygen species (ROS) requires the detoxification actions of both enzymatic and chemical antioxidants. Chemical antioxidants include water-soluble compounds such as ascorbate, urate (13) and reduced glutathione (the key intracellular antioxidant) as well as lipid soluble antioxidants such as vitamin E (tocopherols and tocotrienols) which are the key antioxidants in biological membranes and lipoproteins. Enzymatic antioxidants include catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPX).

3. RETINOPATHY OF PREMATURITY (ROP), OXIDATIVE STRESS AND "OMICS"

3.1. Premature infants are particularly susceptible to oxygen toxicity

Premature infants are often provided with supplementary oxygen since their immature alveoli are lacking surfactant and without additional oxygen they can become seriously hypoxic. Nevertheless, there is a delicate balance between providing therapeutic oxygen and the potential damage done by the increased ROS and reactive nitrogen oxide species (RNOS) resulting from decreased antioxidant enzymes. One of the more serious outcomes associated with supplementary oxygen delivery to premature infants is retinopathy of prematurity (ROP). The retina is particularly sensitive to oxidative stress since it has: (1) a very high content of polyunsaturated fatty acids which are very susceptible to lipid peroxidation (14); (2) a very high level of oxygen consumption and the presence of retinal which is a photosensitizing agent capable of generating free radicals (15).

3.2. A high oxygen saturation level promotes ROP

ROP is a complex ocular disease characterized by initial damage to the retinal microvasculature followed by neovascularization and a potential for subsequent retinal detachment. There are two distinct phases for the development of ROP: phase I is characterized by an inhibition of retinal vascularization (provoked by supplemental oxygen) and phase II is characterized by an accelerated retinal neovascularization caused by hypoxia (16). Early clinical studies were definitive in showing a connection between ROP incidence in premature infants and exposure to unrestricted oxygen (17). These observations lead to studies showing that a reduced level of oxygen saturation could decrease the incidence of ROP (17). Oxygen saturation in infants can now be continuously and non-invasively monitored by pulse oximetry. Recent data analyses suggest that preterm infants targeted with a lower oxygen saturation (85-89% as monitored by a pulse oximeter) have a lower incidence of ROP but a higher morality compared to an oxygen saturation of 91-95% (18). Controversy surrounding the Surfactant, Positive Pressure, and Oxygenation Randomized Trial (SUPPORT) raised by the USA Office of Human Research Protections (OHRP) may make it difficult to pursue future research aimed at finding the safest oxygen saturation levels for extremely premature babies (19,20).

Smith *et al.* (16) have recently published an outstanding review of the pathophysiology of ROP. These authors reviewed the information suggesting that ROP-phase I is caused by a combination of hyperoxia and nutritional deficiencies and phase II by hypoxia driven neovascularization (16). Recent work supports the view that supplemental oxygen during phase I is a significant risk factor for ROP (16). A major conclusion of recent studies is that “new approaches to the prevention of ROP are required ” (17). Considerable evidence supports the view that oxidative stress plays an etiological role in the pathogenesis of ROP (21-25).

3.3. Genetic polymorphisms in antioxidant enzymes are useful in predicting susceptibility to ROP and other NOSDs

A reasonable starting point for a new approach would be the ability to predict which neonates would be particularly susceptible or resistant to ROP (or other neonatal oxidative stress diseases (NOSDs)) (26). An infant with a high-predicted susceptibility to ROP could, for example, be administered therapies designed to protect the retina (more on this below) from high oxygen saturation and thereby avoid the increased mortality risk when suboptimal levels of oxygen saturation are used. One would expect that the genomics of single nucleotide polymorphisms (SNPs) in neonatal antioxidant enzymes could be important in this regard. SNPs are naturally occurring variations in the DNA sequence at a particular locus. While most SNPs are thought to have no particular health related significance there is a subset of SNPs that have the potential for being quite informative with regard to disease susceptibility, progression and outcome.

