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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

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inversely related to birth weight (BW) and gestational age (GA)[13,14] and directly related to intrauterine growth restriction.[15] Male gender contributes to severe ROP.[15] Models for oxygen-induced retinopathy exist,[16] although the duration of mechanical ventilation seems to be a greater predictive factor for ROP development than the total duration of oxygen supplementation.[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.[18–23] However, only a few investigators have aimed to find a relationship between protein levels at birth and ROP development.[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.[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 DAPPR (Ductus arteriosus and pulmonary circulation in premature infants) cohort.[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 postmenstrual age of around 31 weeks and up to at least full-term age. Treatment was performed when indicated (ETROP (Early Treatment for Retinopathy Of Prematurity) 2003).[28] The data on various aspects of screening and treatment for ROP were extracted from SWEDROP (Swedish national register for retinopathy of prematurity), a national web-based ophthalmologic ROP register.[29]

Biochemical Markers

Blood samples were collected from umbilical arterial catheters during the second day of life. A minimum volume of 20 µL was required for proximity extension assay (PEA). After centrifugation at 2400 × *g* for 7 minutes, the supernatant serum was obtained and stored at −80°C until further analysis. In total, 202 biochemical markers were analyzed with the PEA technique using the Proseek Multiplex 96 × 96 CVD I, Oncology I, and Inflammation I biomarker panels (Olink Bioscience, Uppsala, Sweden) as previously described.[30] The list of 202 biochemical markers is presented in Table 1. All data from the PEA analyses are presented as arbitrary units in linear values. Measurements were performed without knowledge of clinical data, and treating physicians were blinded to the results of the biochemical marker analyses.

Cluster Analysis

The biochemical marker levels were analyzed together by two-dimensional hierarchical clustering (Cluster 3.0 freeware)[31] as previously described.[32] The clustering automatically sorted infants into groups depending on the relationship of their clinical and biochemical parameters. All biochemical marker levels were used and weighted equally in the clustering algorithm. The results were visualized by Java Treeview[33] as a map of color pixels. A higher red intensity corresponded to a higher value of a certain parameter.

Statistical Analysis

One-way analysis of variance tests were performed to evaluate differences within a variable and between the hierarchically clustered groups, comparing both clinical data (GA, BW, BW percentile, and BW z score) and biochemical marker levels. Detected differences were analyzed with a two-sided paired Student's t-test for direct comparisons and Bonferroni corrections for multiple comparisons. The level of significance was set at $P < 0.05$ for all tests.

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Table 1. List of All Tested Biochemical Markers ($n = 202$).

4E-BP1	CCL28	CXCL10	ESM-1	hGDNF	IL-1ra	LEP	MMP-7	PTPN22	TM	VE-Statin
ADA	CCL4	CXCL11	EZR	HGF	IL-2	LIF	MPO	PTX3	TNF	VIM
AGRP	CD244	CXCL13	FABP4	hK11	IL-20	LIF-R	MYD88	RAGE	TNFB	
AM	CD40	CXCL16	FADD	HSP 27	IL-20RA	LITAF	NEMO	REG-4	TNF-R1	
AR	CD40-L	CXCL5	FAS	ICOSLG	IL-22 RA1	LOX-1	NRTN	REN	TNF-R2	
ARTN	CD5	CXCL6	FasL	IFN-γ	IL-24	LYN	NT-3	RETN	TNFRSF4	
AXIN1	CD6	CXCL9	FGF-19	IL-1α	IL27-A	mAmP	NT-pro-BNP	SCF	TNFRSF9	
BAFF	CD69	Dkk-1	FGF-21	IL-10	IL-2RB	MB	NTRK3	SELE	TNFSF14	
BDNF	CDCP1	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	EGF	Flt3L	IL-12	IL-5	MCP-3	PAPPA	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 subunit B	ST2	TSLP	
CASP-8	CST5	EN-RAGE	GAL	IL-16	IL-8	MIP-1 α	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	PlGF	TGF-α	U-PAR	
CCL20	CTSL1	ErbB2/HER2	GH	IL-17RB	ITGB1BP2	MMP-10	PRL	THPO	VEGF-A	
CCL23	CX3CL1	ErbB3/HER3	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	

