Cluster Analysis of Early Postnatal Biochemical Markers May Predict Development of Retinopathy of Prematurity

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Purpose: Growth factors and inflammatory and angiogenetic proteins are involved in the development of retinopathy of prematurity (ROP). However, no early biochemical markers are in clinical use to predict ROP. By performing cluster analysis of multiple biomarkers, we aimed to determine patient groups with high and low risk for developing ROP.

Methods: In total, 202 protein markers in plasma were quantified by proximity extension assay from 35 extremely preterm infants on day 2 of life. Infants were sorted in groups by automated two-dimensional hierarchical clustering of all biomarkers. ROP was classified as stages I to III with or without surgical treatment. Predictive biomarkers were evaluated by analysis of variance and detected differences by two-sided paired t-test with Bonferroni corrections for multiple comparisons.

Results: Differences in 39 biochemical markers divided infants without ROP into two control groups (control 1, n = 7; control 2, n = 5; P < 0.05). Sixty-six biochemical markers defined differences between the control groups (n = 13) and all ROP infants (n = 23; P < 0.05). PARK7, VIM, MPO, CD69, and NEMO were markedly increased in control 1 compared to all ROP infants (P < 0.001). Lower TNFRSF4 and higher HER2 and GAL appeared in infants with ROP as compared to control 1 and/or 2 (P < 0.05, respectively).

Conclusions: Our data suggest that early elevated levels of PARK7, VIM, MPO, CD69, and NEMO may be associated with lower risk of developing ROP. Lower levels of TNFRSF4 with higher levels of HER2 and GAL may predict ROP development.

Translational Relevance: Cluster analysis of early postnatal biomarkers may help to identify infants with low or high risk of developing ROP.

Introduction

Retinopathy of prematurity (ROP) is a vasoproliferative retinal disorder affecting premature infants and a leading cause of childhood blindness worldwide.1 The number of infants at risk for ROP has been increasing as the survival rates of premature births improve due to advances in neonatal care.2 The retinal developmental mechanisms behind ROP have been extensively studied in animal models during the past decades.3,4 The dysregulation of retinal vascular development is a major factor in ROP pathogenesis,5 where vascular endothelial growth factor (VEGF) and insulin-like growth factor 1 (IGF-1) are important factors. However, other vascular and inflammatory proteins as well as growth factors, angiogenetic proteins, and neurotrophins have also been suggested to be associated with ROP.6–11 This multitude of ROP-associated factors demonstrates that numerous signaling pathways may be involved in the development of ROP and that maybe not a single biomarker but a combination of biomarkers should be investigated.

There are five stages used to describe ROP progression.12 Stages 1 and 2 are mild and likely to regress spontaneously. From stage 3, ROP is classified as severe since extraretinal neovascularization can become progressive enough to cause total retinal detachment.5 The incidence and severity of ROP are...
inversely related to birth weight (BW) and gestational age (GA)\textsuperscript{13,14} and directly related to intrauterine growth restriction.\textsuperscript{15} Male gender contributes to severe ROP.\textsuperscript{15} Models for oxygen-induced retinopathy exist,\textsuperscript{16} although the duration of mechanical ventilation seems to be a greater predictive factor for ROP development than the total duration of oxygen supplementation.\textsuperscript{17}

Previous studies indicate that plasma proteins can be useful in identifying preterm newborn infants at high risk for developing diseases such as bronchopulmonary dysplasia, respiratory distress syndrome, and persistent ductus arteriosus.\textsuperscript{18–23} However, only a few investigators have aimed to find a relationship between protein levels at birth and ROP development.\textsuperscript{10,24,25} None of these early biochemical markers is in clinical use for the prediction of ROP development. To define predictive biomarkers one by one for ROP may be difficult because of the numerous interacting regulatory mechanisms and the heterogeneity of study populations. Therefore, investigating groups together with expression profiles of proteins, instead of separate protein testing, can be an alternative for detecting predictive protein-level patterns in ROP development.\textsuperscript{26}

Our aim was to find predictive biomarkers for determining patient groups at high and low risk of developing ROP by investigating plasma proteins during the early postnatal period with comprehensive protein screening. The hypothesis was that proteins involved in ROP may be detected and categorized in clusters.

