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# Biomarkers to predict, prevent and find the appropriate treatments of disorders in childhood

**Edited by**
Kristin Skogstrand, Nis Borbye-Lorenzen, Ulrik Lausten-Thomsen and Marie Bækvad-Hansen

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# Biomarkers to predict, prevent and find the appropriate treatments of disorders in childhood

**Topic editors**

Kristin Skogstrand — Department of Congenital Disorders, Statens Serum Institute, Denmark

Nis Borbye-Lorenzen — Department of Congenital Disorders, Statens Serum Institute, Denmark

Ulrik Lausten-Thomsen — Copenhagen University Hospital Rigshospitalet, Denmark

Marie Bækvad-Hansen — Statens Serum Institut (SSI), Denmark

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EDITED AND REVIEWED BY
Tim S Nawrot,
University of Hasselt, Belgium

*CORRESPONDENCE
Kristin Skogstrand
ksk@ssi.dk

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# Editorial: Biomarkers to predict, prevent and find the appropriate treatments of disorders in childhood

Kristin Skogstrand[1]*, Nis Borbye-Lorenzen[1], Marie Bækvad-Hansen[1] and Ulrik Lausten-Thomsen[2]

[1]Danish Center for Neonatal Screening, Department for Congenital Disorders, Statens Serum Institut, Copenhagen, Denmark, [2]Neonatal Intensive Care Unit, Copenhagen University Hospital Rigshospitalet, Copenhagen, Denmark

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Editorial on the Research Topic
Biomarkers to predict, prevent and find the appropriate treatments of disorders in childhood

Biomarkers in its broadest sense refer to (bio)medical signs, i.e., an objective indication of a medical state, that can be accurately and reproducibly measured. Biomarkers play a huge role in clinical practice and research in adults, but for practical and ethical reasons the number of specific pediatric biomarkers has traditionally been fewer. However, neonatal and pediatric biomarkers absolutely can be used to predict, to prevent, or to diagnose disorders, and to find the right treatment, as well as to monitor treatment effects. Biomarkers can be prognostic, by predicting the recurrence of a disorder, or they can be predictive, by identifying which medicine is the best treatment for each patient as also described in The BEST (Biomarkers, EndpointS, and other Tools) guidelines (1). The current special issue presents a 2022 snapshot of pediatric biomarker research through 13 interesting and diverse papers:

Broadly speaking, markers for infection and/or inflammation is still very much a theme in biomarker research. This is exemplified in this Research Topic by papers exploring correlation between inflammatory markers and clinical conditions as variates. About half the included papers describe biomarkers reflecting the immune response and different conditions: Two papers, Brynge et al. and Fabricius et al., presents pediatric immunological markers' association to the mental disorders autism spectrum disorder and obsessive compulsive disorder, respectively. Biomarkers for psychiatric disorders is an underdeveloped field, and the complex and heterogenic topic of psychiatry would likely benefit immensely from the development of predictive, qualitative, and even diagnostic biomarkers, just as other areas of medicine have done over the last century or so. We have previously reported that biomarkers measured in samples taken a few days after birth associate with later diagnosis of autism spectrum disorder (2), but the biomarker differences were not significant

enough to be used as a diagnostic or predictive tool. It is known that the causes of psychiatric disorders are multifactorial, but also broadly genetically dependent (3, 4), but the etiology for the different psychiatric disorders are largely unknown (5–7), and biomarkers can help with the understanding of the disorders' development. Thus, the field of psychiatry both regarding diagnostics and treatments is solely based on symptoms, which can lead to both under- and over-diagnosis, as well as ineffective treatments due to lack of knowledge in personal medicine (8–10). We believe and hope that in the near future, biomarkers will become an important part of psychiatry (2–4).

Inflammatory markers were also analyzed in Faust et al.'s paper, describing a correlation between neonatal inflammation and bronchopulmonary dysplasia, and Ouyang et al. used inflammatory markers as a prognostic tool for pediatric osteosarcoma.

Lamot et al. describes biomarkers as a tool for separating viral from bacterial infections, and Feketea et al. have found a correlation between vitamin D and mean platelet volume in children with viral respiratory infections.

Rossi et al. discusses an important subject for several neonatal screening disorders; how to separate diagnosis with symptoms to asymptomatic cases. As the laboratory technologies get more sensitive, and we are able to analyze about everything in a few drops of blood, the number of disorders in neonatal screening panels all over the world are increasing (11, 12). When is a screen positive sample actually synonymous with a disorder in the child is thus a question more important than ever before. For many disorders, biochemical analyses may be followed up by genotyping to reduce the number of false positive samples (13). This is though not always possible, as the consequence of different genetic variations and combinations sometimes are not known. Thus, the possibility of screening newborns for multiple disorders should be carefully balanced through ethical considerations such as the risk of making otherwise asymptomatic children sick due to a false positive screening result.

Genetics and outcome are presented in one paper by Liu et al., looking at risk for hypospadias with different gene polymorphisms. Xiang et al. have looked at environmental factors by analyzing urine phthalate associations to adolescents' liver function. Mingwen et al. presents the only paper in this issue using biomarkers not measured in body fluids, being parent-reported measures of sleep patterns, to find a model to classify sleep disorders.

Our research topic contains one review paper by Nguyen et al., where the authors have reviewed metabolomics results and lung exacerbations in cystic fibrosis children, and one paper by Tao et al., exploring predictors for syncopal recurrence in children treated with metoprolol. Miller et al. presents another important biomarker topic; biomarkers to predict prognosis after head injury.

As encouraging as these papers are, generally in the area of biomarker research, there is often far between the manuscripts concluding with clinically relevant biomarkers. Several biomarkers may be statistically significant, but cannot be used as either screening or diagnostic markers. The perfect diagnostic biomarker, that is, a biomarker with 100% sensitivity and specificity, does not exist, but biomarkers with very low specificity are of poor value for diagnostic purposes. All statistically significant biomarkers may though help in the understanding of the disorders' etiology. A growing trend is quantity over quality, that is, the more markers the better, employing modern high-throughput laboratory techniques called omics, e.g., genomics, proteomics, transcriptomics, metabolomics, and microbiomics methods. The impressive progress in the field has enabled the fast discovery of candidate biomarkers and consequently large numbers of preclinical reports have been published. The relative complexity of these technologies put extra stress on requirement for well-designed biomarker discovery processes to develop clinically relevant biomarkers. The challenge then is to sort out the useful markers, and to make a mathematic formula that is more than just statistically significant, but also actually clinically useful.

When a biomarker has the potential to become a predictive or diagnostic biomarker, biobank resources such as those found in Denmark and other Scandinavian countries with a wealth of accessible health registers become highly relevant to prove the biomarkers potential (14, 15).

Study design is very important during exploration for biomarkers. Controls should be selected carefully, not only regarding gender, age and BMI, but also regarding treatments, populations etc., and the statistical calculations should be made by people who actually know what they are doing. The more biomarkers available, the more complex the calculations get. Using the wrong statistical methods, almost all studies will find statistically significant biomarkers.

Particularly genomics has clinical appeal: Why use biomarkers in the form of proteins or smaller molecules, when the whole human genome is available? Is it possible to solely use genetic variation either as biomarker for disorders or for personal medicine in the future? Can a few drops of blood and a whole genome sequencing test be enough in the future to both set the diagnosis and to choose the best medicine for the patient? For a few disorders it might be sufficient, but in the majority of cases probably not, as most disorders cannot be explained by genetic variation alone. The lack of effect of medicines can be caused by other molecules in the blood, e.g., environmental factors, or by a combination of both external factors and genetics. In addition, there is the layer of posttranslational modification that is highly tissue-dependent and not directly predictable through a genomic

analysis alone (16). The combination of physical symptoms and blood biomarkers, both protein-based and genetic, are probably for most disorders the best diagnostic combination to avoid both under- and over-diagnosis. Some people claim that all humans can get at least one diagnosis if we get examined thorough enough (17), and this is obviously not what we want as a society. Thus, we are not done yet in the research for good, useful biomarkers for disorders.

## Author contributions

All authors contributed to the article and approved the submitted version.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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# Urine Phthalate Levels and Liver Function in US Adolescents: Analyses of NHANES 2007–2016

*Shiting Xiang*[1], *Jie Dong*[1], *Xun Li*[1]* *and Chao Li*[2]*

[1] Pediatrics Research Institute of Hunan Province, Hunan Children's Hospital, Changsha, China, [2] Department of Epidemiology and Medical Statistics, Xiangya School of Public Health, Central South University, Changsha, China

**Background:** Phthalates are non-persistent chemicals with endocrine-disrupting abilities widely used in a variety of consumer products. Evidence for the effects of phthalate exposure on liver function in adolescents is lacking.

**Methods:** Data were analyzed from the combined 2007–2016 National Health and Nutrition Examination Survey (NHANES). Ultimately, a total of 1,650 adolescents aged 12–19 years were selected as the samples. Weighted linear regression was used to investigate the effects of urinary phthalate metabolites on liver function indexes.

**Results:** Weighted Linear regression models showed that MCOP was negatively associated with TBIL ($\beta = -0.0435$, $P_{FDR} = 0.007$), $\Sigma$DEHP ($\beta = -0.0453$, $P_{FDR} = 0.003$) and MCOP ($\beta = -0.0379$, $P_{FDR} = 0.006$) were negatively correlated with ALB, while MCPP was positively correlated with ALB ($\beta = 0.0339$, $P_{FDR} = 0.024$), and MCOP was negatively correlated with TP ($\beta = -0.0551$; $P_{FDR} = 0.004$).

**Conclusions:** Phthalate metabolites were significantly but weakly associated with changes in liver function indicators among US adolescents. Future work should further examine these relationships.