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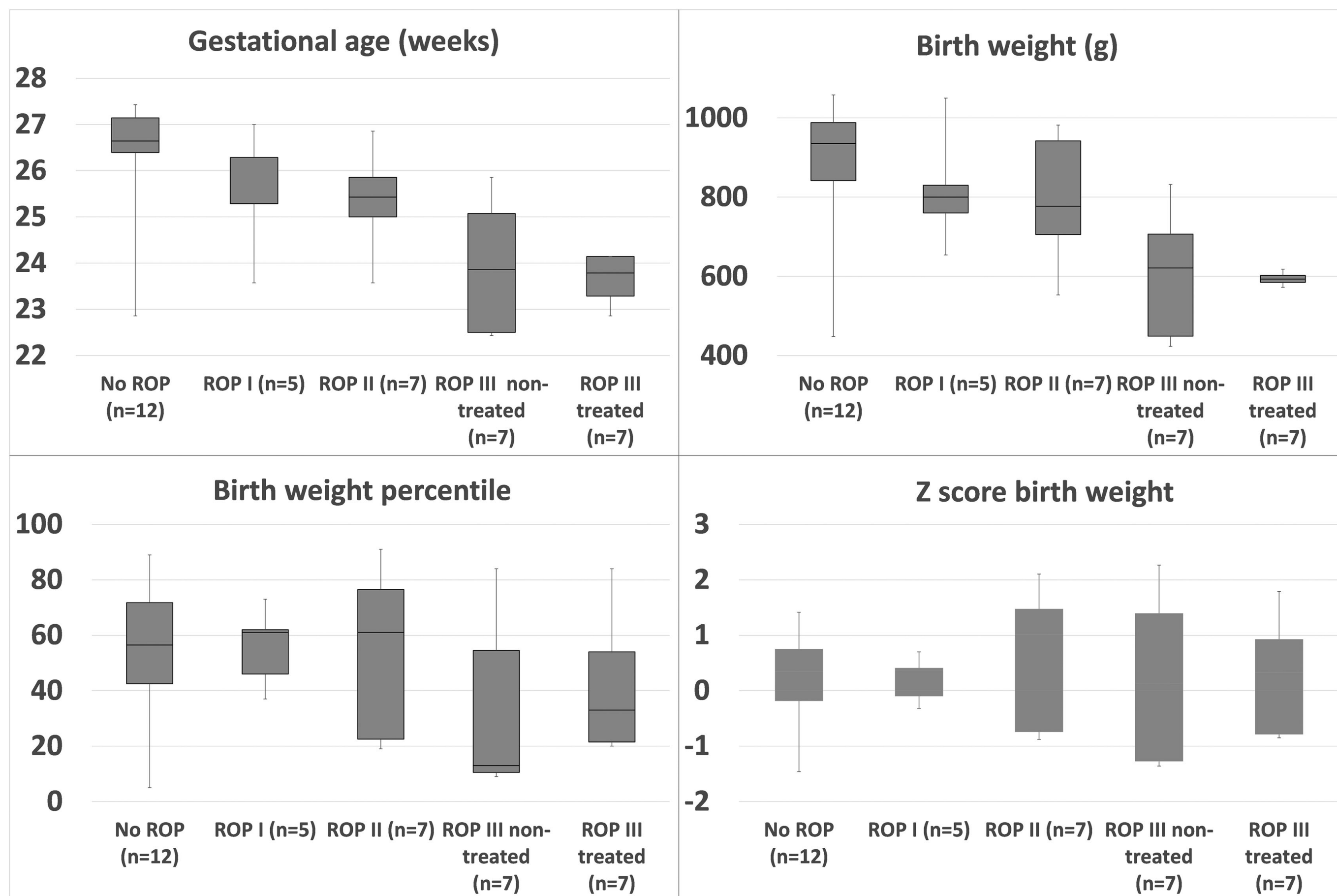

p values	GA	BW	BW %	Z score BW
No ROP vs ROP I	0,416	0,474	0,939	0,946
No ROP vs ROP II	0,117	0,339	0,863	0,938
No ROP vs ROP III	**0,004**	**0,004**	0,153	0,138
No ROP vs ROP III treated	**<0,001**	**<0,001**	0,480	0,523
No ROP vs all ROP	**0,003**	**0,012**	0,306	0,319

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.[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[34] (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