Genetic polymorphisms in the SOD enzymes could be important determinants in ROP. Humans have three forms of superoxide dismutase (SOD) genes called SOD1 (soluble), SOD2 (mitochondrial) and SOD3 (extracellular). These SODs are key antioxidant antioxidants enzymes that cause the dismutation of the superoxide radical into hydrogen peroxide and oxygen. A recent retrospective study by Poggi *et al.* (27) affirms this expectation: these authors found that SOD1 (GG) decreased the risk of ROP as well as RDS and IVH while SOD2 (GT) increased the incidence of BPD and decreased the risk of RDS, IVH and ROP. The rs8192287 SOD3 polymorphism was found to be an independent protective factor for IVH while rs4880 and rs5746136 SOD2 polymorphisms were found to be associated with a lower gestational age and birth weight (27). Poggi *et al.* (27) used DNA from dried blood spots that were routinely collected for screening of inborn errors of metabolism (IEMs) and they only looked at a total of ten SNPs. The importance of SOD in ROP was also underscored in the work of Parad *et al.*(28). These investigators evaluated the effect of intratracheal recombinant Cu/Zn SOD (SOD1) on ROP in extremely low gestational age newborns (ELGANs). ROP was found to be reduced in the ELGANs receiving SOD1 compared to those receiving placebo (28). These investigators did not report the SOD SNPs in their patient population but this could be an important variable that should be looked at in future (and larger) studies. Moreover, the mode of delivery could also be an important variable as discussed further below.

Mohamed *et al.* (29) examined the potential association of ROP with a set of SNPs important in angiogenesis, inflammation and oxygen sensing pathways. These investigators looked at 455 SNPs in 153 genes and found significant associations IHH (Indian hedgehog), AGTR1 (angiotensin II Receptor, Type 1), TBX5 (T-Box 5), CETP (cholesteryl ester transfer protein) and GP1BA (glycoprotein Ib (platelet) alpha polypeptide). These five genes have previously been found to have an association with prematurity (29), which is well-known clinical risk factor associated with ROP, and may not be directly specific to ROP development.

Since vitamin E is potent lipid soluble antioxidant, it is reasonable to suggest that tocopherols could be important in preventing phase I ROP. Tissue levels of vitamin E are influenced by both dietary levels (more on this below) as well as the enzymes involved in vitamin E metabolism. Recent work suggests that polymorphisms in the cytochrome P450 4F2 (CYP4F2) could significantly affect the pharmacokinetics of vitamin E and its use a

Table 1. Genes involved in phase I or II retinopathy of prematurity

	Function	Gene name
Phase I	Oxidative Stress	
	Metabolism of vitamin E	CYP4F2
	Glutathione peroxidase	GPX1 to 8
	Haptoblobin	HP
	NADPH oxidase	NOX1, NOX3 to 5
	Superoxide dismutase	SOD1 to 3
Phase II	Retinal neovascularization	
	Endothelial nitric oxide synthase	NOS3
	Hypoxia inducible factor-1	HIF1A
	Vacular endothelial growth factor	VEGFA
	Insulin like growth factor	IGF1
Each gene has been checked and found to have multiple single nucleotide polymorphisms that measurable by low cost genotyping		

therapeutic agent in children (30). CYP4F2 is the primary enzyme metabolizing vitamin E is it has two prevalent variants (rs2108622 and rs3093105) which can alter the vitamin E activity (30).

3.4. Genetic polymorphisms and the advent of low cost DNA analysis services in neonatal genomics

The advent of low cost personnel (direct-to-consumer) DNA analysis services like 23andMe (www.23andme.com) can provide genome-wide information on hundreds of thousands SNPs including those for the SOD1, SOD2, SOD3, NOX1, NOX4, DUOX1, DUOX2, GPX1, GPX2, GPX3, GPX4, GPX5, GPX6, GPX7 and GPX8 genes (see Table 1). The NADPH oxidase (NOXs and DUOXs) enzymes are a well-known source of superoxide radicals and these pro-oxidant enzymes thought to play important roles in directly damaging the retinal vasculature (important in phase I ROP) and by promoting angiogenesis (25). An increased level of ROS generated from NOX enzymes is known to stabilize transcription factor HIF (hypoxia inducible factor)-1 that can subsequently promote the expression of factors promoting angiogenesis such as VEGF (25). Under normal conditions, HIF-1 is not stabilized, is rapidly degraded by the proteasome and cannot, therefore, initiate transcription. *In vitro* work by Craige *et al.* (31) has demonstrated that hypoxia induced overexpression of NOX4 can promote angiogenesis through activation of endothelial nitric oxide synthase (eNOS). This could be an important triggering mechanism for phase II ROP. The importance of NOX4 in ROP has also been confirmed in a rat model (32).

