Pediatric RESEARCH

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CLINICAL RESEARCH ARTICLE

Very preterm birth results in later lower platelet activation markers

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BACKGROUND: Premature birth entails an adverse cardiovascular risk profile, but the underlying mechanisms are insufficiently understood. Here, we employed an unbiased cardiovascular proteomics approach to profile former very preterm-born preschoolers.
METHODS: This observational study investigated differences in plasma concentrations of 79 proteins, including putative cardiovascular biomarkers between very preterm- and term-born children on average 5.5 years old (53.1% male) using multiple-reaction monitoring mass spectrometry.
RESULTS: Very preterm-born ($n = 38$; median gestational age 29.6 weeks) compared to term-born ($n = 26$; 40.2 weeks) children featured lower plasma concentrations of platelet factor 4 (PLF4; −61.6%, $P < 0.0001$), platelet basic protein (CXCL7; −57.8%, $P < 0.0001$), and hemoglobin subunit beta (−48.3%, $P < 0.0001$). Results remained virtually unchanged when adjusting for complete blood count parameters, including platelet count. Conversely, whole blood hemoglobin was higher (+7.62%, $P < 0.0001$) in preterm-born children.
CONCLUSIONS: Very preterm birth was associated with decreased markers of platelet activation among preschoolers. These findings are consistent with reduced platelet reactivity persisting from very preterm birth to a preschool age.

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IMPACT:

- Former very preterm-born preschoolers featured reduced levels of platelet activation markers.
- While lower platelet reactivity in very preterm-born compared to term-born infants in the first days of life was established, it was unknown when, if at all, reactivity normalizes. The current study suggests that platelet hyporeactivity due to very preterm birth persists at least up to a preschool age.
- "Immaturity of the hemostatic system" may be a persistent sequel of preterm birth, but larger studies are needed to investigate its potential clinical implications.

INTRODUCTION

Former preterm-born subjects suffer from excess cardiovascular risk later in life.[1,2] Effective prevention of cardiovascular disease (CVD) in these subjects becomes increasingly important as their numbers increase,[3] but is hampered by incomplete understanding of the mechanisms mediating excess risk. Mechanisms may pertain to the vasculature, including higher blood pressure[4,5] and higher arterial stiffness,[2,6] or to the heart, such as increased left and right ventricle mass[7,8] and decreased myocardial functional reserve.[8] They may also include alterations in the hemostatic system, in particular of platelet function,[9] and metabolic factors including higher fasting glucose and dyslipidaemia.[5,10]

Decreased functioning of further physiological systems may more indirectly impact cardiovascular risk, including reduced kidney size,[11] a surrogate of nephron mass, and an increased risk for early chronic kidney disease,[12] and hormonal disturbances relating to the hypothalamic-pituitary-adrenal axis[13] and thyroid function.[14]

Given these diverse potential mechanistic links, excess cardiovascular risk due to prematurity may be multifactorial and may best be studied in a more holistic manner. Here, we employ an unbiased proteomics approach to define differences between former preterm- and term-born children at a preschool age in terms of a broad panel, including cardiovascular biomarkers.

MATERIALS AND METHODS

Study design and population

The study was carried out at the Department of Pediatrics, Innsbruck University Hospital, Austria, from May 2012 to March 2015. The survey area, Tyrol, is a state in Western Austria with 680,000 inhabitants and ~7000 live births per year. We investigated a group of former preterm infants born between 1

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January 2007 and 31 July 2009 at <32 weeks gestational age, who were invited to a routine preschool visit at our preterm follow-up clinic. A control group of similarly aged children born at term were recruited through regional kindergardens or while undergoing routine preoperative screening for common surgical procedures (adenotomy, tonsillotomy) at Innsbruck University Hospital. No participants had congenital malformations or chromosomal abnormalities. Children consented to participation orally, and written informed consent was obtained from legal guardians. The study was approved by the local ethics committee, IRB Medical University of Innsbruck (UN 4491).

Perinatal characteristics

Perinatal data for each child belonging to the preterm study group were drawn from the routine preterm follow-up database at our institution. To account for differences in gender and gestational age, birth weight *z*-scores were calculated by means of the Fenton 2013 Growth Calculator for Preterm Infants (available from http://www.peditools.org/fenton2013).[15] Smoking during pregnancy was self-reported by mothers, as was maternal educational status.