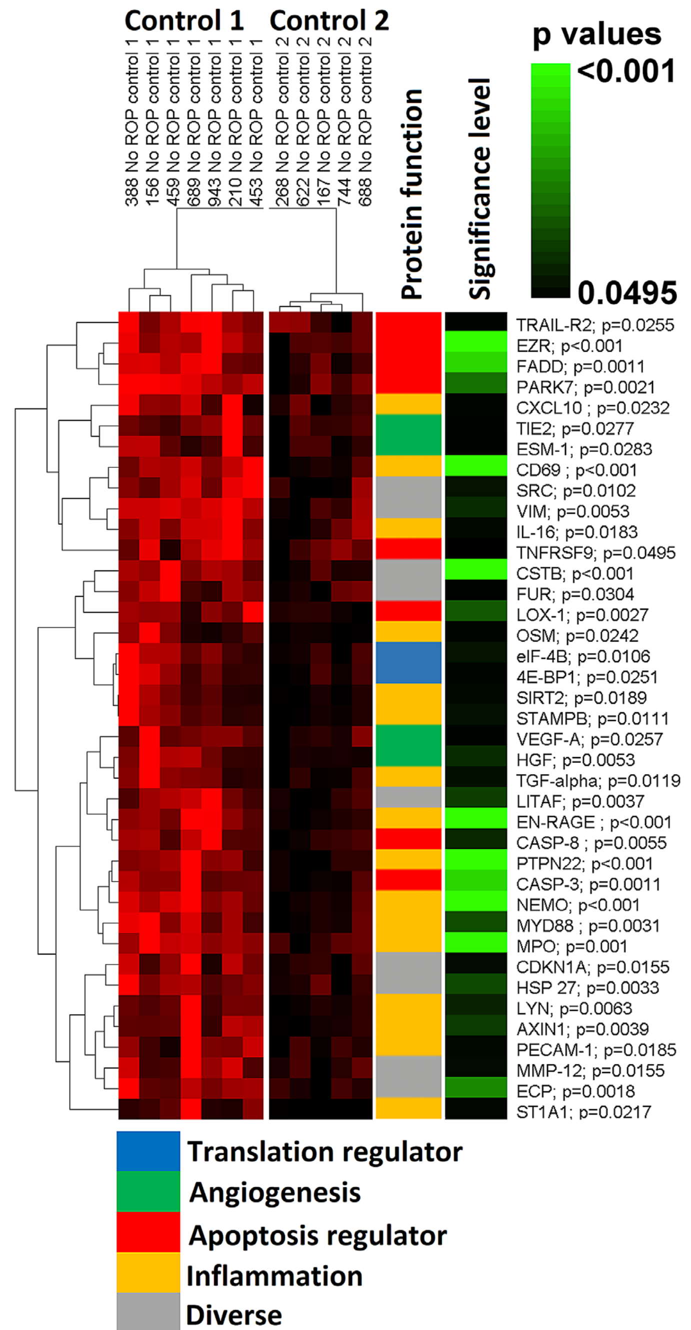

Figure 2. Clustering of infants without ROP (controls 1 and 2) according to biomarker expression ($n = 39$). Two control groups could be defined by clustering. Thirty-nine of 202 biochemical markers showed significant differences between the two control groups (controls 1 and 2). Higher intensity in *red* corresponds to higher value of a certain biochemical marker expression level. The function of the proteins and the significance level of differences between the two controls are labeled with colors. Higher intensity in *green* corresponds to higher level of significance; P values are shown next to each biochemical marker.

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Table 2. Biochemical Markers ($n = 69$) with Significant Differences between Control 1, Control 2, or Both Controls versus All Patients with ROP

Group		Control 1 vs All ROP	Control 1–2 vs All ROP	Control 2 vs All ROP
Group 1	EPO	0.0036	NS	NS
	OSM	0.0348	NS	NS
	4E-BP1	0.0469	NS	NS
	SIRT2	0.0304	NS	NS
	STAMPB	0.0232	NS	NS
	CSTB	0.0013	NS	NS
	PARK7	0.0001	NS	NS
	VEGF-A	0.0194	NS	NS
	ESM-1	0.0255	NS	NS
	SPON1	0.0212	NS	NS
	SRC	0.0037	NS	NS
	MMP-12	0.0267	NS	NS
	TNFRSF9	0.0285	NS	NS
	IL-16	0.0012	NS	NS
Group 2	Flt3L	0.0163	0.0064	NS
	VIM	<0,0001	0.0222	NS
	FADD	0.0013	0.0390	NS
	MYD88	0.0029	0.0160	NS
	CASP-3	0.0021	0.0351	NS
	NEMO	0.0002	0.0142	NS
	HSP 27	0.0014	0.0078	NS
	eIF-4B	0.0111	0.0358	NS
	PTPN22	0.0011	0.0354	NS
	LITAF	0.0036	0.0203	NS
	MPO	0.0001	0.0102	NS
	EN-RAGE	0.0020	0.0237	NS
	CASP-8	0.0050	0.0155	NS
	CD69	0.0002	0.0157	NS
	LYN	0.0067	0.0179	NS
	AXIN1	0.0044	0.0132	NS
	ECP	0.0018	0.0177	NS
	RETN	0.0034	0.0027	NS
	BAFF	0.0051	0.0107	NS
	RAGE	0.0105	0.0088	NS
	MMP-10	0.0170	0.0148	NS
	MMP-3	0.0291	0.0163	NS
	IL-10	0.0268	0.0450	NS
Group 3	FGF-21	NS	0.0303	NS
	ILT-3	NS	0.0454	NS
	mAmP	NS	0.0385	NS
Group 4	KLK6	NS	0.0174	0.0276
	VE-statin	NS	0.0251	0.0315
	FGF-19	NS	0.0197	0.0267
	ErbB4/HER4	NS	0.0122	0.0041
	TIM	NS	0.0303	0.0063
Group 5	TRAIL-R2	NS	NS	0.0074
	TR-AP	NS	NS	0.0410
	TIE2	NS	NS	0.0113