**Keywords: phthalate, liver, adolescents, NHANES, indicators**

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*Marie Bækvad-Hansen, Statens Serum Institut (SSI), Denmark*

***Reviewed by:***
*Moushira Zaki, National Research Centre, Egypt*
*Giovanna Tranfo, National Institute for Insurance Against Accidents at Work (INAIL), Italy*

***Correspondence:**
*Xun Li*
*li.xunxx@qq.com*
*Chao Li*
*jenniferchaoli@csu.edu.cn*

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## BACKGROUND

Phthalates, known as plasticizers, are non-persistent chemicals with endocrine-disrupting abilities widely used in a variety of consumer products (1). High molecular weight phthalates, including di-(2-ethylhexyl) phthalate (DEHP) and di-isononyl phthalate (DiNP), are used primarily as plasticizer for polyvinyl chloride, building and construction materials, and several categories of toys (such as plastic books, ball, doll, and cartoon characters). Low molecular weight phthalates, including di-butyl phthalate (DBP) and diethyl phthalate (DEP), are used primarily as fragrance ingredients in cosmetics, home, and personal care products (2, 3). As phthalates are usually bound to polymers by non-chemical bonds, they are often constantly released from plastic products into the surrounding environment, resulting in food, water, or air pollution (4). Human are exposed to large amounts of phthalates through dietary, inhalation and skin contact (4).

Liver diseases such as non-alcoholic liver disease, alcoholic liver disease and viral hepatitis are major causes of illness and death worldwide. Approximately 2 million people die from it every year in the world (5). Although vaccination and new drugs will reduce the burden of viral-related liver disease, non-alcoholic liver disease continues to rise in general population adolescents (6). In addition to alcohol, viruses, genetics, and unhealthy lifestyles, studies have found that environmental chemicals may play a role in abnormal liver function in adolescents (7).

Liver plays an important role in the detoxification of phthalates (8). The hepatotoxicity of phthalates has been demonstrated in animal models such as mice, zebrafish, and quail (9–11). Phthalate concentrations have been adversely associated with indicators of liver function in adulthood (12), but few studies have examined associations between phthalate exposure and liver function in youth. Changes in liver function are a long-term process of liver injury, early prevention and intervention can reduce the incidence of liver disease in adults.

Therefore, in the present study, we aimed to examine the association between phthalate exposure and indicators of liver function using a nationally representative sample of adolescents aged 12–19 years in the United States.

# METHODS

## Study Population

National Health and Nutrition Examination Survey (NHANES) is a cross-sectional, nationally representative survey in the United States conducted annually by CDC's National Center for Health Statistics (CDC/NCHS). A detailed description of the study design can be found elsewhere (13). The survey uses a multistage stratified probability sample based on selected counties, blocks, households, and persons within households. Survey interviews were conducted in participants' homes by well-trained professionals, while extensive physical examinations, including blood and urine collection, were conducted at mobile exam centers.

The present analysis included five waves of the NHANES from 2007 to 2016, which were publicly shared and downloaded from the CDC official website and combined according to the NHANES tutorials. The 6,598 participants were between the ages of 12 and 19. A one-third subsample were tested for phthalates ($n = 2,076$). We excluded subjects who were serologically positive for hepatitis B virus or hepatitis C virus and did not have complete records, including liver function tests and covariates. Finally, a total of 1,650 adolescents were selected as final samples.

**TABLE 1 |** Demographic characteristics for adolescents aged 12–19 years old in NHANES 2007–2016 ($N = 1,650$).

| Basic characteristics | *N* | % |
|---|---|---|
| **Age (years)** | | |
| 12–14 | 631 | 38.2 |
| 15–17 | 618 | 37.5 |
| 18–19 | 401 | 24.3 |
| **Gender** | | |
| Male | 883 | 53.5 |
| Female | 767 | 46.5 |
| **Race** | | |
| Non-Hispanic White | 485 | 29.4 |
| Non-Hispanic Black | 408 | 24.7 |
| Mexican American | 376 | 22.8 |
| Other Hispanic | 190 | 11.5 |
| Other/Mixed | 191 | 11.6 |
| **Education** | | |
| Less than high school | 1,393 | 84.4 |
| High School graduate or GED | 131 | 7.9 |
| More than High | 126 | 7.6 |
| **PIR** | | |
| ≤1 | 540 | 32.7 |
| >1 | 1,110 | 67.3 |
| **BMI groups** | | |
| Normal/Underweight (<25) | 1,042 | 63.2 |
| Overweight (25 to <30) | 342 | 20.7 |
| Obese (≥30) | 266 | 16.1 |
| **Physical activity** | | |
| No | 365 | 22.1 |
| Yes | 1,285 | 77.9 |

## Liver Function Measure Outcomes

Fasting blood samples were collected in NHANES participants aged 12 years and older at a mobile examination center. The samples were refrigerated and transported to the central laboratory for analysis of serum liver function indicators using the Beckman Coulter DxC800 Synchron clinical system (14).

The liver is rich in alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Serum levels of these two enzymes rise when hepatocytes necrosis or liver cell membrane damage (15). AST/ALT ratio is used for differential diagnosis of acute and chronic liver diseases. The liver is the only place where albumin (ALB) is synthesized. When liver function is impaired, serum albumin (ALB), and total protein (TP) levels decrease (16). Alkaline phosphotase (ALP) and Gamma glutamyl transferase (GGT) are markers of cholestasis (17). The liver has the functions of uptake, combination, and excretion of bilirubin metabolism. The disorder of one or more functions can lead to the increase of total bilirubin (TBIL) (18).

## Measurement of Phthalate

Phthalate metabolites were measured in spot urine samples from a third of study subjects randomly selected from participants 6 years of age and older. The collected samples were frozen at −20°C and then shipped to the CDC's National Center for Environmental Health for analysis. Urine specimens were processed using high performance

**Abbreviations:** DEHP, di-(2-ethylhexyl) phthalate; DiNP, di-isononyl phthalate; DBP, di-butyl phthalate; DEP, diethyl phthalate; NHANES, National Health and Nutrition Examination Survey; CDC, Centers for Disease Control; NCHS, National Center for Health Statistics; ALT, Alanine aminotransferase; AST, Apartate aminotransferase; ALB, Albumin; TP, Total protein; ALP, Alkaline phosphotase; GGT, Gamma glutamyl transferase; TBIL, total bilirubin; HPLC-ESI-MS/MS, high performance liquid chromatography-electrospray ionization-tandem mass spectrometry; LLOD, Lower limit of detection; MCNP, mono-(carboxyisononyl) phthalate; MCOP, mono-(carboxyisoctyl) phthalate; MECPP, mono-2-ethyl-5-carboxypentyl phthalate; MnBP, mono-n-butyl phthalate; MCPP, mono-(3-carboxypropyl) phthalate; MEP, mono-ethyl phthalate; MEHHP, mono-(2-ethyl-5-hydroxyhexyl) phthalate; MEHP, mono-(2-ethylhexyl phthalate; MiBP, mono-isobutyl phthalate; MEOHP, mono-(2-ethyl-5-oxohexyl) phthalate; MBzP, mono-benzyl phthalate; BMI, Body mass index; PIR, Ratio of family income to poverty; FDR, Benjamini-Hochberg false discovery rate.

**TABLE 2 |** Distribution of urinary phthalate metabolites and indicators of liver function for adolescents aged 12–19 years old in NHANES 2007–2016 ($N = 1,650$).

| | ≥LOD% | P25 | P50 | P75 |
|---|---|---|---|---|
| **Urinary phthalate metabolites (μg/mmol Cr)** | | | | |
| MECPP | 99.90 | 6.77 | 7.34 | 7.99 |
| MEHHP | 99.60 | 6.26 | 6.82 | 7.50 |
| MEHP | 71.90 | 4.39 | 5.03 | 5.73 |
| MEOHP | 99.70 | 5.82 | 6.41 | 7.03 |
| MCNP | 98.00 | 4.95 | 5.42 | 6.01 |
| MCOP | 99.60 | 6.27 | 7.09 | 8.04 |
| MnBP | 98.80 | 6.74 | 7.29 | 7.79 |
| MCPP | 93.10 | 4.74 | 5.39 | 6.06 |
| MEP | 99.90 | 7.66 | 8.47 | 9.35 |
| MiBP | 99.40 | 6.33 | 6.84 | 7.31 |
| MBzP | 99.00 | 5.98 | 6.59 | 7.27 |
| **Liver function** | | | | |
| ALT (IU/L) | 100.00 | 14.00 | 17.00 | 21.00 |
| AST (IU/L) | 100.00 | 19.00 | 22.00 | 26.00 |
| GGT(U/L) | 100.00 | 11.00 | 13.00 | 18.00 |
| ALP (IU/L) | 100.00 | 69.00 | 96.00 | 170.00 |
| TBIL (mg/dL) | 100.00 | 0.50 | 0.60 | 0.80 |
| ALB (g/dL) | 100.00 | 4.30 | 4.50 | 4.70 |
| TP (g/dL) | 100.00 | 7.00 | 7.20 | 7.50 |
| AST/ALT | 100.00 | 1.09 | 1.33 | 1.57 |

liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS) for the quantitative detection of phthalate metabolites (14).

We selected 12 metabolites tested in all five rounds and excluded phthalate metabolites whose measured values were more than 40% below the detection limit (LOD). The remaining 11 urinary phthalate metabolites used in our study were mono-(carboxyisononyl) phthalate (MCNP), mono-(carboxyisoctyl) phthalate (MCOP), mono-2-ethyl-5-carboxypentyl phthalate (MECPP), mono-n-butyl phthalate (MnBP), mono-(3-carboxypropyl) phthalate (MCPP), mono-ethyl phthalate (MEP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono-(2-ethylhexyl phthalate (MEHP), mono-isobutyl phthalate (MiBP), mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP), and mono-benzyl phthalate (MBzP). Phthalate metabolites concentrations below LODs were replaced with LOD divided by the square root of two.

Concentrations of MECPP, MEHHP, MEHP, and MEOHP were divided by their respective molar weight (MW) to obtain the molar equivalent. We summed the molar equivalents of these metabolites and multiplied by the molar weight of MEHP (MW = 278) to obtain $\Sigma$DEHP metabolites (19).