**Methods**

**Study Population**

Thirty-five infants were included from our previously described DAPP (Ductus arteriosus and pulmonary circulation in premature infants) cohort.\textsuperscript{23,27} Infants born at Uppsala University Children’s Hospital between November 2012 and May 2015 with a GA of less than 28 weeks and without heart defects or major congenital anomalies were eligible for inclusion. Infants were enrolled after informed and written consent was obtained from the parents. The study was approved by Regional Ethical Review Board.

**ROP Screening**

All infants were screened for ROP in the neonatal period, with weekly examinations from a postmen-
Table 1. List of All Tested Biochemical Markers ($n = 202$).

| 4E-BP1 | CCL28  | CCL10 | ESM-1 | hGDNF | IL-1ra | LEP | MMP-7 | PTPN22 | TM | VE-Statin |
|--------|--------|-------|-------|--------|--------|-----|-------|--------|----|-----------|
| ADA    | CCL4   | CCL11 | EZR   | HGF    | IL-2   | LIF | MPO   | PTX3   | TNF | VIM       |
| AGRP   | CD244  | CCL13 | FABP4 | hK11   | IL-20  | LIF-R| MYD88 | RAGE   | TNF | B         |
| AM     | CD40   | CCL16 | FADD  | HSP-27 | IL-20RA| LITAF| NEMO  | REG-4  | TNF | R1        |
| AR     | CD40-L | CCL5  | FAS   | ICOSLG | IL-22RA | LOX-1| NRTN  | REN    | TNF | R2        |
| ARTN   | CD5    | CCL6  | FasL  | IFN-γ  | IL-24  | LYN  | NT-3  | RETN   | TNF | TRSF-4    |
| AXIN1  | CD6    | CCL9  | FGF-19| IL-1α  | IL-27A | mAmp | NT-pro-BNP | SCF | TNFRSF9 |
| BAFF   | CD69   | Dkk-1 | FGF-21| IL-10  | IL-2RB | MB   | NTRK3 | SELE   | TNFSF14 | |
| BDNF   | CD81   | DNER  | FGF-23| IL-10RA| IL-33  | MCP-1| OPG   | SIRT2  | t-PA |          |
| Beta-NGF| CDH3  | ECP   | FGF-5 | IL-10RB| IL-4   | MCP-2| OSM   | SLAMF1 | TRAIL |          |
| BNP    | CDKN1A | EGFR  | Flt3L | IL-12  | IL-5   | MCP-3| PAPP  | SPON1  | TRAIL-R2|          |
| CA-125 | CEA    | EGFR  | FR-α  | IL-12B | IL-6   | MCP-4| PAR-1 | SRC    | TRANCE |          |
| CAIX   | CHI3L1 | eIF-4B| FS    | IL-13  | IL-6RA| MIA  | PARK7 | ST1A1  | TR-AP  |          |
| CASP-3 | CSF-1  | EMMPRIN| FUR   | IL-15RA| IL-7   | MIC-A| PDGF | ST2    | TSLP  |          |
| CASP-8 | CST5   | EN-RAGE| GAL   | IL-16  | IL-8   | MIP-1α| PD-L1 | STAMPB | TWEAK |          |
| CCL11  | CSTB   | Ep-CAM| Gal-3 | IL-17A | ILT-3  | MK   | PECAM-1| TF    | uPA   |          |
| CCL19  | CTSD   | EPO   | GDF-15| IL-17C | ITGA1  | MMP-1| PIGF  | TGF-α  | U-PAR |          |
| CCL20  | CTSL1  | ErbB2/H2R2| GH   | IL-17RB| ITGB1BP2 | MMP-10| PRL   | THPO   | VEGF-A |          |
| CCL23  | CX3CL1 | ErbB3/H3R3 | HB-EGF| IL-18  | KLK6   | MMP-12| PRSS8 | TIE2   | VEGF-D |          |
| CCL25  | CXCL1  | ErbB4/HER4| HE4  | IL-18R1| LAP TGF-β-1| MMP-3| PSGL-1| TIM   | VEGFR-2 |          |
Figure 1. Infants’ clinical characteristics. The patients without ROP showed significant differences compared to patients with ROP III nontreated or ROP III treated in GA and BW (marked with gray). No significant differences were found in BW percentile (BW%) or in z score BW between any of the studied groups.