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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	0.0462
	HGF	NS	NS	0.0001
	TGF-α	NS	NS	0.0191
	EZR	NS	NS	0.0007
	PTX3	NS	NS	0.0378
	LOX-1	NS	NS	0.0005
	IL-17C	NS	NS	0.0186
	ST1A1	NS	NS	0.0397
	ITGB1BP2	NS	NS	0.0357
Group 6	TNFRSF4	0.0333	0.0082	0.0303
	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 NF-κB essential modulator (NEMO) show the most

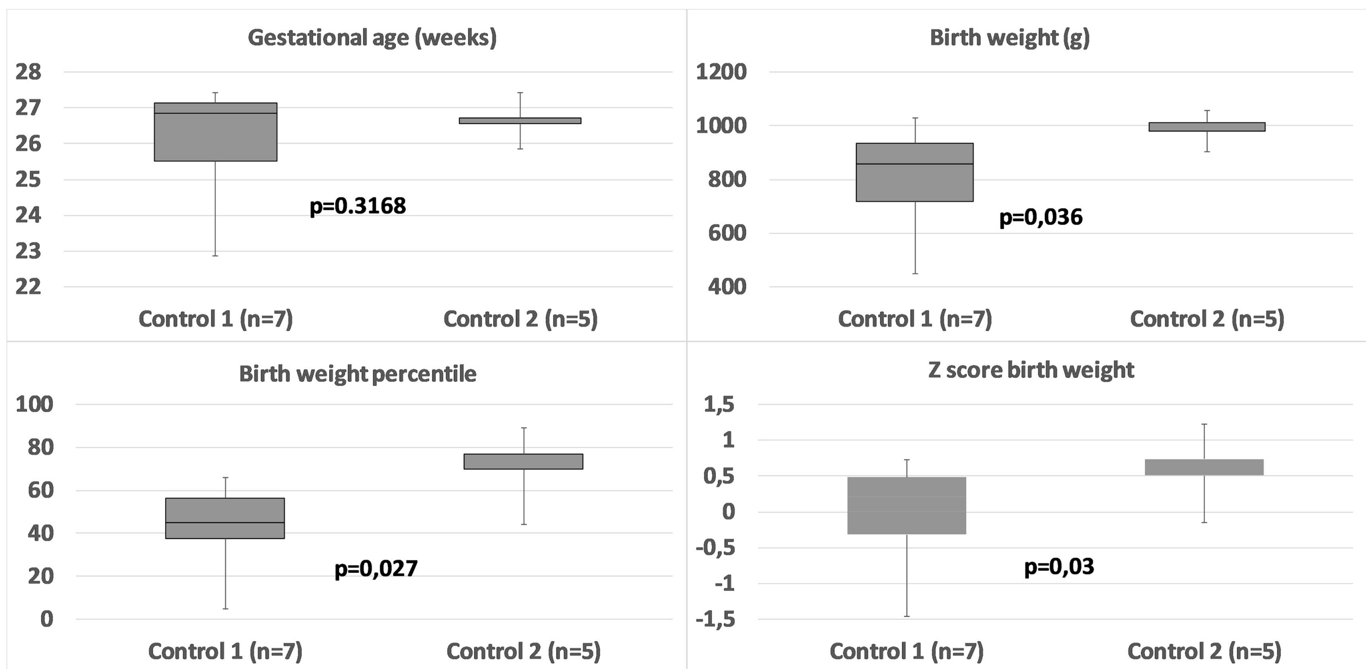

Figure 3. Differences in GA and BW between the two control groups (controls 1 and 2). The control groups did not differ in GA. However, infants in control 2 had higher BW, higher *z* score, and higher percentile values in BW.