## Measurements of Covariates

Covariates were selected as potential confounders by referencing to previous publications (20, 21). Covariates were age, gender, race, education, ratio of family income to poverty (PIR), physical activity, body mass index (BMI), and total daily

**TABLE 3 |** Association between log-transformed phthalate metabolites and indicators of liver function for adolescents aged 12–19 years old in NHANES 2007–2016 ($N = 1,650$).

| | β (95% CI) | *P*-value | $P_{FDR}$ |
|---|---|---|---|
| **ALT** | | | |
| ∑DEHP[a] | −0.3339 (−0.7630 0.0952) | 0.437 | 0.863 |
| MCNP | 0.2414 (0.3108, 0.7936) | 0.662 | 0.887 |
| MCOP | −0.2438 (−0.8074, 0.3198) | 0.665 | 0.887 |
| MnBP | −0.5485 (−1.2658, 0.1688) | 0.445 | 0.863 |
| MCPP | −0.1163 (−0.5285, 0.2959) | 0.778 | 0.916 |
| MEP | 0.1591 (−0.1512, 0.4694) | 0.608 | 0.887 |
| MiBP | 0.0998 (−0.7522, 0.9518) | 0.907 | 0.936 |
| MBzP | 0.5276 (−0.1920, 1.2472) | 0.464 | 0.873 |
| **AST** | | | |
| ∑DEHP[a] | 0.0460 (−0.2511, 0.3431) | 0.877 | 0.920 |
| MCNP | 0.0793 (−0.3238, 0.4842) | 0.844 | 0.916 |
| MCOP | −0.4631 (−0.8743, −0.0519) | 0.260 | 0.693 |
| MnBP | −0.6381 (−1.6631, 0.3869) | 0.534 | 0.877 |
| MCPP | 0.1178 (−0.2362, 0.4718) | 0.739 | 0.916 |
| MEP | 0.2225 (0.0047, 0.4403) | 0.307 | 0.728 |
| MiBP | 1.7819 (−0.0886, 3.6524) | 0.341 | 0.753 |
| MBzP | −0.3969 (−1.3026, 0.5088) | 0.661 | 0.877 |
| **GGT** | | | |
| ∑DEHP[a] | −0.2084 (−0.5071, 0.0903) | 0.485 | 0.887 |
| MCNP | 0.1705 (−0.1851, 0.5261) | 0.632 | 0.887 |
| MCOP | 0.1373 (−0.2632, 0.5378) | 0.732 | 0.916 |
| MnBP | 1.1198 (0.5187, 1.7209) | 0.063 | 0.310 |
| MCPP | −0.5597 (−0.8166, −0.3028) | 0.030 | 0.192 |
| MEP | −0.1097 (−0.3595, 0.1401) | 0.661 | 0.887 |
| MiBP | −0.7511 (−1.1897, −0.3125) | 0.087 | 0.352 |
| MBzP | −0.0995 (−0.3384, 0.5374) | 0.820 | 0.916 |
| **ALP** | | | |
| ∑DEHP[a] | 1.2759 (−0.8193, 3.3711) | 0.543 | 0.887 |
| MCNP | 4.6037 (1.4705, 7.7369) | 0.142 | 0.488 |
| MCOP | −1.4999 (−3.7503, −0.7505) | 0.508 | 0.887 |
| MnBP | −0.8977 (−4.6925, 2.8971) | 0.813 | 0.816 |
| MCPP | −0.7931 (−3.6079, 2.0217) | 0.778 | 0.816 |
| MEP | −0.2738 (−1.8363, 1.2887) | 0.861 | 0.918 |
| MiBP | 6.3493 (3.1975, 9.5013) | 0.044 | 0.256 |
| MBzP | 3.1292 (0.5445, 5.7139) | 0.226 | 0.629 |
| **TBIL** | | | |
| ∑DEHP[a] | −0.0197 (−0.0330, −0.0064) | 0.140 | 0.488 |
| MCNP | 0.0153 (0.0005, 0.0301) | 0.301 | 0.728 |
| MCOP | −0.0435 (−0.0557, −0.0313) | **<0.001** | **0.007** |
| MnBP | 0.0273 (0.0086 0.0460) | 0.145 | 0.488 |
| MCPP | 0.0466 (0.0300, 0.0632) | 0.005 | 0.053 |
| MEP | 0.0159 (0.0075, 0.0243) | 0.058 | 0.309 |
| MiBP | −0.0381(−0.0537, −0.0225) | 0.015 | 0.120 |
| MBzP | 0.0075 (−0.0050, 0.0200) | 0.549 | 0.887 |
| **ALB** | | | |
| ∑DEHP[a] | −0.0453 (−0.0564, −0.0342) | **<0.001** | **0.003** |
| MCNP | −0.0026 (−0.0145, 0.0093) | 0.827 | 0.916 |
| MCOP | −0.0379 (−0.0483, −0.0275) | **<0.001** | **0.006** |

*(Continued)*

**TABLE 3** | Continued

| | β (95% CI) | *P*-value | $P_{FDR}$ |
|---|---|---|---|
| MnBP | −0.0151 (−0.0294, −0.0008) | 0.298 | 0.728 |
| MCPP | 0.0339 (0.0230, 0.0448) | **0.0019** | **0.024** |
| MEP | −0.0183 (−0.0262, −0.0114) | 0.0082 | 0.075 |
| MiBP | 0.0006 (−0.0140, 0.0128) | 0.966 | 0.966 |
| MBzP | −0.0149 (−0.0269, −0.0029) | 0.215 | 0.625 |
| **TP** | | | |
| ∑DEHP[a] | −0.0202 (−0.0351, −0.0053) | 0.176 | 0.536 |
| MCNP | 0.0017 (−0.0162, 0.0196) | 0.925 | 0.940 |
| MCOP | −0.0551 (−0.0694, −0.0408) | **<0.001** | **0.004** |
| MnBP | −0.0472 (−0.0682, −0.0262) | 0.025 | 0.178 |
| MCPP | 0.0036 (−0.0144, 0.0216) | 0.839 | 0.916 |
| MEP | 0.0121 (−0.0004, 0.0246) | 0.335 | 0.753 |
| MiBP | 0.0108 (−0.0091, 0.0307) | 0.588 | 0.887 |
| MBzP | −0.0120 (−0.0273, 0.0033) | 0.432 | 0.863 |
| **AST/ALT** | | | |
| ∑DEHP[a] | 0.0026 (0.0017, 0.0035) | 0.774 | 0.916 |
| MCNP | −0.0184 (−0.0290, −0.0078) | 0.084 | 0.352 |
| MCOP | −0.0050 (−0.0143, 0.0043) | 0.595 | 0.887 |
| MnBP | 0.0070 (−0.0075, 0.0215) | 0.630 | 0.887 |
| MCPP | 0.0098 (−0.0014, 0.0210) | 0.383 | 0.817 |
| MEP | 0.0022 (−0.0043, 0.0087) | 0.731 | 0.916 |
| MiBP | 0.0195 (0.0058, 0.0332) | 0.154 | 0.493 |
| MBzP | −0.0182 (−0.0289, −0.0075) | 0.088 | 0.352 |

[a] *ΣDEHP indicates the creatinine corrected molar sum of DEHP metabolites including: MECPP, MEHHP, MEHP, and MEOHP (expressed as MEHP, molecular weight 278).* $P_{FDR}$ *is the P-value adjusted by the method of Benjamini-Hochberg false discovery rate (FDR) correction to adjust for multiple testing. All models were adjusted for PIR(≤1/>1), BMI (<25/25–30/≥30), age (12–14/15–17/18–19 years), gender (male/female), race/ethnicity (Mexican American/Other Hispanic/Non-Hispanic White/Non-Hispanic Black/Other Race), education (Less than high school/ High School graduate or GED/ More than High), physical activity (Yes/No), and total daily protein intake. Values in bold are statistically significant (*$P_{FDR} < 0.05$*).*

protein intake. Physical activity was a dichotomous variable, with yes representing moderate or vigorous intensity sports, fitness, or recreational activities in a typical week. BMI was calculated as weight (kg) /height$^2$ (m$^2$) measured in the physical examination and categorized into three levels: <25 kg/m$^2$ (Normal/Underweight), 25 to <30 kg/m$^2$ (overweight) and ≥30 kg/m$^2$ (obese) (22). Data on total daily protein intake were measured through a 24-h food recall interview.

## Statistical Analysis

Demographic characteristics were reported as percentages. Phthalate metabolite concentrations and liver function levels were described in quartile range. We used urine creatinine to adjust the concentrations of phthalate metabolites in all statistical analyses (23, 24). Creatinine-adjusted phthalate metabolites concentrations and indicators of liver function were natural log-transformed to make them normally distributed. Spearman's coefficients were used to test the pairwise correlations of phthalate metabolite concentrations (**Supplementary Table 2**). We performed survey-weighted linear regression to assess the associations of the urinary phthalate metabolites with indicators of liver function. Benjamini-Hochberg false discovery rate (FDR) correction was used to adjust *P*-values to adjust for multiple testing.

All models were adjusted for PIR, BMI, age, gender, race, education, physical activity, and total daily protein intake. All analyses were performed using phthalate-specific subsample weight to account for the complex sampling design and non-response of NHANES. Weights for combined NHANES survey cycles were calculated according to NHANES guidelines. All statistical analyses were performed using R 3.5.3. All test values were 2-sided and $P < 0.05$ was considered significant.

# RESULTS

## Study Population

Characteristics of the study subjects are shown in **Table 1**. Of the 1,650 participants, the average age was 15.49 ± 2.266 years, with female subjects accounting for 46.5%. Most of the participants are Non-Hispanic White, 84.4% of the participants had education less than high school, 67.3% had a ratio of family income to poverty >1, 16.1% were obese, and 77.9% were physically active.

## Levels of Urinary Phthalate Metabolites and Liver Function Indicators

Descriptive statistics for phthalate metabolites and liver function indicators are presented in **Table 2**. The detection rates for the 11 phthalate metabolites ranged from 71.90 to 99.90%. The median concentrations of MECPP, MEHHP, MEHP, MEOHP, MCNP, MCOP, MnBP, MCPP, MEP, MiBP, and MBzP were 7.34, 6.82, 5.03, 6.41, 5.42, 7.09, 7.29, 5.39, 8.47, 6.84, and 6.59 μg/mmol Cr, respectively. Spearman correlation analysis showed that except for MCOP and MEP, all of them were significantly correlated (**Supplementary Table 2**).