Results

Infants’ Clinical Characteristics

The infants’ clinical characteristics are presented in Figure 1. ROP was classified as stages I to III according to the International Classification of ROP.\textsuperscript{12} Infants with ROP stage III were divided into nontreated and treated groups. Prenatal steroids were used in all cases.

No significant differences were found in BW percentile (BW%) or z score in BW between any of the groups. Infants with ROP stage I or ROP stage II were similar to infants in the group without ROP in GA and BW. Nontreated or treated infants with ROP stage III had lower GA and BW than infants without ROP (Fig. 1). The gender ratio of infants with ROP (male, n = 13; female, n = 10) was similar to infants without ROP (male, n = 6; female, n = 6).
Clustering of Infants without ROP (Controls 1 and 2)

The two-dimensional hierarchical clustering sorted infants without ROP into two control groups (controls 1 and 2). Both control groups manifested a unique and individual visual pattern of red pixels, mainly related to significant differences in 39 of 202 biochemical markers ($P < 0.05$). The levels of all 39 biochemical markers were higher in control 1 than in control 2 (Fig. 2). Of these 39 markers, 5 were associated with angiogenesis, 8 with apoptosis, and 16 with inflammation$^{14}$ (Fig. 2).

Clinical Characteristics of Infants without ROP (Controls 1 and 2)

There was no significant difference in the gender ratio between the two control groups, and they did not differ in GA ($P = 0.316$). However, infants in control 2 had higher BW ($P = 0.036$), higher $z$ score ($P = 0.03$), and higher percentile values in BW ($P = 0.027$). None of the controls were growth restricted ($z$ minimum: $-1.46$), but these findings could indicate that infants in control 2 had a higher intrauterine growth (Fig. 3).

Biochemical Markers Associated with ROP

The levels of 66 biochemical markers were significantly different between control 1, control 2, or both controls and all ROP infants, and the combination of respectively significant differences ($P < 0.05$) resulted in six groups (Table 2):

- Group 1. Control 1 versus all ROP
- Group 2. Control 1 and all controls versus all ROP
- Group 5. Control 2 versus all ROP
- Group 4. Control 2 and all controls versus all ROP
- Group 3. All controls versus all ROP
- Group 6. Control 1, control 2, and all controls versus all ROP