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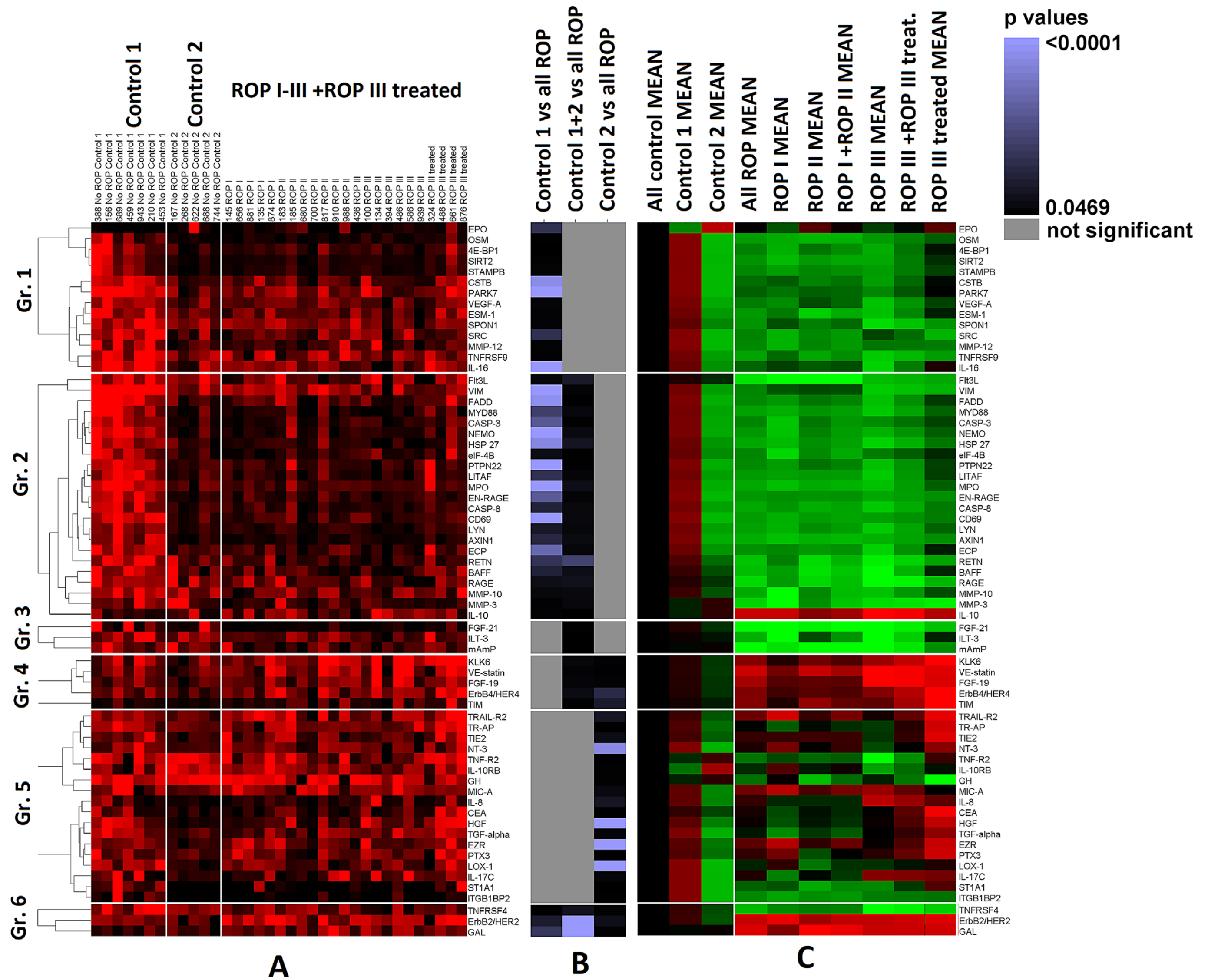