## Survey-Weighted Liner Regression Analyses

The results of survey-weighted linear regression are shown in **Table 3**. MCOP was negatively associated with TBIL (β = −0.0435, $P_{FDR}$ = 0.007). ΣDEHP (β = −0.0453, $P_{FDR}$ = 0.003) and MCOP (β = −0.0379, $P_{FDR}$ = 0.006) were negatively correlated with ALB, while MCPP was positively correlated with ALB (β = 0.0339, $P_{FDR}$ = 0.024). MCOP was negatively correlated with TP (β = −0.0551, $P_{FDR}$ = 0.004).No significant linear relationships were found between ALT, AST, GGT, ALP, and ALT/AST with phthalate metabolites.

# DISCUSSION

In this cross-sectional, population-based analysis of US adolescents aged 12–19, we found significantly but weakly associations between several phthalate metabolites and TBIL, ALB, and TP. We observed null associations between phthalate metabolites and ALT, AST, GGT, ALP, and ALT/AST. To our knowledge, this is the first study examined the association between urine phthalate metabolites with liver function indexes in the adolescents population.

ALT was mainly distributed in liver. AST was mainly distributed in myocardium, followed by liver. Serum ALT can be sharply increased before the onset of clinical symptoms in patients with acute liver injury, while AST is significantly increased in cases of chronic hepatitis, cirrhosis, and liver cancer (15). ALP and GGT are also abundant in liver cells. Serum ALP and GGT are significantly increased when cholestasis caused by cirrhosis, cholelithiasis, and tumor (17). Yu et al. (12) reported that ΣDEHP was positively correlated with ALT, GGT, and ALP, and MBP was positively correlated with AST. Wang et al. (25) reported that ALT, AST, GGT were significantly raised as compared to the controls with increasing plasma DEHP residues. Our study found phthalate exposure was not significantly associated with ALT, AST, GGT, and ALP. There could be several reasons for these differences. First, it may be because our study only included participants aged from 12 to 19 years old, and the other two studies were based on adults. Previous studies on animal reported that the liver toxicity of phthalates was related to dose and time-dependent (8). Second, we used urine creatinine to adjust the concentrations of phthalate metabolites. Although it is an acceptable urine dilution adjustment when measuring non-persistent chemicals, more precise methods for calculating biomarkers should be considered. Finally, we used single-point urine samples instead of 24-h urine samples to measure phthalate exposure, which may also increase the measurement error. Further studies are needed to replicate these findings.

Bilirubin usually increases with excess bilirubin production (such as hemolysis), hepatocyte injury (such as hepatitis, cirrhosis, and fatty liver), or obstructed bile drainage (such as bile duct stones, pancreatic cancer, and bile duct cancer) (18). Previous studies have reported that phthalate exposure is associated with cholestasis (26, 27). However, our study found that MCOP was negatively correlated with TBIL. This negative correlation may be related to the fact that phthalates are thought to be involved in inducing oxidative stress and inflammation, while TBIL is thought to have potent antioxidant properties (28).

Hepatocytes are the main site of protein synthesis. The decrease of serum albumin and total protein levels indicates the gradual decrease of normal hepatocytes and the poor function of hepatocyte protein synthesis (16). Our study found that ΣDEHP and MCOP were negatively correlated with ALB, as well as MCOP and TP. This finding is consistent with previous studies that showed exposure to phthalates can lead to hepatocyte apoptosis and accelerate liver damage (29–31). Our results also showed that MCPP was positively correlated with ALB. We lack the detailed knowledge to explain this positive correlation, additional studies will be required to clarify the mechanistic link between phthalate exposure and ALB.

The main strength of this study is that we included a representative sample of US adolescents and we used the data that had been consolidated for 10 years. To our knowledge, this is the first study that summarized the urine phthalate levels and seven liver function indicators in adolescents. The study provides more evidence for further studies to demonstrate a correlation between phthalate exposures with liver dysfunction.

Our study has several limitations. First, the NHANES data were cross-sectional, which did not allow us to make causal inferences. Therefore, all relationships are related and further prospective research should be done to overcome this methodological limitation. Regardless, this study provides important information regarding how phthalate levels change in association with subclinical changes in liver function indicators in the US adolescents which have not been previously reported. Second, because we had no information about the subjects' alcohol consumption and smoking, we did not control for these underlying variables and only adjusted for covariates such as age, BMI, and sex. Finally, we measured phthalate exposure using a single-spot urine sample from each subject, possibly without taking into account changes in the human body over time. This may prevent us from obtaining a more precise exposure assessment to reduce exposure misclassification.

## CONCLUSIONS

Phthalate metabolites were significantly but weakly associated with changes in liver function indicators among US adolescents. Future work should further examine these relationships.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.cdc.gov/nchs/nhanes/index.htm.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by http://www.cdc.gov/nchs/nhanes/irba98.htm. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

## AUTHOR CONTRIBUTIONS

STX contributed to designing this article, performing the statistical analyses, and drafting the manuscript. CL provided the statistical analyses. XL and JD provided critical revision of the manuscript. All authors read and gave final approval of the version to be published.

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## SUPPLEMENTARY MATERIAL

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# Serum Calprotectin Is a Valid Biomarker in Distinction of Bacterial Urinary Tract Infection From Viral Respiratory Illness in Children Under 3 Years of Age

*Mirta Lamot[1]*, Marijana Miler[2], Nora Nikolac Gabaj[2,3], Lovro Lamot[4,5†], Milan Milošević[6], Miroslav Harjaček[4,5†] and Slaven Abdović[7]*

[1] Division of Neonatology, Department of Gynecology and Obstetrics, Sestre Milosrdnice University Hospital Center, Zagreb, Croatia, [2] University Department of Chemistry, Sestre Milosrdnice University Hospital Center, Zagreb, Croatia, [3] Faculty of Pharmacy and Biochemistry, University of Zagreb, Zagreb, Croatia, [4] Department of Pediatrics, Sestre Milosrdnice University Hospital Center, Zagreb, Croatia, [5] Department of Pediatrics, University of Zagreb School of Medicine, Zagreb, Croatia, [6] Andrija Štampar School of Public Health, University of Zagreb School of Medicine, Zagreb, Croatia, [7] Division of Pediatric Nephrology, Department of Pediatrics, Children's Hospital Zagreb, Zagreb, Croatia

**Background:** Febrile illnesses in young children can be a major diagnostic challenge, despite the routine use of various laboratory markers. Recent advancements in the understanding of inflammatory processes have highlighted the role of calprotectin, a heterodimer consisting of S100A8 and S100A9 proteins, with many studies suggesting its clinical value as a biomarker of inflammation. This research aimed to evaluate the usefulness of serum calprotectin (sCal) as a biomarker of urinary tract infection (UTI), which was due to its high pooled prevalence and feasibility of urine culture as a diagnostic reference standard selected for a model of bacterial infection in children.

**Methods:** Febrile children aged 0–36 months with suspected UTI based on positive urinalysis or viral respiratory tract infection were included. Children with significant bacteriuria in urine culture were labeled as cases ($n = 58$), while those with confirmed viral infection ($n = 51$), as well as those with suspected UTI but sterile urine culture who went on to develop symptoms consistent with viral respiratory infection ($n = 7$), were labeled as controls. sCal levels were determined by a commercial immunoassay. Conventional inflammation markers (C-reactive protein, procalcitonin, white blood cell count, absolute neutrophil count, and neutrophil percentage) were measured on the day of the clinical examination. Differences in measured inflammatory markers between cases and controls were analyzed with Mann-Whitney *U*-test. ROC analysis reported cut-off values with the best sensitivity and specificity to distinguish bacterial UTI from viral respiratory infection.

**Results:** All analyzed inflammatory biomarkers, including sCal, were significantly higher in cases than in controls. Median concentration of sCal was 4.97 μg/mL (IQR 3.43–6.42) and 2.45 μg/mL (IQR 1.63–3.85) for cases and controls, respectively ($p < 0.001$). For identifying bacterial UTI, sensitivity and specificity of sCal were 77.6 and 69.0%, respectively, at an adjusted cut-off point of >3.24 μg/mL (AUC 80.2%).

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*Kristin Skogstrand, Statens Serum Institute, Denmark*

***Correspondence:**
*Mirta Lamot*
*mirta.lamot@gmail.com*

**†Present addresses:**
*Lovro Lamot, Division of Pediatric Nephrology, Dialysis and Transplantation, Department of Pediatrics, University Hospital Centre Zagreb, Zagreb, Croatia*
*Miroslav Harjaček, Department of Pediatrics, College of Medicine and Health Sciences, United Arab Emirates University, Al Ain, United Arab Emirates*

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**Conclusion:** sCal could have substantial added value in the management of a child with fever and positive urinalysis and is a promising biomarker in distinction between bacterial UTI and viral respiratory causes of febrile illness in children under the age of 3 years.

**Keywords: calprotectin, urinary tract infection, bacterial infection, biomarker, pediatrics, respiratory viral diseases**

## INTRODUCTION

A number of recent studies have shown a significant interest in comprehending the clinical diagnostic potential of monitoring calprotectin concentrations in blood and body fluids (1). Due to its ubiquity during infection and/or inflammation and stability at room temperature, it comes as no surprise that calprotectin has been by now isolated from feces, urine, saliva, cerebrospinal fluid, meconium, synovia, and serum/plasma (1, 2). Fecal or blood-based calprotectin seems to be an effective diagnostic and follow-up biomarker in inflammatory bowel disease (IBD), rheumatoid arthritis (RA), spondyloarthritis, juvenile idiopathic arthritis (JIA), ANCA associated vasculitis, systemic lupus erythematosus (SLE), and Kawasaki disease, where it is used as a predictor of disease relapse, response to treatment, and structural damage (3–6). Additionally, it has been shown that plasma calprotectin levels might be used to distinguish the bacterial from viral infection (7). In adults, calprotectin exhibited a great sensitivity and specificity in the discrimination between bacterial pneumonia and viral respiratory infections, while in preterm and term infants with culture proven or high probable sepsis, its sensitivity, as well as positive and negative predictive values, were higher than those reported for conventional infection markers (8–10).