Work in an animal model shows that GPX1 is protective against ROP (22). Holmstrom *et al.* (33) have done an excellent job of reviewing the evidence supporting a genetic component to ROP susceptibility. These authors have emphasized the potential roles of polymorphisms in the vascular endothelial growth factor (VEGF) genes and the insulin-like growth factor 1 (IGF1) gene (33). The 23andme DNA service also provides SNPs for the VEGF genes (VEGFA, VEGFB and VEGFC) as well as IGF1.

Assuming that issues related to turn-around time, sampling, privacy and other bioethical concerns can be solved, the genomic SNP information provided by large-scale DNA testing services could be a very cost-effective approach to potentially identifying those neonates susceptible to NOSDs as well as host of other diseases. The ability to identify infants at high risk for ROP by genetic criteria may allow for the development of more targeted and cost-effective clinical screening criteria by reducing unnecessary additional clinical screening in infants with a low ROP genetic risk. Clearly, there is a need to perform large-scale multisite clinical studies using the latest cost-effective DNA testing services with state-of-art bioinformatic analyses. Moreover, looking at genetic polymorphisms in both neonates and their mothers might also be useful since oxidative stress also occurs during fetal development (34). In the USA it is also possible that State-screening services for IEMs (inborn errors of metabolism) will eventually utilize large-scale cost effective DNA testing services to help guide preventive medical decisions for all newborns (see below). Nevertheless, this is topic of intense and ongoing bioethical debate.

The bioinformatic tools necessary to study the association of genome-wide SNPs to complex multigenetic diseases, like ROP, are rapidly advancing (35). Bakit-Gungor *et al.* (35) have developed a web server called PNAOGA (Pathway **a**nd Network Oriented GWAS (Genome-Wide Association Study) Analysis) that advances conventional GWAS by emphasizing the functional effect of a SNP and its potential impact of biological pathways and protein interaction networks. In order for GWAS to be useful for pediatricians (and clinicians in general) there must be an ongoing update of relevant SNP- disease correlations as well as some uniformity in the statistical methods used to uncover such associations.

Although more expensive then measuring genome-wide SNPs, genome and exome sequencing can provide an enormous amount of medically relevant information. The Eunice Kennedy Shriver National Institute of Child Health (NICHD) along with the National Genome Research Center (NHGRI, www.genome.gov) are now funding a program that will explore the medical potential of genomic sequencing in newborn healthcare (DOI: news/health/sep2013/nhgri-04.htm). This NIH program is primarily focused on diagnosing inborn errors

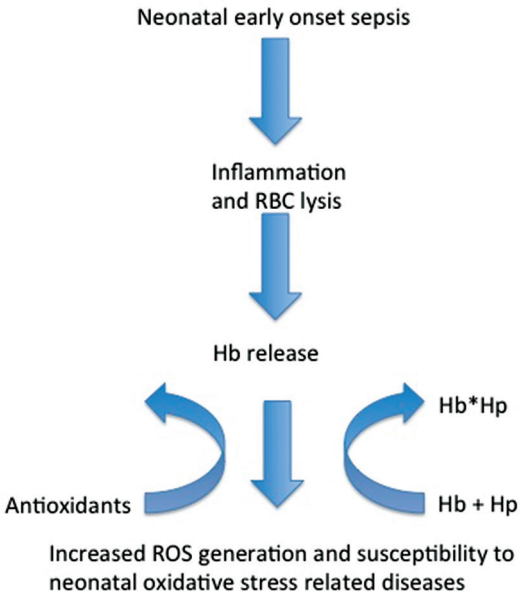

Figure 1. Inflammation caused by neonatal early onset species may produce an oxidative stress contributing to erythrocyte hemolysis, which, in turn would promote yet more oxidative stress if not counteracted by haptoglobin (Hp) which forms a complex with hemoglobin (Hb).

of metabolism but the genomic information gained could eventually be relevant to the susceptibility of newborns to NOSDs and provide genetic biomarkers for timely interventions. It is relevant that issues related to the turn-around time for genome sequencing may not be a major issue. Quite amazingly, work done at the Children's Mercy Hospital (Kansas MO) suggests that the genomes of ill newborns can be sequenced in less than 24 hours (36). The Children's Mercy Hospital is one of the grantees of the NICHD-NHGRI Newborn Genome Sequencing Program.