Study visit

Examinations were carried out either at Innsbruck University Hospital or at provisional medical posts installed in participating kindergardens between 8 a.m. and 10 a.m. by trained personnel. After a routine clinical examination, weight was measured by means of calibrated medical precision scales and height was determined by a Harpenden stadiometer. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. In order to account for gender- and age-specific differences, BMI *z*-scores were calculated for each study participant by means of a reference data set.[16] Blood samples were collected after a minimum overnight fasting period of 8 h. All children and their parents were asked to fill out a questionnaire regarding family history of CVD. A positive family history of CVD was defined as a diagnosis of coronary heart disease, angina, heart attack, or stroke in first-degree male relative of child and/or parent under the age of 55 years, or first-degree female relative of child and/or parent under the age of 65 years. In children born at term not previously registered at our hospital, basic perinatal data were collected at study visit. In the preterm group, data not available from our routine preterm follow-up database were filled in during the study visit. Remaining missing data were classified as "unknown."

Proteomic measurements

Proteomic measurement methods are described in detail in the online-only Supplementary Material. In brief, they were carried out at King's College London applying a mass spectrometry-based method with commercial authentic reference standards (PlasmaDive kits; Biognosys AG, Schlieren, Switzerland). Results on apolipoproteins were already published in a separate paper,[17] thus apolipoproteins were excluded from analysis.

Statistical analysis

Between-subjects differences in perinatal characteristics and differences in characteristics at study visit between term and preterm study groups were analyzed using the *t* test, Mann–Whitney–Wilcoxon test, chi-squared test, or Fisher's exact test as appropriate.

Analyses of Proteomic data were conducted using linear models under adjustment for age and sex (Model A), for age, sex, whole blood hemoglobin, platelet count (Model B), or for age, sex, birth weight *z*-score, BMI, maternal smoking during pregnancy, maternal education, and family history of CVD (Model C). Protein concentrations were log transformed before analysis due to right skew and because they were on differing orders of magnitude, and for analyses of group differences, results are reported as % difference. Proteomic analyses were corrected for multiplicity using the Bonferroni procedure over all 79 proteins.

Gene ontology term enrichment analysis was conducted using GOrilla,[18] using the ranked list approach, and regarding enriched terms significant based on a false discovery rate *q* value <0.05.

There were five missing values each for whole blood hemoglobin and for platelet count, which were median imputed. All *P* values are two-sided and an alpha level of 0.05 is used. Analysis was conducted using R version 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Characteristics of the study population are shown in Table 1. Former very preterm-born ($n = 38$, mean gestational age 28.9 weeks) preschoolers were more likely than their former term-born counterparts ($n = 26$, 39.8 weeks) to be born to mothers who had smoked during pregnancy and who underwent <12 years of formal education, but apart from expected

Table 1. Characteristics of the study groups.

	Term born	Very preterm born	*P* value
n	26 (40.6)	38 (59.4)	
Male sex	14 (53.8)	20 (52.6)	1
Perinatal characteristics			
Gestational age (weeks)	39.8 ± 1.2	28.9 ± 2.5	<0.001
Birth weight (g)	3333 ± 436	1248 ± 405	<0.001
Birth weight *z*-score	−0.28 ± 0.79	0.11 ± 0.75	0.053
Small for gestational age at birth	2 (8.3)	2 (5.3)	0.637
Smoking in pregnancy	3 (11.5)	6 (15.8)	0.021
Maternal education status <12 years	12 (46.2)	25 (65.8)	0.012
Mainly breastfed (>50%)	20 (80.0)	25 (65.8)	0.349
Erythropoietin administration		13 (34.2)	
Red blood cell transfusion		6 (15.8)	
Platelet transfusion		0 (0.0)	
Characteristics at study visit			
Age (years)	5.53 ± 0.61	5.42 ± 0.28	0.338
Body mass index	14.9 ± 1.3	14.5 ± 1.5	0.292
Body mass index *z*-score	−0.34 ± 0.85	−0.72 ± 0.94	0.103
Family history of cardiovascular disease	2 (7.7)	3 (7.9)	0.4
Favorable childhood nutrition profile	16 (69.6)	15 (44.1)	0.105
Erythrocytes (trillion/L)	4.67 ± 0.33	4.89 ± 0.28	0.01
Hemoglobin (g/L)	126 ± 7	135 ± 7	<0.001
Hematocrit (%)	35.6 ± 1.7	37.8 ± 1.9	<0.001
Platelets (billion/L)	322 (87)	310 (87)	0.624
Mean corpuscular hemoglobin (pg)	27.5 (1.6)	27.6 (1.1)	0.247
Mean corpuscular volume (fL)	76.4 ± 4.6	77.2 ± 2.3	0.365
Mean corpuscular hemoglobin concentration (g/L)	352 ± 9	357 ± 8	0.03

Values are presented as mean ± standard deviation, median (interquartile range), or count (percentage).