Clustering of Infants without ROP

The biochemical markers were clustered separately in each group and are presented together in Figure 4A. Figure 4B presents the level of significant differences between controls and ROP infants. The direction (±) and amplitude of differences in biochemical marker levels of the two controls and ROP infants were compared to the joint mean value of all control infants (Fig. 4C). Thus, the existence of groups 1 to 6 could be confirmed both with the individual pattern of marker levels and with the direction of differences from the clustering (Figs. 4A–C). Figure 4 shows that most
| Group  | Control 1 vs All ROP | Control 1–2 vs All ROP | Control 2 vs All ROP |
|--------|---------------------|------------------------|---------------------|
| Group 1| EPO 0.0036 NS NS NS | OSM 0.0348 NS NS NS | 4E-BP1 0.0469 NS NS NS |
|        | SIRT2 0.0304 NS NS NS | STAMPB 0.0232 NS NS NS | CSTB 0.0013 NS NS NS |
|        | PARK7 0.0001 NS NS NS | VEGF-A 0.0194 NS NS NS | ESM-1 0.0255 NS NS NS |
|        | SPON1 0.0212 NS NS NS | 4E-BP1 0.0304 NS NS NS | SRC 0.0037 NS NS NS |
|        | MMP-12 0.0267 NS NS NS | TNFRSF9 0.0285 NS NS NS | CSTB 0.0013 NS NS NS |
|        | Flt3L 0.0163 0.0064 NS | VIM <0.0001 0.0222 NS | MyD88 0.0013 0.0390 NS |
|        | FADD 0.0013 0.0390 NS | CASP-3 0.0029 0.0160 NS | HSP27 0.0002 0.0142 NS |
|        | MYD88 0.0029 0.0160 NS | NEMO 0.0002 0.0142 NS | eIF-4B 0.0111 0.0358 NS |
|        | CASP-3 0.0021 0.0351 NS | GSK3B 0.0002 0.0142 NS | PTEN 0.0011 0.0354 NS |
|        | NEMO 0.0002 0.0142 NS | TNFRSF9 0.0014 0.0078 NS | LITAF 0.0036 0.0203 NS |
|        | HSP27 0.0014 0.0078 NS | eIF-4B 0.0111 0.0358 NS | MPO 0.0001 0.0102 NS |
|        | KLF4 0.0020 0.0237 NS | CASP-8 0.0050 0.0155 NS | EN-RAGE 0.0020 0.0237 NS |
|        | CD69 0.0002 0.0157 NS | CD69 0.0003 0.0157 NS | TNFRSF9 0.0014 0.0078 NS |
|        | LYN 0.0067 0.0179 NS | LYN 0.0067 0.0179 NS | BIRC5 0.0004 0.0132 NS |
|        | AXIN1 0.0044 0.0132 NS | AXIN1 0.0044 0.0132 NS | TET1 0.0018 0.0177 NS |
|        | ECP 0.0018 0.0177 NS | ECP 0.0018 0.0177 NS | RETN 0.0034 0.0027 NS |
|        | RETN 0.0034 0.0027 NS | RETN 0.0034 0.0027 NS | BAFF 0.0005 0.0107 NS |
|        | BAFF 0.0005 0.0107 NS | BAFF 0.0005 0.0107 NS | RAGE 0.0010 0.0088 NS |
|        | RAGE 0.0010 0.0088 NS | RAGE 0.0010 0.0088 NS | MMP-10 0.0017 0.0148 NS |
|        | MMP-10 0.0017 0.0148 NS | MMP-10 0.0017 0.0148 NS | MMP-3 0.0029 0.0163 NS |
|        | MMP-3 0.0029 0.0163 NS | MMP-3 0.0029 0.0163 NS | IL-10 0.00268 0.0450 NS |
| Group 3| FGF-21 NS 0.0303 NS | ILT-3 NS 0.0454 NS | mAmP NS 0.0385 NS |
|        | FGF-19 NS 0.0303 NS | FGF-19 NS 0.0303 NS | FGF-19 NS 0.0303 NS |
|        | ErbB4/HER4 NS 0.0410 NS | ErbB4/HER4 NS 0.0410 NS | ErbB4/HER4 NS 0.0410 NS |
| Group 4| KLK6 NS 0.0174 0.0276 | VE-statin NS 0.0251 0.0315 | KLK6 NS 0.0174 0.0276 |
|        | TIM NS 0.0303 0.0063 | TIM NS 0.0303 0.0063 | TIM NS 0.0303 0.0063 |
| Group 5| TRAIL-R2 NS NS NS | TR-AP NS NS NS | TR-AP NS NS NS |
|        | TIE2 NS NS NS | TIE2 NS NS NS | TIE2 NS NS NS |
Table 2. Continued

| Group       | Control 1 vs All ROP | Control 1–2 vs All ROP | Control 2 vs All ROP |
|-------------|----------------------|------------------------|----------------------|
| NT-3        | NS                   | NS                     | 0.0014               |
| TNF-R2      | NS                   | NS                     | 0.0338               |
| IL-10RB     | NS                   | NS                     | 0.0296               |
| GH          | NS                   | NS                     | 0.0297               |
| MIC-A       | NS                   | NS                     | 0.0145               |
| IL-8        | NS                   | NS                     | 0.0076               |
| CEA         | NS                   | NS                     | 0.0378               |
| HGF         | NS                   | NS                     | 0.0001               |
| TGF-α       | NS                   | NS                     | 0.0191               |
| EZR         | NS                   | NS                     | 0.0007               |
| PTX3        | NS                   | NS                     | 0.0357               |
| LOX-1       | NS                   | NS                     | 0.005                |
| IL-17C      | NS                   | NS                     | 0.0186               |
| ST1A1       | NS                   | NS                     | 0.0397               |
| ITGB1BP2    | NS                   | NS                     | 0.0357               |
| Group 6     | TNFRSF4              | 0.0333                 | 0.0082               |
| ErbB2/HER2  | 0.0059               | 0.0009                 | 0.0099               |
| GAL         | 0.0032               | 0.0008                 | 0.0306               |

NS, not significant.