3.5. Proteomics and ROP

3.5.1. Genomic data alone is insufficient to accurately predict ROP and NOSDs

Genomic information, while very useful, has limitations in so much as it provides minimal information on the actual levels of expressed proteins or their posttranslational modifications. Moreover, genomic data generally does not provide information on infectious agents or dynamic responses to major environmental stressors such as smoking during pregnancy or free radical stress caused by the intestinal microbiome. Proteomics is therefore a critical supplement to genomics. It is, however, much less amenable to high throughput technology. Moreover, in the case of ROP, there are very few clinical proteomic studies.

3.5.2. The proteomics of early-onset neonatal sepsis, ROP and oxidative stress

Buhimschi *et al*. (37) have noted that early-onset neonatal sepsis (EONS) is associated with ROP, RDS, NEC, BPD and IVH which are all neonatal oxidative stress diseases (NOSDs). EONS in newborns is caused by the acquisition of maternal microorganisms and its onset is very quick in premature infants. EONS is difficult to predict yet early identification is extremely important for timely treatment and prevention of NOSDs. Buhimschi *et al.* (37) used proteomics to identify EONS biomarkers in cord blood (CB). CB is an extremely valuable biofluid for both proteomics and genomics since CB has a DNA profile identical to that obtained from a heel stick (38). Two-dimensional differential gel electrophoresis (2D-DIGE) showed that haptoglobin (Hp) and haptoglobin related protein (Hpr) are markedly elevated in newborns with EONS compared to non-EONS newborns (37). Healthy newborns were, in fact, found to have "near-absent Hp" (37).

Both Hp and Hpr exhibit a high affinity binding for hemoglobin (Hb) released by the lysis of erythrocytes (e.g., by hemolytic anemia) but only the Hp-hemoglobin complex is rapidly removed from circulation by the CD163 scavenger receptor of the reticuloendothelial system (39). Hp can be considered an antioxidant by virtue of its ability to prevent heme-iron from promoting the generation of ROS (c.f. (40)). As outlined in Figure 1, it is plausible, therefore, that that the inflammation caused by EONS produces an oxidative stress contributing to erythrocyte hemolysis, which, in turn would promote yet more oxidative stress if not counteracted by Hp. This hypothesis provides a reasonable link between EONS and NOSDs. The ability to use Hpr and Hr plasma protein levels to quickly diagnose EONS would be extremely valuable since such data would be useful in deciding whether or not to administer antibiotics and/or antioxidant agents (more on this below).

Hp also plays a critical bacteriostatic role by sequestering iron (in Hb) that could otherwise fed the growth of many types of bacteria (e.g. *Escherichia Coli*), i.e. "nutritional immunity" (41). There are however, a number of microbial species that are able to obtain iron from the HpHb complex (41) and this could obviously be problematic for preterm infants with EONS. Knowing exactly (and quickly) what microorganisms might be contributing to EONS would be clinically very useful in formulating strategies for preventing both sepsis and ROP. In particular, this information could permit a more targeted therapy by helping to limit "unnecessary" antibiotic use in those infants do not need it and thereby minimizing the problematic development of multi-drug resistant bacteria in the infants' intestinal microbiome.

3.5.3. The potential of nucleic acid programmable protein arrays (NAPPA) in pediatric proteomics

Mass spectrometry and/or 2-D gel electrophoresis are not high throughput technologies and their turn-around times can be sufficiently long so

as to diminish their usefulness for making rapid clinical decisions. Nucleic acid programmable protein arrays (NAPPAs) show the promise of immense clinical utility since this technology is very amenable to "bedside" application with rapid turnaround and minimal investment in laboratory infrastructure. Standard protein microchips/ microarrays requires that many hundreds of individually purified proteins (usually monoclonal antibodies) be placed as spots on a slide and then probed with fluorescently tagged proteins from the biofluid sample (e.g. cord blood) to be analyzed. This is a daunting task fraught with many technical difficulties such as the long-term stability of spotted proteins to be probed.

In NAPPA, cDNA templates are first spotted on microarray slides with each spot containing a template coding for a specific target protein. The cDNA is constructed so as to express the gene of interest as a fusion protein with a glutathione-s-transferase (GST) tag. The cDNA is spotted on the slide with each spot containing an anti-GST antibody. After application of an *in vitro* transcription/translation system, the newly expressed target-GST fusion protein is tethered to the spot by the anti-GST antibody and can be subsequently probed for an interacting protein in the biofluid to be assayed. This technology is adept at looking for serum antibodies or autoantibodies but should eventually prove useful where a serum protein has a specific interacting protein that be expressed from the cDNA template. It could be useful for the early detection of plasma antibodies to specific bacterial antigens important in neonatal sepsis and therefore relevant to ROP.