Figure 4. Clustered biochemical markers with significant differences ($n = 66$) between controls and all ROP patients. Patients labeled with ROP III are nontreated ROP cases. Sixty-six biochemical markers were significantly different between control 1, control 2, or both controls versus all ROP patients. Six groups (groups 1–6) could be defined depending on their combination of significances: Group 1. Control 1 versus all ROP. Group 2. Control 1 and all controls versus all ROP. Group 3. All controls versus all ROP. Group 4. Control 2 and all controls versus all ROP. Group 5. Control 2 versus all ROP. Group 6. Control 1, control 2, and all controls versus all ROP. (A) Biomarkers in each group clustered separately and presented together. (B) Level of significant differences between the two controls and all ROP patients. Increased *lighter purple* corresponds to higher level of significance; *gray*, not significant (numeric values can be followed in Table 2). (C) The direction (±) and amplitude of differences in biochemical marker levels of the two control groups. All ROP patients and combinations of patients with grade I to III ROP were compared to all control patients. Higher intensity in *red* corresponds to higher positive value; higher intensity in *green* corresponds to lower negative value.

significant differences between control 1 and all ROP infants ($P < 0.001$; 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 ($P < 0.001$; Table 2) between control 2 and all ROP infants, but the expression levels were highly heterogonous 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.

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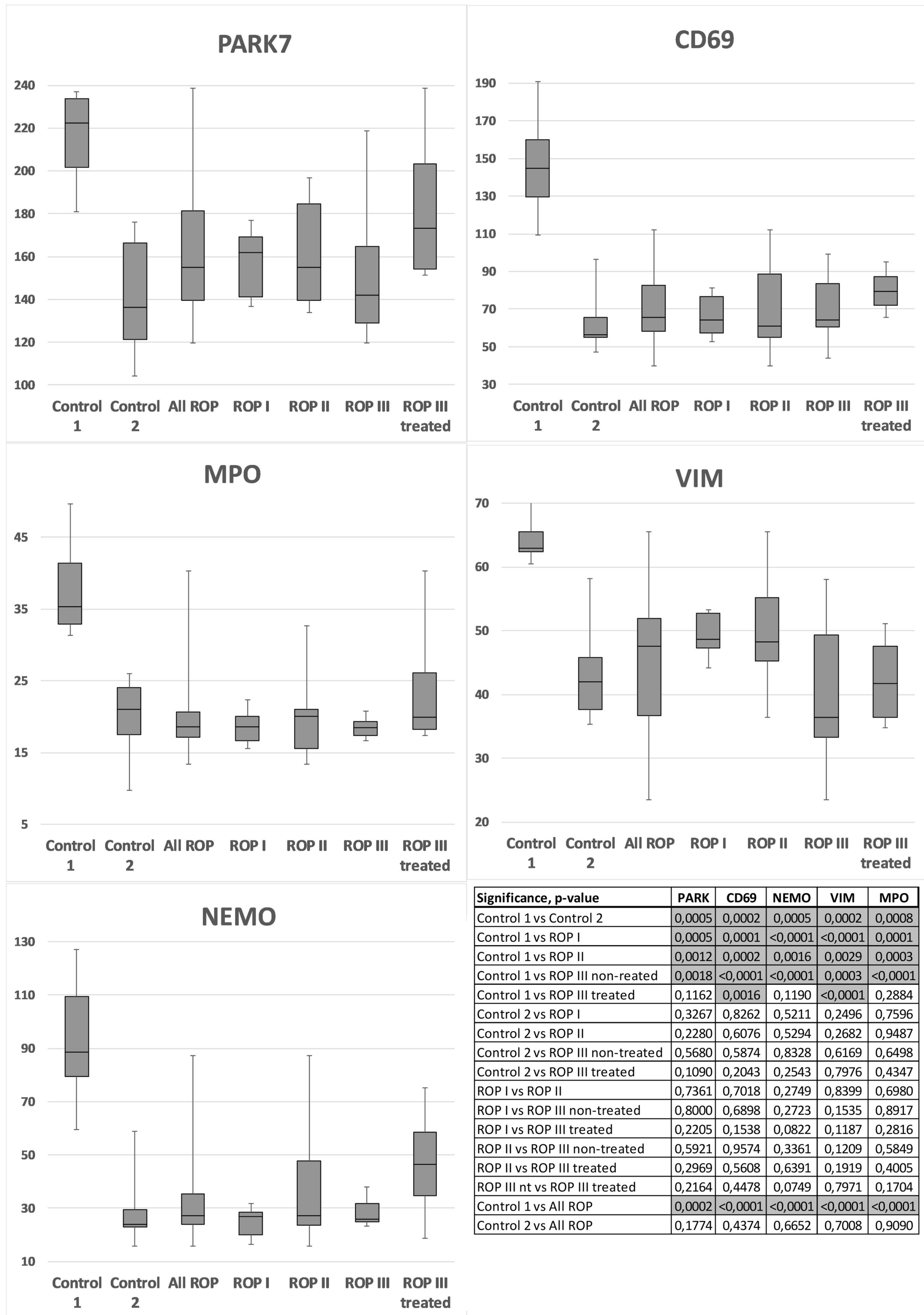