differences in birth weight were similar in clinical characteristics. With regard to complete blood count, they showed higher concentrations of erythrocytes, whole blood hemoglobin, hematocrit, and higher mean corpuscular hemoglobin concentration, but no significant difference in platelet count. When investigating differences due to prematurity in a panel of 79 proteins mostly representing cardiovascular biomarkers, very preterm-born compared to term-born children featured lower plasma concentrations of platelet factor 4 (PLF4; −61.6%, $P < 0.0001$), platelet basic protein (CXCL7; −57.8%, $P < 0.0001$), and hemoglobin subunit beta (HBB; −48.3%, $P < 0.0001$) (Fig. 1). Ten other proteins reached an unadjusted $P < 0.05$, but lost statistical significance with adjustment for multiplicity. Detailed results on all proteins are presented in Supplemental Table S1.

These results under adjustment for age and sex remained virtually unchanged under further multivariable adjustment, including adjustment for platelet count or whole blood hemoglobin, or for further maternal or perinatal characteristics, as shown in Table 2.

No significant association emerged between gestational age and any of these three proteins within either the term or preterm group (all $P > 0.05$). Furthermore, red blood cell transfusion or administration of erythropoietin in preterm infants during their stay at the neonatal intensive care unit were not associated with PLF4, CXCL7, or HBB (all $P > 0.05$), and no children are known to have received platelet transfusions (Table 1).

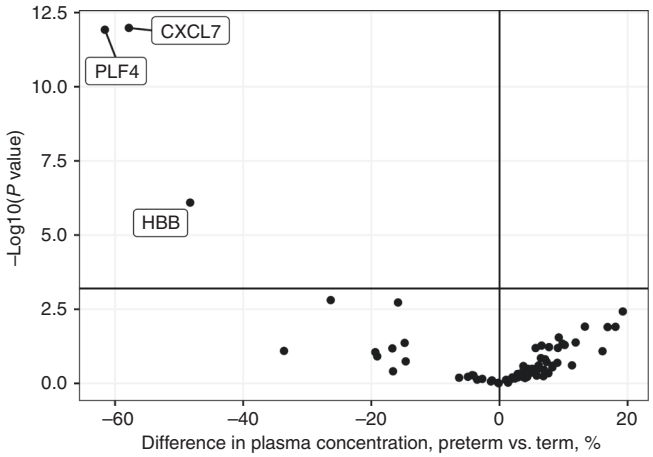

Fig. 1 Differences in plasma concentrations of 79 proteins between former very preterm-born and former term-born preschoolers under adjustment for age and sex. Effect size is shown on the *x*-axis, and significance level on the *y*-axis. The horizontal solid line indicates a Bonferroni-corrected *P* value of 0.05. Three proteins emerged as significant and are labeled. CXCL7 platelet basic protein, PLF4 platelet factor 4, HBB hemoglobin beta chain.

Correlations among the three significant proteins were $r = 0.95$ between PLF4 and CXCL7, $r = 0.51$ between PLF4 and HBB, and $r = 0.49$ between CXCL7 and HBB (all $P < 0.0001$).

Gene ontology term enrichment analysis yielded no significantly enriched terms (all $q > 0.05$).

DISCUSSION

PLF4 and the closely related platelet basic protein (CXCL7) are chemokines stored in platelet alpha granules and released abundantly upon platelet activation.[19] The biosynthesis of PLF4 is almost exclusively limited to megakaryocytes,[20] from which mature circulating platelets are derived. Thus, substantially lower concentrations here observed in very preterm-born children at a preschool age indicate marked differences pertaining to platelet activation compared to term-born children.

Alterations in blood coagulation may predispose to cardiovascular events,[21] but have so far attracted less attention than did hemodynamic, metabolic, and cardiac[5,8] sequelae of preterm birth. Because this study did not assess clinical outcomes, it does not inform on the relevance for cardiovascular risk of the platelet markers that were found associated with former preterm birth. However, it provides indirect evidence against excess cardiovascular risk due to prematurity related to one of the other proteins that were also measured, but did not differ between former preterm- and term-born children, including several plasmatic coagulation factors (Supplementary Table 1).