markers from groups 1 and 2 had higher levels in control 1 compared to control 2 or infants with ROP independent of grade. There were no significant differences in these markers between control 2 versus all the ROP infants (Fig. 4B, Table 2). Furthermore, the pattern for these markers seems to be homogeneous within control 2 and ROP infants. Parkinson disease protein 7 (PARK7), vimentin (VIM), myeloperoxidase (MPO), CD69, and NFκB essential modulator (NEMO) show the most

Figure 3. Differences in GA and BW between the two control groups (controls 1 and 2). The control groups did not differ in GA. However, infants in control 2 had higher BW, higher z score, and higher percentile values in BW.
significant differences between control 1 and all ROP infants \((P < 0.001; \text{Table 2})\). No correlation could be found between the levels of these five markers and the GA or the BW (data not shown). The levels of these five biochemical markers are represented in Figure 5.

The levels of hepatocyte growth factor (HGF), ezrin (EZR), and lectin-like oxidized-low-density lipoprotein receptor 1 (LOX-1) in group 5 showed strong significant differences \((P < 0.001; \text{Table 2})\) between control 2 and all ROP infants, but the expression levels were highly heterogenous in the separate ROP groups (Fig. 4). Thirty-four of the 39 biochemical markers that distinguish the two controls from each other were represented in groups 1, 2, and 5 but not in group 3, 4, or 6.
Figure 5. Biochemical markers (PARK7, VIM, MPO, CD69, NEMO) with the most significant differences ($P < 0.001$) between control 1 and all ROP groups. The significant differences are marked with dark gray in the table.
Postnatal Biochemical Markers Associated with ROP

Figure 6. Biochemical markers (ErbB2/HER2, GAL, TNFRSF4) in group 6. All three biochemical markers from group 6 show significant differences between all combinations of the controls (control 1, control 2, or both controls) and all patients with ROP. The values correspond to significant differences between controls and all patients with ROP. Note that ErbB2/HER2 and GAL are higher in controls, whereas TNFRSF4 is lower than in ROP.

Group 6 included three biochemical markers with significant differences between any combination of the controls (control 1, control 2, or both controls) and all ROP infants; tumor necrosis factor receptor superfamily member 4 (TNFRSF4) had lower expression in all ROP infants, whereas human epidermal growth factor receptor 2 (HER2) and galanin (GAL) had higher expression in all ROP infants Figure 6. There was no significant difference between the expression levels of these three markers and the different ROP stages, that is, no correlation to future severity of ROP could be observed (data not shown). Although GA and BW correlate negatively to the severity of ROP (Fig. 1), no correlation was seen between these three markers and the GA or BW (data not shown).

Discussion

Although numerous proinflammatory cytokines in the systemic circulation are associated with ROP,35 several studies have failed to find early predictive markers for ROP development with clinical relevance. This is likely due to the process being multifactorial and that the group of infants with potential risk of developing ROP is highly heterogeneous. Clustering of infants and plasma protein levels enabled us to identify biochemical markers with possible predictive value on ROP development in the early postnatal period in extremely premature infants. Our study has two main findings: first, simultaneously elevated levels of PARK7, VIM, MPO, CD69, and NEMO were associated with a protective effect on ROP development, and second, decreased levels of TNFRSF4 and elevated levels of HER2 and GAL were associated with ROP development independently of the final stage of ROP. All these proteins are involved in inflammatory or vascularizing processes; however, no study has previously associated any of them with ROP development. We propose the following possible explanations for the role these eight biomarkers might play in the development of ROP.

PARK7 is as a multifunctional protein with transcriptional regulation, protein chaperone, protease, and antioxidative functions.36 Xu et al.37 showed that PARK7 has the ability to protect neurons against oxidative stress and apoptosis. PARK7 may also influence angiogenesis indirectly by inhibiting production of reactive oxygen species and by enhancing the antioxidant capacity to reduce apoptosis in retinal pericytes.38 As pericytes regulate vessel permeability and endothelial cell proliferation through direct contact with endothelial cells,39 a lack of
pericytes might lead to abnormal vascular morphogenesis. 

Choi et al. reported that the proper amount of pericytes is crucial for the normal retinal angiogenesis by controlling endothelial cell proliferation. These findings are in accordance with our results that PARK7 may have a protective effect on ROP development.