Despite the routine use of various clinical and laboratory markers, febrile illnesses in children younger than 3 years of age can be a major diagnostic challenge for physicians caring for children (11). Although self-limiting viral respiratory infections are the principal cause of fever in this group, a considerable portion of children will develop a bacterial urinary tract infection (UTI) that requires antibiotic treatment. The most commonly used inflammatory markers such as white blood cell count (WBC), C-reactive protein (CRP), and procalcitonin (PCT) aid in identifying children at risk for bacterial infection, though their sensitivity and predictive ability are limited. Generally, both CRP and PCT perform better than WBC, while in the youngest children with serious bacterial infection PCT outperforms CRP in the very first hours from fever onset, but with much higher cost (11).

Calprotectin (referred to by various authors as L1, 27E10 antigen, CFA, MRP8/14, calgranulin A/B, and S100A8/S100A9) is a calcium binding protein named after its protective, antimicrobial properties (12, 13). It is expressed primarily in neutrophils and to a much lesser extent in monocytes, and its production is induced by the pro-inflammatory cytokines TNFα and IL1β *via* transcriptional factor C/EBPα (14, 15). The pathways which induce calprotectin expression and secretion during bacterial infection start with bacterial lipopolysaccharides (LPS) binding to a toll-like receptor 4 (TLR4) on phagocytes (16). On the other hand, calprotectin is designated as a damage associated molecular pattern protein (DAMP) which acts by binding to two pattern-recognition receptors, TLR4 and RAGE (receptor for advanced glycation end products), on innate immune cells, releasing numerous inflammatory mediators, including calprotectin. Hence, calprotectin acts in paracrine and autocrine manner to amplify acute immune response, and has a potential to indicate inflammation (16, 17).

To the best of our knowledge, no study has investigated the possible role of serum calprotectin (sCal) in differentiating viral from the bacterial cause of febrile illnesses in infants and children. This research aimed to evaluate the usefulness of sCal as a biomarker of bacterial UTI in children younger than 3 years of age, hypothesizing that sCal is significantly higher in children with proven bacterial UTI than in children with respiratory viral disease.

## MATERIALS AND METHODS

### Participants and Procedures

This was a prospective study performed between October 2018 and February 2020 (before the first confirmed case of SARS-CoV-2 infection in Croatia) at the Department of Pediatrics in Sestre milosrdnice University Hospital Center, Zagreb, Croatia. The study involved children aged 0–36 months who were brought to Emergency Department due to a fever $\geq$38°C lasting $<$72 h and admitted to the inpatient or outpatient ward for further follow-up and treatment of suspected UTI (based on urinalysis) or respiratory tract infection. None of the participants had a history of chronic illness or ongoing antibiotic use at the time of visit.

Patients who turned out to have a UTI were regarded as cases, while those with proven viral respiratory infection, as well as those with sterile urine culture who went on to develop symptoms suggestive of viral respiratory infection, were regarded as controls. All of the participants had other source of bacterial infection excluded by negative pharyngeal swab, normal otoscopy finding, sterile blood culture, and/or negative chest radiography (if performed). The urine was collected as per the institution's protocol, with a sterile plastic bag attached to the perineum after thorough cleaning. Diagnosis of UTI was made with both positive urinalysis (leukocyte esterase greater than a trace amount and/or any positive nitrite detected by dipstick test) and significant bacteriuria in urine culture [100,000 colony forming units (CFU) of a single urinary tract pathogen per milliliter] (18–20). The diagnosis of viral respiratory infection was confirmed with rapid influenza or respiratory syncytial virus (RSV) immunoassays.

Every participant of the study underwent a medical history taking and clinical examination. Conventional inflammatory markers, such as CRP, PCT, WBC, absolute neutrophil count

**TABLE 1 |** Microbiological isolates and their distribution in study participants.

| Participants | Diagnosis | Microbial etiology | No. of subjects |
|---|---|---|---|
| Bacterial infection ($N = 58$) | UTI | *Escherichia coli* | 55 |
| | | *Klebsiella pneumoniae* | 2 |
| | | *Enterobacter* spp. | 1 |
| Controls ($N = 58$) | Confirmed respiratory viral infection | RSV | 9 |
| | | Influenza A or B | 42 |
| | Symptomatic respiratory viral infection with sterile pyuria | Unknown | 7 |

*UTI, urinary tract infection; RSV, respiratory syncytial virus.*

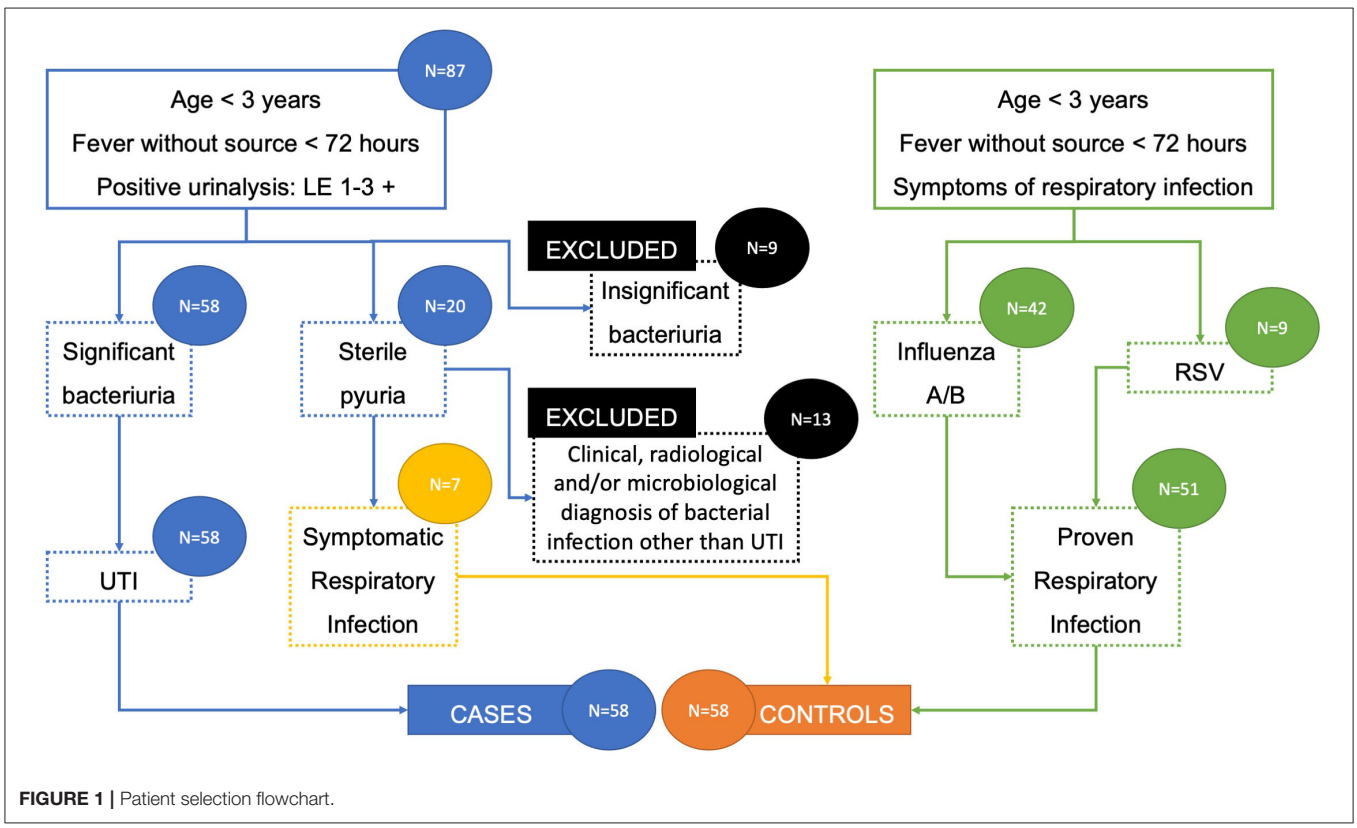


**FIGURE 1 |** Patient selection flowchart.

(ANC), and neutrophil percentage (N%), were measured on the day of the clinical examination with routine standards of the hospital laboratory.

WBCs were evaluated using Sysmex XN-1000 hematology analyzer (Sysmex, Kobe, Japan), CRP was measured by immunoturbidimetry method on Architect c8000, and PCT was determined by chemiluminescent microparticle immunoassay on Architect i2000sr, both Abbott, Abbott Park, IL, USA. The serum was frozen and preserved at −20°C until 69 samples were collected and analyzed for calprotectin at the same time. Two cycles were performed, which makes a total of 138 tested serum samples. SCal levels were determined using a commercial Quantum Blue® sCal quantitative lateral flow assay on Quantum Blue reader (BÜHLMANN Laboratories AG, Schönenbuch, Switzerland). Test required 20 μL of serum, samples were diluted 1:10, and results were available after 20 min. Sensitivity of the test is <0.5 μg/mL.

## Ethical Considerations

All included patients provided a written informed consent to participation signed by their parent/guardian. The study protocol was approved by the institutional ethics committee and all procedures performed were following the ethical standards of the institutional ethics committee and the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

## Statistical Analysis

Data distribution was checked with the Smirnov-Kolmogorov test. Where the assumption of normality was not upheld, the non-parametric tests were used. Continuous variables were expressed as the median and interquartile range (IQR). Data were analyzed

**TABLE 2 |** Differences in variables of interest between cases with bacterial infection and respiratory viral controls.

| | Bacterial UTI cases (*N* = 58*) | Respiratory viral controls (*N* = 58*) | *P*-value |
|---|---|---|---|
| Age (months) | 3.75 (2.0–6.13) | 14.0 (5.0–21.25) | <0.001 |
| Duration of fever (hours) | 12.0 (5.75–24.5) | 24.0 (12.0–48.0) | 0.006 |
| sCal (μg/mL) | 4.97 (3.43–6.42) | 2.45 (1.63–3.85) | <0.001 |
| CRP (mg/L) | 41.8 (21.1–104.38) | 6.4 (1.68–16.28) | <0.001 |
| PCT (ng/mL) | 0.31 (0.11–2.05) | 0.09 (0.06–0.2) | <0.001 |
| WBC ($10^9$/L) | 17.95 (13.08–22.73) | 7.8 (5.40–11.7) | <0.001 |
| ANC ($10^9$/L) | 8.99 (6.35–12.01) | 3.42 (2.02–5.19) | <0.001 |
| N% | 52.45 (45.28–61.43) | 45.55 (33.73–55.9) | 0.001 |