Lower platelet activation as compared to adults is well established in healthy full-term neonates, including both lower reactivity of platelets to various agonists,[22] as well as lower expression of activation markers on their surface.[23] Neonatal platelet hyporeactivity assessed by flow cytometry has been conclusively demonstrated to be present 3–4 days after birth,[24] but some uncertainty revolves around its persistence thereafter. Several studies found partial or complete normalization after 10–14 days of life,[23–25] while others found persistent hyporeactivity,[23,26] with one study reporting lower expression of integrin beta 3 on platelets as compared to adults in term-born infants and children up to age 15 years.[26]

Lower platelet reactivity in preterm-born compared to term-born infants has been reported for very preterm-born infants, but less consistently for less preterm infants, suggesting a correlation of gestational age with platelet reactivity.[9,23,27] In the first days of life, infants born before 30 weeks of gestation expressed lower levels of membrane glycoproteins, and their platelets were less reactive after stimulation,[23] while preterm infants born with a gestational age between 31 and 37 weeks showed no significant difference in whole blood platelet aggregometry compared to term-born infants.[25] The duration of preterm platelet hyporeactivity is unclear, with one study reporting reduced platelet adhesion in preterm (mean gestational age 32.1 years) compared to term-born infants persisting until at least the tenth week of life.[27]

Table 2. Differences in significant proteins between former preterm- and term-born preschoolers under multivariable adjustment.

Protein	Model A		Model B		Model C	
	Effect	*P* value	Effect	*P* value	Effect	*P* value
CXCL7	−57.8 (−65.2, −48.9)	<0.001	−56.8 (−64.4, −47.7)	<0.0001	−56.2 (−65.2, −44.7)	<0.001
HBB	−48.3 (−59.3, −34.3)	<0.001	−55.2 (−65.7, −41.4)	<0.0001	−44.2 (−56.7, −27.9)	0.002
PLF4	−61.6 (−69.0, −52.4)	<0.001	−59.5 (−67.7, −49.5)	<0.0001	−59.2 (−68.4, −47.4)	<0.001

Effects are given as % difference in plasma concentration in preterm vs. term group. *P* values are corrected for multiple comparisons by the Bonferroni procedure. Model A: adjustment for age and sex; Model B: adjustment for age, sex, whole blood hemoglobin, platelet count; Model C: adjustment for age, sex, birth weight *z*-score, body mass index, maternal smoking during pregnancy, maternal education, family history of cardiovascular disease.

The current study adds to this body of evidence by demonstrating substantially reduced markers of platelet activation in former very preterm infants at a preschool age (Table 2), suggesting that platelet hyporeactivity as compared to term-born infants persists at least up to age 5 years in former very preterm-born infants.

Platelet activation and aggregation is a dynamic cascade, including binding of platelets to exposed collagen, activation and degranulation, and cross-linking with fibrinogen.[28] This proteomic study was not designed to provide a comprehensive view of these dynamics and allows only limited conclusions regarding platelet function. It does not inform on the impact of endothelial factors or of plasmatic coagulation. Reductions in fibrinogen chains in former very preterm-born infants were not significant after correction for multiplicity (Supplementary Table 1), and fibrinogen function was not assessed. Our results reflect conditions under no shear stress, although shear stress is an important determinant of platelet function.[28]

With these caveats in mind, some connection to prior reports may be possible. As CXCL7 and PLF4 are exocytosed during platelet degranulation, our findings may be more likely to reflect effects of preterm birth on platelet binding to exposed collagen or activation and degranulation than on fibrinogen cross-linking. This would be broadly in line with prior reports that found reduced levels of glycoprotein Ib internalization,[26] which is important for platelet adhesion to subendothelium, and of P-selectin after platelet activation by thrombin receptor-activating peptide, a marker of platelet degranulation,[23,26] after preterm birth[23] or from preterm birth to 15 years of age.[26]

Very preterm-born preschoolers exhibited higher whole blood hemoglobin (Table 1), consistent with results of prior studies for young adult males[29,30] and females.[29] Although hemoglobin levels were higher, erythropoietin levels were similar to those of term-born subjects, suggesting a sustained upregulation of erythropoiesis.[29] As an alternative, if less likely, potential explanation, in conjunction with observed lower plasma concentrations of free HBB (Table 2), higher whole blood hemoglobin might also hypothetically reflect lower thrombotic activity and hemolysis secondary to reduced platelet activation. Together with hemoglobin alpha chain, HBB makes up alpha 2 beta 2 hemoglobin, the dominant form of hemoglobin after the first year of life.[31]

The clinical relevance of our findings is unclear. In term-born neonates, the so-called "immaturity of the hemostatic system" due to lower platelet reactivity does not predispose to bleeding,[9] likely because platelet hyporeactivity is counteracted by enhanced platelet/vessel wall interaction related to higher hematocrit, higher mean corpuscular volume, and higher von Willebrand factor concentration.[32] While bleeding times performed on the first day of life in very preterm (<33 weeks) neonates are approximately twice as long, by day of life 10, they were found similar to those of term-born neonates.[33] Notably, studies using an in vitro test of primary hemostasis (the platelet function analyzer PFA-100) demonstrated shorter closure times in term-born neonates[34] and shorter or similar closure times in preterm neonates,[35] compared to adults, indicating adequate primary hemostasis in both. Importantly, our current findings do not demonstrate impaired hemostasis in former preterm infants and the presence of a qualitatively different, but well-functioning hemostatic system may be more likely. Likewise, our results do not suggest alterations in platelet function to cause excess cardiovascular risk in preterm-born subjects.