VIM, a major type III intermediate filament protein, is expressed in cells with mesenchymal origin such as astrocytes and endothelial cells. In addition to the effects that retinal astrocytes have on blood vessels, a reverse interaction also occurs, where the developing vessels influence retinal astrocyte development by promoting their differentiation. Extracellular VIM can be secreted by unstimulated astrocytes and activate insulin-like growth factor 1 receptor (IGF1R) as an alternative ligand in neurons and thus promote axonal growth. Interestingly, Hellström et al. showed that sufficient postnatal level of IGF-1 (the main ligand in IGF1R activation) is associated with normal vessel development and reduced risk of developing ROP. VIM-deficient mice show decreased flow-induced dilation during arterial remodeling, suggesting that VIM plays an important role in regulating structural responses of arteries in chronic blood flow and hypoxia-induced retinal neovascularization.

Our finding that higher levels of MPO may be associated with a reduced risk of developing ROP is in contrast with observations from previous studies. Inflammation is in general found to be associated with ROP, and sepsis is closely related to the development of any stage of ROP. Bulka et al. found that greater CpG methylation (repressed gene transcription) of MPO in the placenta serum at birth was associated with a lower risk of prethreshold ROP, and increased MPO activity and lipid peroxide levels have been demonstrated in patients with diabetic retinopathy. However, the time point for inflammatory response may be of importance, in which late neonatal bacteremia appears to be a more prominent risk factor for severe ROP than early neonatal bacteremia. Holm et al. found that the risk for ROP increased when vascular endothelial growth factor receptor 1 (VEGFR-1) was elevated on day 1 but not when MPO was elevated at them same time. Although MPO is known as a local mediator of tissue damage by catalyzing the formation of reactive oxygen intermediates, it has multifaceted functions. Studies in murine models of inflammation show that MPO has an anti-inflammatory role, which appears even in complex inflammatory responses without present infectious agents. Odobasic et al. showed that MPO inhibits the generation of adaptive immunity by suppressing dendritic cell activation, antigen uptake/processing, and migration to lymph nodes to limit pathologic tissue inflammation. Furthermore, infants with total or subtotal lack of MPO have an increased incidence of chronic inflammatory conditions. These findings do not exclude the possibility that elevated early postnatal levels of MPO together with the other four biomarkers might have an anti-inflammatory and modulatory effect on angiogenesis in ROP.

CD69 is an early inflammation marker, which is expressed on leukocytes, and the expression increases following cell stimulation. There are indirect associations between lower CD69 expression and neovascularization. CD69 may act as a negative regulator of endothelial cell activation, and Falk et al. found a lower percentage of CD4+CD69+CXCR3+ T cells in patients with neovascular age-related macular degeneration compared to age-matched controls. The coexpression of CD69 and chemokine receptor CXCR3 can have further consequences, as CXCR3 plays an important role in C-X-C motif chemokine ligand 10 (CXCL10)–mediated inhibition of VEGF-induced angiogenesis, a vascular factor implicated in the development of ROP.

NEMO is a key regulator in NF-κB–mediated signaling. The mutation in the gene of NEMO is a cause of incontinentia pigmenti (IP) as NF-κB activation is defective in IP cells. The eye involvement in infants with IP can manifest as areas of ischemia and reactive neovascularization, similar to the development of ROP.