4. THERAPEUTIC APPROACHES TO ROP BASED ON SYSTEMS BIOLOGY

4.1. Prenatal vitamins, an untapped resource

A reasonable approach for dealing with ROP would be to help reduce the occurrence of prematurity and mitigate low birth weight by the use of interventions targeted to high-risk pregnant women identified by known clinical risk factors and validated biomarkers. It is also reasonable to suggest that antioxidant status of the pregnant mother could be an important determinant of antioxidant status of the developing fetus as well as the neonate and thereby be a factor in the susceptibility to ROP and other NOSDS (42). Since most pregnant women are advised to take, or are provided with, a prenatal dietary supplement this could be an ideal opportunity to provided key antioxidant vitamins/ nutrients along with folate. Se in a prenatal supplement might be useful for preventing episodes of sepsis in preterm infants and by providing a source of Se for GPX synthesis. Se is not present in many prenatal supplements.

Vitamin E is often present in prenatal vitamins but is usually present as only all-rac-alpha-tocopheryl acetate. RRR-alpha-tocopherol is the natural form of alpha-tocopherol and is the predominant form found in plasma. RRR-gamma-tocopherol is the primary dietary from of vitamin E and *in vitro* studies show this form to be preferentially taken up by cells (43) Alpha- and gamma-tocopherol are both antioxidants but have unique biochemical properties and abilities to regulate signal transduction pathways. In a key large-scale study, Scholl *et al.* examined the influence of alpha- and gamma-tocopherols on fetal growth (42). These investigators found that maternal alpha-tocopherol levels during pregnancy were correlated with increased fetal growth and a decreased risk of small-for-gestational-age births. Maternal gamma-tocopherol levels were positively correlated with dietary fat intake and negatively correlated with multivitamin use. The gamma-tocopherol data makes sense since multivitamins usually have only the alpha-tocopherol isoform, which is known to suppress plasma levels of gamma-tocopherol. These data are important since they suggest tocopherol should be added to prenatal vitamin in an attempt to increase fetal growth, which could be critical for extremely low birth infants.

4.2. The antioxidant status of premature infants and the effect of antioxidant supplementation on ROP

4.2.1. Vitamin E and ROP

The evidence reviewed above suggests that the overproduction of ROS is a contributing factor for ROP. It is reasonable, therefore, to expect that preterm infants with a robust antioxidant status at birth or supplemented with antioxidants would have a decreased risk of ROP or other NOSDs: this would be a second "line of defense". Vitamin E is the major lipid soluble antioxidant in plasma. Vitamin E is, however, not a single compound and comprises at least eight different naturally occurring isoforms -RRR-alpha, RRR-beta-, RRR-gamma- and RRR-delta-tocopherols and the corresponding tocotrienols). Dietary vitamin E is primarily RRR-gamma-tocopherol yet most clinical research has been done with all-racemic alpha-tocopherol (eight stereoisomers), which is only one eighth the naturally occurring RRR-alpha-tocopherol, and is often mislabeled as d,l-alpha-tocopherol (two stereoisomers). Newborns are known to have lower plasma tocopherol levels then their mothers as well as higher levels of tocopheryl quinone which is a biomarker for oxidative stress (44). Recent work by Bell *et al.* (45) confirms previous findings (e.g. (46,47)) that premature infants have a profound and functional tocopherol deficiency. Bell *et al*. (45) have found that a single enteral dose of "d,l-alpha-tocopheryl acetate" could increase the plasma level of tocopherol in premature infants 24 hours after administration as well as 7 days after administration. Since d,l-alpha-tocopheryl acetate has not been commercially available for a least a decade, it is likely that all-racemic alpha-tocopheryl acetate was actually used by Bell *et al.* (45).