One further potential mechanistic link between former preterm birth and reduced PLF4 is due to heparin-induced thrombocytopenia (HIT), which is mediated by antibodies against complexes of PLF4 with heparin[36] that bind to FcγRIIA receptors on platelets, eliciting platelet activation.[37] While this immune reaction may be as common as 8–50%, only 0.2–3% of subjects exposed to unfractionated heparin develop thrombocytopenia and thrombosis,[36] suggesting considerable prevalence of subclinical antibody formation after exposure to heparin. Notably, formation, but not binding of antibodies, is dependent on the presence of heparin,[38] and PLF4 bound to glycosaminoglycans on the surface of endothelial cells, monocytes, and platelets can present the HIT antibody target antigen in the absence of heparin.[39] All children in the very preterm group received small doses of unfractionated heparin during their stay at the neonatal intensive care unit to ensure patency of catheters during administration of parenteral nutrition. Thus, increased elimination of PLF4 due to increased prevalence of persisting HIT antibodies in former preterm infants after postnatal heparin exposure may be an alternative, hypothetical explanation for lower PLF4 levels in these infants, although HIT is less common in children than in adults[39] and persistence of HIT antibodies for multiple years has not been reported.[40]

Ten proteins differed significantly between former preterm- and term-born infants before, but not after correction for multiple testing (Supplementary Table S1), including further proteins involved in hemostasis (fibrinogen gamma chain, kininogen-1), immunity (complement component C8 alpha chain), and several transport proteins (transthyretin, sex hormone-binding globulin, corticosteroid-binding globulin). These exploratory results could inform future studies.

Gene ontology term enrichment analysis using the ranked list approach yielded no significantly enriched terms (all $q > 0.05$). This type of analysis makes use of annotations (gene ontology terms) that map proteins to biological processes and tests whether proteins that are more strongly associated with former preterm birth are enriched for annotation with individual terms. Interpretation of negative results of this analysis should consider that it is less sensitive to strong effects concentrated on a small number of proteins, as were found here, and that correction for multiple testing is stringent due to the large number of terms tested, possibly limiting statistical power.

Strengths of the current study include the novelty of applying an unbiased, comprehensive cardiovascular proteomics approach to former very preterm infants at a preschool age, with protein measurement using highly sensitive multiple-reaction monitoring mass spectrometry. Key conclusions are based on strong, highly significant, and robust results.

Weaknesses include that no direct assay of platelet activation was performed. Reduced levels of PLF4 and CXCL7 may be due to differences in platelet activation, but could also be due to differences in protein content of alpha granules, or in alpha granule content of platelets. Likewise, the present data cannot define whether differences observed relate to platelet activation before or after sampling of blood. One further weakness pertains to limited sample size, which was mostly due to the difficulty of obtaining blood samples from healthy children and which prevented subgroup analysis.

Clinical relevance of findings could be elucidated by future studies examining primary hemostasis, for example, using the platelet function analyzer-100,[9,28] and fibrinolysis, for example, by D-dimer. Hemoglobin electrophoresis could clarify whether differences in hemoglobin types due to preterm birth exist, and measurement of haptoglobin or lactate dehydrogenase whether hemolysis is a plausible explanation for found differences in hemoglobin and hemoglobin beta chain levels.

In summary, former very preterm-born infants at a preschool age showed markedly lower plasma levels of platelet activation markers.

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AUTHOR CONTRIBUTIONS

Designed the study: U.K.-K. and A.P. Obtained funding: U.K.-K. and S.K. Supervised the study: U.K.-K. Analyzed the data: R.P. Drafted the manuscript: R.P. Revised the manuscript for important intellectual content: A.P., X.Y., S.A.P., S.J.K., M.M., S.K., and U. K.-K. Performed proteomics measurements: X.Y. and S.A.P. Approved the final version of the manuscript submitted: all authors.

ADDITIONAL INFORMATION

The online version of this article (https://doi.org/10.1038/s41390-020-1070-8) contains supplementary material, which is available to authorized users.

Competing interests: The authors declare no competing interests.

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