We could not find any clear explanation for the possible connection between TNFRSF4 (synonyms: CD40, CD134) and ROP in the literature. TNFRSF4 is expressed by T cells that are largely dependent on antigen recognition and participate in activation, clonal expansion, and differentiation of both CD4+ and CD8+ T cells. T cells can facilitate postischemic angiogenesis by recruiting macrophages to ischemic tissues, promoting secretion of cytokines in murine hindlimb ischemic models. OX40 ligand (OX40L) expression has been observed on antigen-presenting dendritic cells, B cells, microglia cells, and endothelial cells. Cunningham et al. showed that OX40L abolishes the retinal pigment epithelium–mediated immunosuppression. TNFRSF4 is involved in the pathogenesis of autoimmune vasculitis diseases (e.g., systemic lupus erythematosus, Behcet disease, and Henoch-Schönlein purpura). Inherited TNFRSF4 deficiency in a patient was associated with childhood-onset classic Kaposi sarcoma (an endothelial tumor with inflammatory origin), in which TNFRSF4 was not able to bind to its ligand (OX40L) expressed by endothelial cells.
et al.\textsuperscript{78} found upregulated TNFRSF4 gene expression in active fibrovascular membranes of proliferative diabetic retinopathy. Nakano et al.\textsuperscript{79} observed that VEGF-induced angiogenesis was suppressed by the genetic deletion of the OX40/OX40L signaling in a mouse model. Angiogenic cytokines can recruit immune cells that cause continuous secretion of cytokines and further recruitment of immune cells.\textsuperscript{80} OX40/OX40L interaction induces the phospholipase C signal transduction pathway,\textsuperscript{81} which induces diacylglycerol-protein kinase C and the inositol trisphosphate (IP(3))-intracellular free calcium ([Ca(2+)](i)) pathway.\textsuperscript{82} These pathways are also known as downstream signal pathways for VEGF-induced angiogenesis,\textsuperscript{83} which could be in conflict with our results in which ROP development was associated with lower TNFRSF4 expression. However, markers such as VEGF can be suppressed immediately after birth and increased later during development of ROP.\textsuperscript{11}

HER2 (synonym: ErbB2, CD340) is known as an oncogene and a member of the human epidermal growth factor receptor family. HER2 activation is followed by several intracellular signaling pathways such as the phospholipase C pathway, which ultimately affects proliferation, survival, motility, and adhesion of different cells.\textsuperscript{84} As discussed above, the phospholipase C pathway has a connection to VEGF-induced angiogenesis,\textsuperscript{83} and studies have shown consequently that HER2 signaling is involved in angiogenesis.\textsuperscript{85,86} HER2 overexpression correlates with increased expression of VEGF in human breast carcinoma\textsuperscript{87,88} and ovarian carcinoma,\textsuperscript{89} whereas neutralizing antibodies against HER2 result in the reduction of VEGF production in a dose-dependent manner in cancer cells.\textsuperscript{90} HER2 signaling induces protein synthesis of hypoxia-inducible factor 1\textalpha, which is playing a predominant role in the direct transcriptional upregulation of VEGF.\textsuperscript{91} Furthermore, hypoxia-inducible factor 1 was presented earlier as a potential target in the treatment of ocular neovascular diseases.\textsuperscript{92} These findings correspond well with our results that higher HER2 levels may be associated with ROP development.

GAL is a vasoactive neuropeptide acting via three known receptors: GALR 1, 2, and 3.\textsuperscript{93} GAL receptors have been detected in various ocular tissues and cells in normal human eye structures, but the role of GAL in the eye is not fully understood.\textsuperscript{94} GAL is involved in the regulation of inflammatory processes, and the expression of GAL receptors is upregulated in inflammatory conditions.\textsuperscript{95} There are potential associations to ROP development since it is involved in both vascular homeostasis\textsuperscript{96} and neuronal differentiation,\textsuperscript{97} and several authors suggest that GAL is an important angiogenetic factor in cancer.\textsuperscript{98,99} No previous reports are in conflict with our results that ROP development may be associated with elevated GAL levels.

Our study has the following limitations: we performed semiquantitative measurements of plasma proteins, which make the values not comparable with other studies. Furthermore, there are no reference intervals available for extremely premature infants, which makes it difficult to interpret and compare the measured protein levels to values in physiologic circumstances. A larger span in maturational ages and sequential samplings might have also given correlations between the tested biomarkers and GA and/or PMA (postmenstrual age), which we did not see in our study. The small number of infants in each group did not allow us to perform multivariate analysis.

The strength of our study is that we analyzed and could interpret the levels of over 200 plasma proteins simultaneously. We also present clustering as a powerful tool to describe multidimensional associations between the plasma levels of our biomarkers and to find possible relevant early markers associated with ROP development.

**Conclusions**

Our data suggest that simultaneously elevated plasma levels of PARK7, VIM, MPO, CD69, and NEMO after birth may help to identify extremely preterm infants at low risk of developing ROP. Lower levels of TNFRSF4 and simultaneously higher levels of HER2 and GAL may predict ROP development. The signaling networks of these proteins may help to understand the mechanisms of ROP development. Further studies are needed on larger populations to confirm the predictive relevance of the presented biochemical markers, using cluster analysis, in which comprehensive protein screening on small blood samples enables sequential testing.

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