**Number of participants with determined PCT levels were 45 and 36 for patients with bacterial infection and controls, respectively.*
*sCal, serum calprotectin; CRP, C-reactive protein; PCT, procalcitonin; WBC, white blood cell count; ANC, absolute neutrophil count; N%, neutrophil percentage.*
*For continuous variables, medians and interquartile ranges are shown.*
*For two-group comparison, significance was defined as $P \leq 0.05$.*

**TABLE 3 |** Correlation between sCal values and variables of interest.

| | $r_s$ | *N* | *P*-value |
|---|---|---|---|
| Age (months) | 0.416 | 58 | 0.001 |
| Duration of fever (hours) | 0.231 | 58 | 0.081 |
| CRP (mg/L) | 0.446 | 58 | <0.001 |
| PCT (ng/mL) | 0.263 | 45 | 0.081 |
| WBC ($10^9$/L) | 0.491 | 58 | <0.001 |
| ANC ($10^9$/L) | 0.611 | 58 | <0.001 |
| N% | 0.601 | 58 | <0.001 |

*CRP, C-reactive protein; PCT, procalcitonin; WBC, white blood cell count; ANC, absolute neutrophil count; N%, neutrophil percentage.*
*Strong positive correlation was defined as $r_s$ (Spearman correlation coefficient) >0.5 and $P \leq 0.05$.*

for statistically significant differences using Mann-Whitney U test or Fisher exact test. The Kruskal Wallis test was used for determining whether the medians of sCal in 2 or more age sub-groups differ significantly, after which a Dwass-Steel-Critchlow-Fligner *post-hoc* test was performed to find out which of these groups differ from each other. Spearman correlation coefficient ($r_s$) tested the significance of the correlation between sCal and values of standard inflammatory biomarkers. ROC analysis reported cut-off values with the best sensitivity and specificity to distinguish bacterial from viral infection. Univariate and multivariate binary logistic regression analysis were made for prediction of bacterial infection among febrile children. Statistical analysis was performed using SPSS for Windows (version 25; IBM Corporation, Chicago, IL, USA). ROC curve analysis was performed using MedCalc (version 19.1.7; MedCalc Software Ltd, Ostend, Belgium). Two-sided tests were used, with the level of statistical significance set at 0.05.

# RESULTS

## Patient Selection and Demographics

A total of 138 patients were included in the study: 87 with suspected UTI and 51 with proven respiratory viral infection. Out of the patients with suspected UTI, 9 with insignificant bacteriuria (<100,000 CFU/mL) were immediately excluded, while 20 with sterile pyuria (no identified urinary tract pathogen) went for further assessment. Among these patients with sterile pyuria, 13 had clinical, radiological, and/or microbiological diagnosis of bacterial infection other than UTI and were excluded, while 7 with no such diagnosis of bacterial infection who further developed symptoms suggestive of viral respiratory infection were included in the control group. Therefore, the final number of participants was 116, divided into UTI cases group and respiratory viral infection control group, each consisting of 58 participants (**Table 1**). There were no significant differences in gender distribution between cases and controls, with 30 (51.7%) and 29 boys (50%), respectively. **Figure 1** outlines the patient selection for the study enrollment.

## Inflammatory Markers Levels

A statistically significant difference was found between participants and controls for all measured inflammatory biomarkers, including sCal. There was a strong positive correlation in cases between sCal and neutrophil count and percentage ($r_s = 0.611$ and $r_s = 0.601$, respectively, $P < 0.001$) and moderate between sCal and WBC and CRP ($r_s = 0.491$ and $r_s = 0.446$, respectively, $P < 0.001$). **Table 2** shows differences in variables of interest between participants with bacterial infection and controls. There was no significant relationship between sCal and PCT ($P = 0.081$) in patients with proven bacterial infection (**Table 3**).

## Performance of Serum Calprotectin in Bacterial Infection

ROC analysis (**Figure 2**) showed that sCal > 3.24 μg/mL has the best sensitivity (77.6%) and specificity (69.0%) according to the Youden index ($J = 0.46$; AUC = 80.2%, 95% confidence interval, 0.717–0.870). With the cut-off value set, a new binary variable (>3.24 or ≤3.24) was analyzed with the binary regression model. A significant prediction of bacterial infection was found for the value of sCal > 3.24 μg/mL with odds ratio 7.69 (95%CI: 3.35–17.65; $P < 0.001$). **Table 4** summarizes AUC results and, at optimal cut-off value, sensitivity and specificity of all

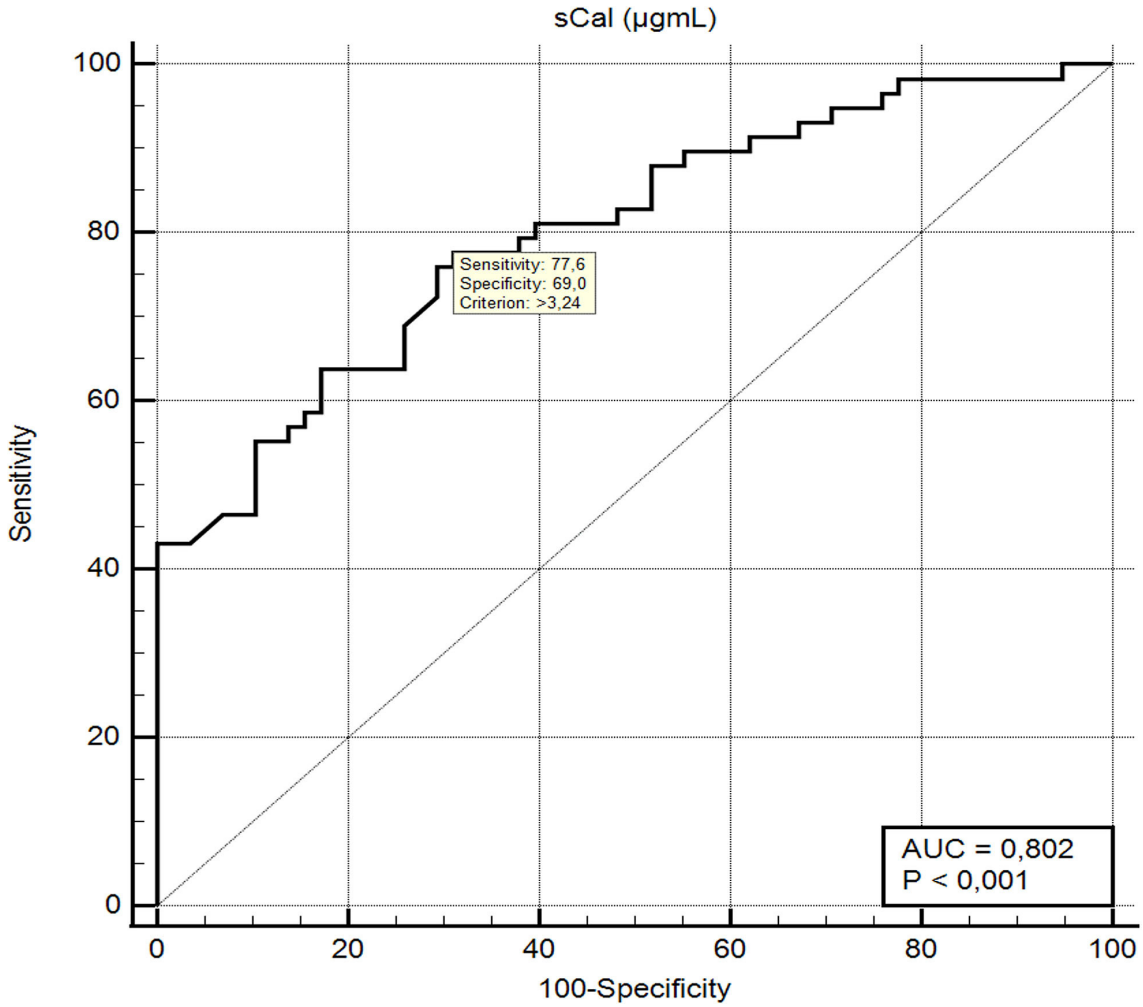


**FIGURE 2 |** ROC curve and diagnostic performance of serum calprotectin in differentiation between bacterial UTI and respiratory viral infections.

tested blood-based inflammatory biomarkers for the distinction between diagnosis of bacterial UTI and viral infection.

## Serum Calprotectin Analysis Across Age Sub-groups of Participants With Bacterial Infection

Children with UTI were divided into 4 age groups: neonates (under 28 days of age), young infants (1–3 months), infants (4–12 months), and toddlers (13–36 months) (19, 21, 22). As shown in **Tables 5**, **6**, sCal values in patients with bacterial infection were statistically significantly lower in neonates than infants ($W = 4.140$, $P = 0.018$).

## Binary Logistic Regression Analysis for Predicting Bacterial Infection Among Febrile Children

The results of univariate binary logistic regression analysis for association of all measured biomarkers (based on their AUC) with UTI in febrile children under 3 years of age are presented in hierarchical order in **Table 7**. WBC was the best predictor of UTI with $R^2$ 0.32, while $R^2$ of sCal and CRP were 0.24 and 0.21, respectively. In multivariate binary logistic regression analysis, when sCal was combined with WBC, its predictive value for UTI improved ($R^2 = 0.37$, AUC 0.876). The model with CRP added to WBC and sCal was only slightly better ($R^2 = 0.40$, AUC 0.882).