In a well designed study, Phelps *et al.* (48) demonstrated that "d,l-alpha-tocopherol", provided

initially as IV and then followed up by oral administration, did not prevent ROP. Moreover, the IV administration of the "d,l-alpha-tocopherol" to preterm infants appeared to increase incidence of hemorrhagic complications (48). It should be noted that the "d,l-alpha-tocopherol" used by Phelps *et al.* (48) was emulsified in a vehicle containing Emulphor, a polyethoxylated vegetable oil as well as propylene glycol. The Emulphor could have transiently lysed RBCs (during the IV administration) thereby causing the release of heme-iron, which is a pro-oxidant (see below for more details). Clearly, careful consideration must be given to the mode of administration of any lipid soluble antioxidant. Johnson *et al.* (49) have also explored the use of vitamin E supplementation to treat ROP. In contrast to the work of Phelps *et al.* (48) these investigators present preliminary results suggesting that pharmacological levels of IV free alpha-tocopherol, also in a vehicle containing Emulphor, and found positive results in treating severe ROP in VLBW infants. These investigators found no clinical complications to be associated IV vitamin E.

Human milk feeding has recently been found to significantly prevent ROP in VLBW neonates compared to VLBW infants fed formula (50). In addition to antioxidant enzymes, human milk contains lutein and zeaxanthin, which are carotenoid antioxidants found at high levels in the retina and macula. Nevertheless, a small clinical trial found that supplementation with both lutein and zeaxanthin did not prevent ROP in preterm infants and did not affect patient outcomes (51).

4.2.2. Preterm antioxidant enzyme status, glutathione peroxidase (GPX) and selenium (Se) supplementation

Davis and Auten (12) have provided an excellent review of the fetal and neonatal maturation of antioxidant defenses. Under normal circumstances, fetal increases in key antioxidant enzymes, such as SOD, occurs only late in gestation. Premature infants are, therefore, particular susceptible to oxidative stress related pathologies since they can be lacking in key antioxidant enzymes as well as chemical antioxidants.

Selenium (Se) is a micronutrient that is a necessary component of selenoproteins such as GPX, which is a key antioxidant enzyme: GPX converts lipid peroxides (or H_2O_2) into the corresponding alcohols (or H_2O). In an animal model, dietary Se deficiency results in a decreased level of retinal GPX activity and an increased level of retinal oxidative stress (52). A Cochrane Neonatal Review has summarized the effects of Se supplementation on short-term morbidity in preterm neonates (53). Preterm infants were found to have low blood Se levels compared to normal newborns or their mothers. Se supplementation, while showing a significant reduction in the number of sepsis episodes, did not affect survival, neonatal chronic lung disease or ROP (53). Moreover, Se supplementation doubled the level of blood Se but did not influence the level of protein carbonyls or malondialdehyde (MDA) in the supplemented versus un-supplemented preterm control group (54).

Protein carbonyl levels are an excellent systemic biomarker for *in vivo* oxidative stress (55) but MDA levels are more problematic as measure of *in vivo* oxidative stress since this method involves heating the plasma sample, i.e., the MDA could be generated during the heating process and may not represent *in vivo* levels. It is also important to note that plasma GPX levels were not different between the Se supplemented or un-supplemented group at birth but were higher in the Se supplemented group only after seven days of Se supplementation (no earlier measurements were made). RBC glutathione peroxidase, which is a very sensitive indicator of dietary Se intake was not measured in the studies of Darlow (53) or Winterbourn (54). While the results of Se-supplementation on ROP appear discouraging, it may well be that GPX is only effective when the preterm infants are oxidatively stressed by administration of supplemental oxygen. This clinical study has not yet been attempted.

5. NANOTECHNOLOGY APPROACH TO THE DELIVERY OF ANTIANGIOGENIC DRUGS/ NUTRACEUTICALS TO THE RETINA

Phase II of ROP is characterized by a hypoxia driven neovascularization of the retina. This can eventually lead to rupture of the newly formed vessels with bleeding into the vitreous humor, retinal detachment and progressive loss of visual acuity. It is reasonable to assume that antiangiogenic drugs or nutraceuticals could, therefore, be therapeutic in combating this phase of ROP. Vascular endothelial growth factor (VEGF) is a key signaling protein for the stimulation of vascular growth and monoclonal antibody against VEGF (bevacizumab) has been used to inhibit angiogenesis in tumors. The intravitreal injection of bevacizumab has also shown promise in the treatment of ROP in both small clinical trials (56,57) and in a well-controlled mouse model of ROP (58). Nevertheless, intravitreal injections can have side effects (59) as well as major compliance issues, particularly in newborns that will not be still. There is also a concern about the potential negative effects of systemic VEGF absorption on the developing brain.