Significance, p-value	PARK	CD69	NEMO	VIM	MPO
Control 1 vs Control 2	0,0005	0,0002	0,0005	0,0002	0,0008
Control 1 vs ROP I	0,0005	0,0001	<0,0001	<0,0001	0,0001
Control 1 vs ROP II	0,0012	0,0002	0,0016	0,0029	0,0003
Control 1 vs ROP III non-reated	0,0018	<0,0001	<0,0001	0,0003	<0,0001
Control 1 vs ROP III treated	0,1162	0,0016	0,1190	<0,0001	0,2884
Control 2 vs ROP I	0,3267	0,8262	0,5211	0,2496	0,7596
Control 2 vs ROP II	0,2280	0,6076	0,5294	0,2682	0,9487
Control 2 vs ROP III non-treated	0,5680	0,5874	0,8328	0,6169	0,6498
Control 2 vs ROP III treated	0,1090	0,2043	0,2543	0,7976	0,4347
ROP I vs ROP II	0,7361	0,7018	0,2749	0,8399	0,6980
ROP I vs ROP III non-treated	0,8000	0,6898	0,2723	0,1535	0,8917
ROP I vs ROP III treated	0,2205	0,1538	0,0822	0,1187	0,2816
ROP II vs ROP III non-treated	0,5921	0,9574	0,3361	0,1209	0,5849
ROP II vs ROP III treated	0,2969	0,5608	0,6391	0,1919	0,4005
ROP III nt vs ROP III treated	0,2164	0,4478	0,0749	0,7971	0,1704
Control 1 vs All ROP	0,0002	<0,0001	<0,0001	<0,0001	<0,0001
Control 2 vs All ROP	0,1774	0,4374	0,6652	0,7008	0,9090

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.