# DISCUSSION

The present study is the first to report the efficacy of sCal as a biomarker for the differentiation between bacterial UTI and viral respiratory infection in febrile children younger than 3 years of age. Moreover, it explored the performance of sCal relative to other inflammatory markers in children with bacterial UTI. A febrile bacterial UTI and viral respiratory infection were used as a model of bacterial and viral infection in children, respectively, due to its high pooled prevalence and feasibility of urine culture and nasopharyngeal aspirates as a diagnostic reference standard

**TABLE 4 |** Performance of all analyzed inflammatory biomarkers.

| Inflammatory marker | Cases/controls | AUC | Optimal cut-point | Sensitivity | Specificity | Accuracy | Youden index |
|---|---|---|---|---|---|---|---|
| sCal | 58/58 | 0.802 | 3.24 μg/mL | 77.6 | 79.0 | 73.28 | 0.4655 |
| CRP | 58/58 | 0.8176 | 19.7 mg/L | 75.86 | 77.59 | 76.72 | 0.5345 |
| PCT | 45/36 | 0.758 | 0.28 ng/mL | 53.33 | 86.11 | 67.9 | 0.3944 |
| WBC | 58/58 | 0.8585 | 12.1 × $10^9$/L | 84.48 | 77.59 | 81.03 | 0.6207 |
| ANC | 58/58 | 0.8433 | 6.18 × $10^9$L | 79.3 | 84.48 | 81.9 | 0.6379 |
| N% | 58/58 | 0.6828 | 48.35% | 63.79 | 67.24 | 65.52 | 0.3103 |

*sCal, serum calprotectin; CRP, C-reactive protein; PCT, procalcitonin; WBC, white blood cell count; ANC, absolute neutrophil count; N%, neutrophil percentage.*

**TABLE 5 |** sCal median values across age sub-groups of participants with UTI.

| Age group | *N* | Median sCAL (IQR) |
|---|---|---|
| Neonates | 13 | 2.40 (1.65–5.10) |
| Young Infants | 16 | 5.20 (3.63–5.93) |
| Infants | 24 | 5.61 (3.84–8.70) |
| Toddlers | 5 | 6.40 (4.19–8.76) |

*Significant difference in median values of sCal between two or more age sub-groups was detected with Kruskal-Wallis non-parametric test ($X^2 = 11.1$, $P = 0.001$).*
*sCAL, serum calprotectin.*
*Neonates: under 28 days of age; Young infants: 1–3 months; Infants: 4–12 months; Toddlers: 13–36 months.*

**TABLE 6 |** Pairwise comparisons of sCAL median values between age sub-groups of participants with UTI using the Dwass-Steel-Critchlow-Flinger (DSCF) *post-hoc* test.

| Pairwise comparison | | *W* | *P* |
|---|---|---|---|
| Neonates | Young Infants | 3.071 | 0.131 |
| **Neonates** | **Infants** | **4.140** | **0.018** |
| Neonates | Toddlers | 3.137 | 0.118 |
| Young Infants | Infants | 1.445 | 0.737 |
| Young Infants | Toddlers | 2.103 | 0.446 |
| Infants | Toddlers | 0.653 | 0.967 |

*DSCF post-hoc test showed significant difference ($P \leq 0.05$) in median values of sCal between neonates and infants (bold).*
*W, Wilcoxon rank sum test statistic.*
*Neonates: under 28 days of age; Young infants: 1–3 months; Infants: 4–12 months; Toddlers: 13–36 months.*

(23–25). Besides, clinical manifestation of febrile UTI in this age group often lack any signs and symptoms typical for children after 5 years of age, making UTI in a younger age group somewhat of a diagnostic dilemma (26).

According to AAP (American Academy of Pediatrics) and NICE (National Institute for Health and Care Excellence) guidelines, urinalysis is an important part of managing infants and young children presenting with unexplained fever of 38°C or higher (19, 20). Urine dipstick test is the recommended screening method for UTI in febrile children from 3 months to 3 years of age, and in age group 2 to 24 months has diagnostic sensitivity of 67–94% and specificity of 64–92% (19, 20). On top of that, readily available serum markers CRP and PCT are often used to prognosticate pyelonephritis in children with UTI. While CRP has been studied with conflicting results, more studies confirmed that PCT is a valuable indicator of acute renal involvement and the best predictor of permanent renal scarring in children under 2 years of age with first febrile UTI (27). Nevertheless, there is no compelling evidence to recommend the routine use of any of these tests in clinical practice (28). Moreover, as mentioned above, the reference standard for diagnosis of UTI is urine culture, which is time consuming and frequently leads to engagement of antibiotic treatment while pending for results, often not in line with the rules of rational pharmacotherapy (29). Finally, despite the increased availability of laboratory testing and development of clinical scores reachable online, such as UTIcalc, there is still no entirely accurate early predictor of UTI in children (30). Hence, establishing timely and reliable diagnosis of UTI in children presents an unmet clinical need.

The main presented finding in our study was that children with proven bacterial urinary tract infection had significantly higher sCal concentration than children with viral respiratory tract infection. This result is supported by Havelka et al. who recently reported that in adults with acute respiratory infections, plasma calprotectin levels were significantly higher in patients with bacterial pneumonia, mycoplasma pneumonia, and streptococcal tonsillitis compared to plasma calprotectin levels in patients with proven viral infections (10). Based on results of our multivariate binary logistic regression analysis, sCal has the best predictive value for UTI when combined with WBC ($R^2 = 0.37$), while CRP has little added value ($R^2 = 0.40$) in that combination. Moreover, the strongest correlation was observed between sCal values and neutrophils (absolute count and percentage), which can be explained by the fact that calprotectin is almost exclusively restricted to neutrophils and by now is already known as a marker for neutrophil mediated inflammation (2, 10, 15, 31).

In addition, sCal values correlated with CRP moderately, and there was no correlation with PCT, which reflects different aspects of the body's response to infection. Moreover, PCT in our study showed noticeably lower AUC for recognizing bacterial UTI than sCal (0.758 vs. 0.802), but this can be attributed to the selection bias, since PCT was routinely analyzed in only 62% of controls and 77.6% of cases (who all were probably more ill-appearing than the others) compared with 100% of study participants with defined sCal values. On the other hand, our results showed that other routinely measured inflammatory biomarkers (CRP, PCT, WBC, ANC, and N%) were significantly higher in cases of bacterial UTI than in controls with respiratory

**TABLE 7 |** The results (in hierarchical order) of univariate binary logistic regression analysis for association of all measured biomarkers (based on their AUC values) with UTI in febrile children under 3 years of age.

| Predictor | OR | 95% CI | $R^2$ (%) | $R^2$ adj (%) | AUC | *P* |
|---|---|---|---|---|---|---|
| WBC | 1.2898 | 1.18–1.41 | 32.26 | 31.64 | 0.8585 | ≤0.001 |
| ANC | 1.4334 | 1.25–1.64 | 28.60 | 27.98 | 0.8433 | ≤0.001 |
| sCal | 1.9494 | 1.5–2.55 | 24.2 | 23.58 | 0.8017 | ≤0.001 |
| CRP | 1.0362 | 1.02–1.05 | 21.33 | 20.71 | 0.8176 | ≤0.001 |
| PCT | 1.9988 | 0.93–4.29 | 12.64 | 11.74 | 0.7580 | 0.076 |
| N% | 1.0522 | 1.02–1.08 | 8.45 | 7.83 | 0.6828 | 0.001 |

*sCal, serum calprotectin; CRP, C-reactive protein; PCT, procalcitonin; WBC, white blood cell count; ANC, absolute neutrophil count; N%, neutrophil percentage.*

viral infection, with similar observed high performance AUC for identifying bacterial UTI.

We analyzed sCal values in four different physiologically meaningful age sub-groups of children under 3 years of age with bacterial UTI and recognized that neonates with UTI have lower sCal concentrations than infants with UTI. Unfortunately, the number of cases in each sub-group was too small to make relevant conclusions based on our findings. Nevertheless, this has raised an important point of how age influences sCal kinetics. Since currently the number of reliable studies on the matter is limited, a new study investigating reference interval of sCal in various age groups of children, especially neonates, is warranted.

Interestingly, Terrin et al. reported that in very low birth weight newborns with suspected sepsis, the diagnostic accuracy of sCal was greater (at cut-off value of 1.7 μg/mL sensitivity was 89% and specificity 96%) than the performance of CRP, WBC, and ANC (8). A few years later, Decembrino et al. published similar results in term infants with the same diagnosis: at cut off value of 2.2 μg/mL sCal was identified to distinguish between infants with and without sepsis with sensitivity and specificity of 62.5 and 69.7%, respectively, whereas CRP for a cut-off of 6.0 mg/L showed a sensitivity of 50% and specificity of 66.7% (9). The provided explanation was that CRP concentration increases rather slowly in the initial phase of inflammatory response to pathogens and many peripartum factors influence its kinetics. Considering there is no need for *de novo* synthesis and its rapid release from neutrophils, sCAL could be an earlier marker of bacterial infection, although this should be confirmed by a new study as well (32).

In our study, the participants were selected keenly and divided into two well-defined homogeneous groups according to the reference standard for the diagnosis of bacterial UTI or viral respiratory infection, which is the main strength of the study. Besides, comparison to other inflammatory markers was performed, providing data on the performance of sCal relative to the routinely used biomarkers.

The most important limitation of the study is a small number of participants from a single center. Thus, caution should be exercised, especially when interpreting findings of subgroups with small sample size. Additionally, instead of commonly recommended invasive urethral catheterization or bladder puncture, non-invasive bag technique was performed, thus the positive urine culture/significant bacteriuria was defined as presence of $\geq 10^5$ CFU of single urinary tract pathogen per milliliter of urine, while patients with lower bacterial counts or multiple pathogens were excluded from the study (18, 19). Nevertheless, this non-invasive collection technique could possibly influence the results of the study. Furthermore, the design of the study with only one, initial laboratory analysis on the first day of admission is insufficient for evaluating kinetics of inflammatory biomarkers, which makes the hypothesis that sCal as a biomarker of bacterial infection reaches its maximum level in blood before CRP and other routinely used inflammatory biomarkers only speculative. Also, there were some significant differences in age and duration of fever among participants with bacterial UTI and viral respiratory infection, with the former being of younger age and shorter duration of fever (**Table 2**). Considering the above-mentioned observation that neonates have lower sCAL concentration than infants, as well as weak correlation of sCAL with age (**Table 3**), the differences in sCAL among these two groups might be even higher, as probably are the differences in clinical appearance, prompting caregivers of children with UTI to visit an emergency department earlier and hence the lower duration of fever. Finally, 7 patients who initially presented as possible UTI due to the positive urinalysis, developed symptoms suggestive of respiratory viral infection while waiting for the results of urine culture, which came back negative. This situation is not uncommon in everyday clinical practice and therefore those participants were regarded as symptomatic respiratory viral infection with sterile pyuria and included in controls group. Nevertheless, similar sCAL concentrations were observed among these and other patients with proven viral respiratory infection and no significant differences were noticed if they are excluded from the analysis (data not shown).