The facile non-invasive delivery of drugs to the posterior tissues of the retina has been a long-term goal of ocular pharmacology. Recent work suggests that the topical application (eye drops) of liposome encapsulated of drugs is a viable method for their delivery to the vitreous humor and the posterior tissues of retina (60,61). Topical eye drop application can be done on an outpatient basis and multiple applications per day would not be a major problem. Unilamellar liposomes are spherical

shaped aggregates of phospholipid molecules where the "skin" of the liposome is made of lipid bilayer with a hydrophobic interior (62). The lipid bilayer encapsulates an aqueous interior, which can accommodate water-soluble drugs or water-soluble macromolecules such as an antibody (e.g. bevacizumab). Hydrophilic water-soluble agents in the aqueous phase of the liposomes do not cross the lipid bilayer and are thus "encapsulated." Moreover, hydrophobic nutraceuticals/drugs can also be sequestered in the hydrophobic domain of the liposomal bilayer.

5.1. The optimal form of vitamin E for a liposomal preparation to prevent/treat ROP

Vitamin E can be readily incorporated into liposomes (63) but little is known about what might be the optimal form to use. "D,l-alpha-tocopherol" is probably not the optimal form of vitamin E for this purpose since, in addition to RRR-alpha-tocopherol, it contains stereoisomers not found in any dietary source. Using an animal model Tanito *et al.* (64) studied the distribution of tocopherols and tocotrienols after topical application of 5 microliter of the pure free antioxidant. Interestingly, they found that alpha-tocotrienol levels were increased in every ocular tissue analyzed after topical administration but this did not occur for alpha-tocopherol. Moreover, rats orally provided with the same amount of vitamin E (5 microliter) did not show any increase in ocular vitamin E levels. These data certainly suggest that: (1) direct topical application of vitamin E is far superior to dietary vitamin E at increasing ocular vitamin E levels and; (2) tocotrienols are superior to alpha-tocopherol (64). Delta-tocotrienol might be the optimal form for preventing ROP since this form of vitamin E blocks VEGF induced angiogenesis in marked contrast to alpha-tocopherol which has no such effect (65).

6. SUMMARY

A systems biology approach to treating ROP and other NOSDs is ideal since it can encompass and track many of the variables that could dictate clinical decisions and therapeutic strategies. Most clinical intervention studies are done in the absence of omics data and the outcome followed is a positive or negative response. With complementary omics data it is also possible to collect detailed information on why one patient could be responder to therapy and anther be a non-responder, e.g., variations in relevant SNPs. In the setting of a neonatal intensive care unit the application of omics technology should be at its best since an enormous number of environmental and nutritional parameters are monitored and controlled over time.

7. ACKNOWLEDGEMENTS

This research was supported in part by the National Institutes of Health grant C06RR0306551 and the East Tennessee State University Robert W. Summers Pediatric Research Endowment. All authors contributed equally to this paper.

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Abbreviations: 2D-DIGE: two dimensional differential electrophoresis, GTR1: angiotensin II receptor, type 1, BPD: bronchopulmonary dysplasia, CB: cord blood, CETP: cholesteryl ester transfer protein, CYP4F2: cytochrome P450, family 4, subfamily F, polypeptide 2, eNOS: endothelial nitric oxide synthase, ELGAN: extremely low gestational age children, EONS: early onset neonatal sepsis, GP1BA: glycoprotein Ib (platelet), alpha polypeptide, GPX: glutathione peroxidase,

GST: glutathione S-transferase, GWAS: genome wide association study, Hb: hemoglobin, HIF-1: hypoxia inducible factor-1, Hp: haptoglobin, Hpr: haptoglobin related protein, IEM: inborn error of metabolism, IGF1: insulin-like growth growth factor 1, IHH: Indian hedgehog, IVH: intraventricular hemorrhage, MDA: malondialdehyde, NAPPA: nucleic acid programmable protein array, NEC: necrotizing enterocolitis, NOSD: neonatal oxidative stress disease, NOX: NADPH oxidase, RDS: respiratory distress syndrome, RNOS: reactive nitrogen oxide species, ROP: retinopathy of prematurity, ROS: reactive oxygen species, SNP: single nucleotide polymorphism, SOD: superoxide dismutase, TBX5: T-Box 5, VEGF: vascular endothelial growth factor

Key Words: Systems Biology, Premature Infants, Proteomics, Retinopathy Of Prematurity, Antioxidants, Vitamin E, Glutathione Peroxidase, Superoxide Dismutase, Single Nucleotide Polymorphism, Liposomes, Review

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