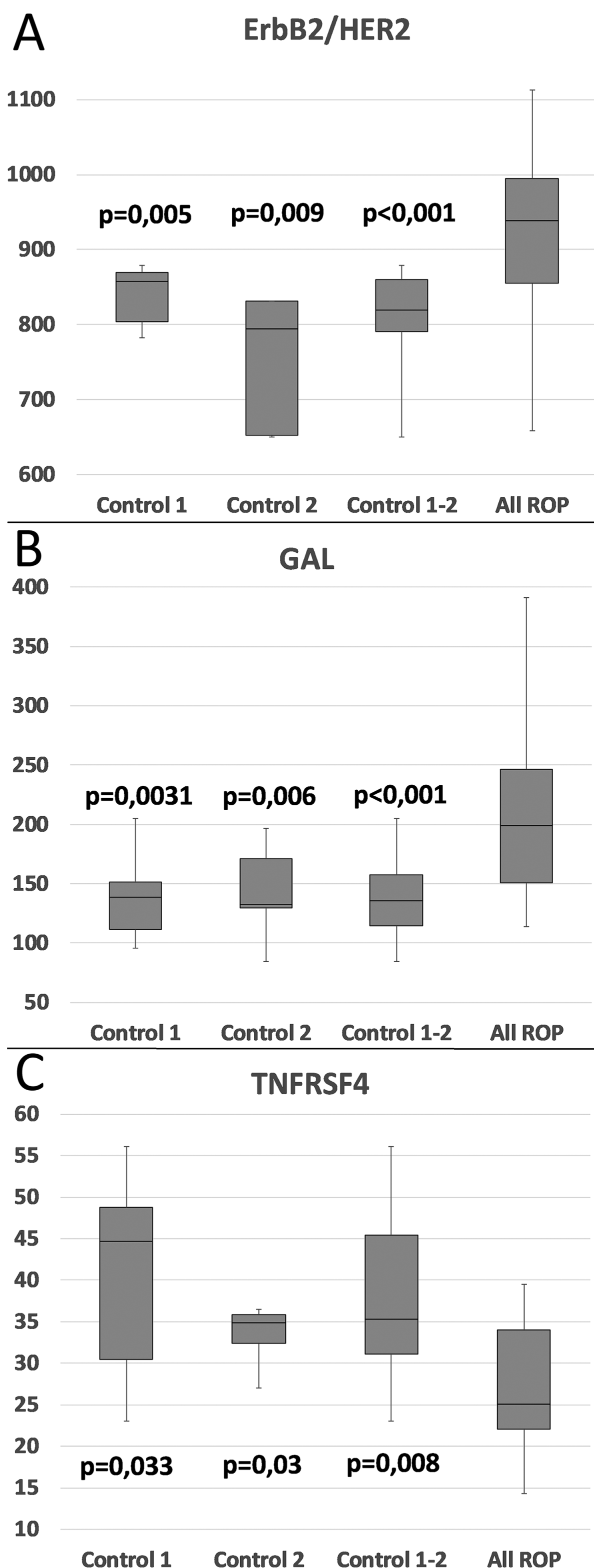

Figure 6. Biochemical markers (ErbB2/HER2, GAL, TNFRSF4) in group 6. All three biochemical markers from group 6 show significant differences between all combinations of controls (control 1, control 2, and all controls) and all patients with ROP. *P* values correspond to significant differences between 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.[39] Choi et al.[40] 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[41] and endothelial cells.[42] 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.[43] Extracellular VIM can be secreted by unstimulated astrocytes[41] and activate insulin-like growth factor 1 receptor (IGF1R) as an alternative ligand in neurons and thus promote axonal growth.[44] Interestingly, Hellström et al.[45] 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[46] and hypoxia-induced retinal neovascularization.[47]

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,[35] and sepsis is closely related to the development of any stage of ROP.[48] Bulka et al.[49] 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.[50] 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.[51] Holm et al.[6] 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,[52] it has multifaceted functions.[53] Studies in murine models of inflammation show that MPO has an anti-inflammatory role,[53,54] which appears even in complex inflammatory responses without present infectious agents.[54] Odobasic et al.[55] 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.[56] 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.[57] There are indirect associations between lower CD69 expression and neoangiogenesis. CD69 may act as a negative regulator of endothelial cell activation,[58] and Falk et al.[59] 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,[60] a vascular factor implicated in the development of ROP.

NEMO is a key regulator in NF-κB–mediated signaling.[61] The mutation in the gene of NEMO is a cause of incontinentia pigmenti (IP) as NF-κB activation is defective in IP cells.[62] The eye involvement in infants with IP can manifest as areas of ischemia and reactive neovascularization, similar to the development of ROP.[63–65]

We could not find any clear explanation for the possible connection between TNFRSF4 (synonyms: OX40, 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.[66] T cells can facilitate postischemic angiogenesis by recruiting macrophages to ischemic tissues, promoting secretion of cytokines in murine hindlimb ischemic models.[67,68] OX40 ligand (OX40L) expression has been observed on antigen-presenting dendritic cells,[69] B cells,[70] microglia cells,[71] and endothelial cells.[72] Cunningham et al.[73] 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,[74] Behcet disease,[75] and Henoch-Schönlein purpura).[76] 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.[77] Gong

et al.[78] found upregulated TNFRSF4 gene expression in active fibrovascular membranes of proliferative diabetic retinopathy. Nakano et al.[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.[80] OX40/OX40L interaction induces the phospholipase C signal transduction pathway,[81] which induces diacylglycerol-protein kinase C and the inositol trisphosphate (IP(3))-intracellular free calcium ([Ca(2+)](i)) pathway.[82] These pathways are also known as downstream signal pathways for VEGF-induced angiogenesis,[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.[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.[84] As discussed above, the phospholipase C pathway has a connection to VEGF-induced angiogenesis,[83] and studies have shown consequently that HER2 signaling is involved in angiogenesis.[85,86] HER2 overexpression correlates with increased expression of VEGF in human breast carcinoma[87,88] and ovarian carcinoma,[89] whereas neutralizing antibodies against HER2 result in the reduction of VEGF production in a dose-dependent manner in cancer cells.[90] HER2 signaling induces protein synthesis of hypoxia-inducible factor 1α, which is playing a predominant role in the direct transcriptional upregulation of VEGF.[91] Furthermore, hypoxia-inducible factor 1 was presented earlier as a potential target in the treatment of ocular neovascular diseases.[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.[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.[94] GAL is involved in the regulation of inflammatory processes, and the expression of GAL receptors is upregulated in inflammatory conditions.[95] There are potential associations to ROP development since it is involved in both vascular homeostasis[96] and neuronal differentiation,[97] and several authors suggest that GAL is an important angiogenetic factor in cancer.[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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