In conclusion, compared to febrile patients with respiratory viral infection, sCal was significantly elevated in patients with bacterial UTI. Although it is clear that the diagnosis of UTI cannot be based on serum biomarkers and that urine culture remains the gold standard for diagnosis, our results suggest that sCal could have substantial added value in the early management of a child with fever and positive urinalysis and serve as an accurate biomarker in distinction between bacterial UTI and respiratory viral causes of febrile illness in children under 3 years of age.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Zagreb School of Medicine Institutional Ethics Committee and Sestre Milosrdnice University Hospital Institutional Ethics Committee. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

## AUTHOR CONTRIBUTIONS

ML, LL, SA, and MH contributed to the study conception and design. Material preparation and data collection were performed by ML and LL. Formal analysis were performed by MMile, NN, MMilo, and SA. The resources were managed by MMile, NN, and MH. The first draft of the manuscript was written by ML and all authors commented on previous versions of the manuscript. SA was a supervisor. All authors read and approved the final manuscript.

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# *HAAO* rs3816183 Polymorphisms [T] Increase Anterior/Middle Hypospadias Risk in Southern Han Chinese Population

*Yanqing Liu[1†], Wen Fu[2†], Kai Fu[2], Xiaoyu Zuo[3], Wei Jia[2], Ning Wang[3], Yan Zhang[3], Guochang Liu[2] and Fuming Deng[2*]*

[1] Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangzhou, China, [2] Department of Urology, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangzhou, China, [3] Guangdong Provincial Key Laboratory of Research in Structural Birth Defect Disease, Department of Pediatric Surgery, Guangzhou Women and Children's Medical Center, Guangzhou Institute of Pediatrics, Guangzhou Medical University, Guangzhou, China

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***Correspondence:**
*Fuming Deng*
*fm_deng@126.com*

*[†]These authors have contributed equally to this work*

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Hypospadias is one of the most common congenital external genital malformations, which is characterized by abnormal urethral meatus. However, the etiology remains to be incompletely understood. *HAAO* is a gene that encodes a protein, which catalyzes the synthesis of quinolinic acid, and has been identified as a risk gene for hypospadias. Thus, this study was conducted to elaborate the association between *HAAO* gene polymorphism rs3816183 T>C and hypospadias in the largest hypospadias cohort from Asia, including 577 patients and 654 healthy controls in China. The strength of interrelation was evaluated using 95% confidence intervals (CIs) and odds ratios (ORs). Based on the stratified analysis of hypospadias subtypes, it was found that the *HAAO* risk allele rs386183[T] enhances the susceptibility for hypospadias among patients with anterior/middle hypospadias subtypes (adjusted OR = 1.31, 95% CI = 1.05–1.64, $p = 0.017$). Enhanced risk of hypospadias in the entirety could not be demonstrated (OR = 1.20, 95% CI = 1.00–1.47, $p = 0.054$). In summary, our study found that the rs3816183[T] polymorphism is associated with increased risk of anterior/middle hypospadias among Southern Han Chinese children. The mechanisms by which the variations in the *HAAO* gene require further research.

**Keywords: hypospadias, *HAAO*, single-nucleotide polymorphism (SNP), genetics, urethral abnormalities**

## BACKGROUND

Hypospadias is one of the most common congenital external genital malformations, which is characterized by abnormal urethral meatus (1), and affects approximately 20.9 out of every 10,000 births and has shown significant increases worldwide (2). Over the past decade, an increasing trend in the prevalence of hypospadias has been observed in China (3, 4). The clinical characteristics of hypospadias include proximal urethral opening, ventrally deficient hooded prepuce, and chordee (5). Hypospadias can be classified into two subgroups based on the urethral meatus location: anterior/middle hypospadias and posterior hypospadias (6). The meatus localization is best evaluated during surgery when chordee is corrected.

Although the surgical approach to hypospadias treatment has a great progress over the past decades, its etiology remains incompletely understood (1, 7–9). Individual phenotypic differences, such as disease susceptibility, survival, and treatment response, were identified to be associated with different genetic variants (10). Genetic variants have been observed to be associated with hypospadias risk (11, 12). However, very few studies have focused on variants in potential genes, such as *DGKK, MAMLD1, MID1, CYP1A1, GSTM1*, and *GSTT1,* which are associated with susceptibility to hypospadias (9). Some single-nucleotide polymorphisms (SNPs) have been reported in association to hypospadias. Nevertheless, recent studies used small sample sizes and have not been consistently replicated (13, 14).

Geller et al. conducted a genome-wide association study (GWAS) and reported that 17 SNPs were independently associated with hypospadias (15). Yoshiyuki validated these 17 SNPs in a Japanese cohort. However, only *HAAO* rs3816183 T>C was significantly associated with an increased risk toward hypospadias (16). Considering that ethnic differences exist at some loci, it would prove meaningful to evaluate the effect of SNPs on hypospadias susceptibility in different ethnic groups. Thus, we conducted this study to validate the association of *HAAO* rs3816183 T>C polymorphism with hypospadias susceptibility.

# MATERIALS AND METHODS

## Study Population

We recruited 557 isolated hypospadias patients at the Guangzhou Women and Children's Medical Center from January 2016 to December 2019, all of whom were Han Chinese, and the diagnosis was confirmed by pediatric urologists before surgery repair. Hypospadias classification was performed by experienced pediatric urologists at our center. The meatus localization is best evaluated during surgery when chordee is corrected. Based on the urethral orifice, the patients were divided into two groups: patients with anterior/middle hypospadias were defined as having a urethral opening in glanular, subcoronal, distal penile, and midshaft penile areas, while patients with posterior hypospadias were identified as having the urethral opening in penoscrotal, scrotal, and perineal areas. The control group included 654 male children without a medical history of hypospadias, who were selected from the Guangzhou Women and Children's Medical Center. Since hypospadias can be inherited, all the patients and controls group with a first-degree relative who suffers from hypospadias were excluded.

Informed consent was obtained from all patients' parents or legal guardians. This study was approved by the Ethics Committee of Guangzhou Women and Children's Medical Center in China.

## DNA Extraction and Genotyping

Genomic DNA was extracted from venous blood samples using TIANamp Blood DNA kits (Catalog No. DP335-02; TIANGEN Biotech Co. Ltd., Beijing, China) following the manufacturer's instructions (17). NanoPhotometer® N50 (Implen GmbH., Munich, Germany) was used to assess DNA purity and concentration. Genomic DNA was amplified using the ABI-7900 real-time quantitative PCR instrument (Applied Biosystems, Foster City, CA, USA) and was subjected to *HAAO* rs3816183 TaqMan genotyping (18). PCR reactions were run as described in the previous study (19) using TaqMan® SNP Genotyping Assays (Catalog No: 4351379,C_180222_20, Thermo Fisher, USA) and TIANexact genotyping qPCR PreMix (Probe) (Catalog No. FP211-02; TIANGEN Biotech Co. Ltd., Beijing, China). In addition, 10% of DNA samples were selected randomly for second genotyping. The accuracy of data was ensured by the replicated samples with 100% consistency (19).

## Statistical Analysis

SAS 9.4 software (SAS Institute Inc., Cary, NC, USA) and GraphPad Prism version 8 (GraphPad Software, Inc., La Jolla, California, USA) were used to perform statistical analyses. Hardy–Weinberg equilibrium (HWE) test was performed in the control group using a goodness-of-fit chi-squared test. SNPs were analyzed for association with hypospadias susceptibility by comparing the risk of allele frequency (allelic test) in patients and controls, along with other tests using PLINK 1.9 (20). Association was stratified by subgroup through comparing controls with cases with a certain subgroup. A *p*-value of 0.05 was considered statistically significant (21).

**TABLE 1 |** Association between HAAO rs3816183 T>C polymorphism and hypospadias susceptibility.

| Genotype | Cases ($n = 557$) | Controls ($n = 654$) | Crude OR (95% CI) | *p* | Adjusted OR (95% CI)1 | $p^a$ |
|---|---|---|---|---|---|---|
| CC | 288 | 376 | 1.0 | | | |
| TC | 204 | 232 | 1.09 (0.85–1.39) | 0.52 | 1.09 (0.87–1.39) | 0.52 |
| TT | 42 | 35 | 1.57 (1.12–2.19) | **0.008** | 1.57 (1.12–2.19) | **0.008** |
| Genotypic | | | | 0.13 | | 0.11 |
| Dominant (TT+TC vs. CC) | 246/288 | 267/376 | 1.20 (0.95–1.52) | 0.12 | 1.19 (0.94–1.52) | 0.15 |
| Recessive (TT vs. CC+TC) | 42/492 | 35/608 | 1.48 (0.93–2.36) | 0.10 | 1.59 (0.99–2.57) | 0.06 |

*Values are shown as numbers. Significant p values (<0.05) are in bold. CC, homozygous protective; TC, heterozygous; TT, homozygous risk for rs3816183; OR (95% CI), odds ratio and confidence interval. [a]Adjusted for age.*

## RESULTS

### Association Between *HAAO* rs3816183 Polymorphism and Hypospadias Susceptibility

In the present study, 534 of 557 patients and 634 of 654 controls could be successfully genotyped. The frequencies of controls and patients group genotypes are shown in **Table 1**. The frequency distribution of the rs3816183[T] genotype in the control groups was consistent with HWE ($p = 0.64$). The HAAO rs3816183 TT phenotype was associated with an increased risk of hypospadias (TT vs. CC: OR = 1.57, 95% CI = 1.12–2.19, $p = 0.008$). Nevertheless, the results showed that the *HAAO* rs3816183[T] polymorphism may not be associated with hypospadias susceptibility in dominant and recessive models (adjusted OR = 1.19, $p = 0.15$/adjusted OR = 1.59, $p = 0.06$).

### Stratification Analysis of *HAAO* Gene Polymorphism With Hypospadias Susceptibility

Hypospadias can be divided into different subtypes based on the urethral meatus location after penile degloving. The *HAAO* risk allele rs3816183[T] was associated with an increased susceptibility toward anterior/middle hypospadias (OR = 1.35, 95% CI = 1.08–1.68, $p < 0.01$). Nevertheless, no significant association was found between the *HAAO* risk allele rs3816183 T

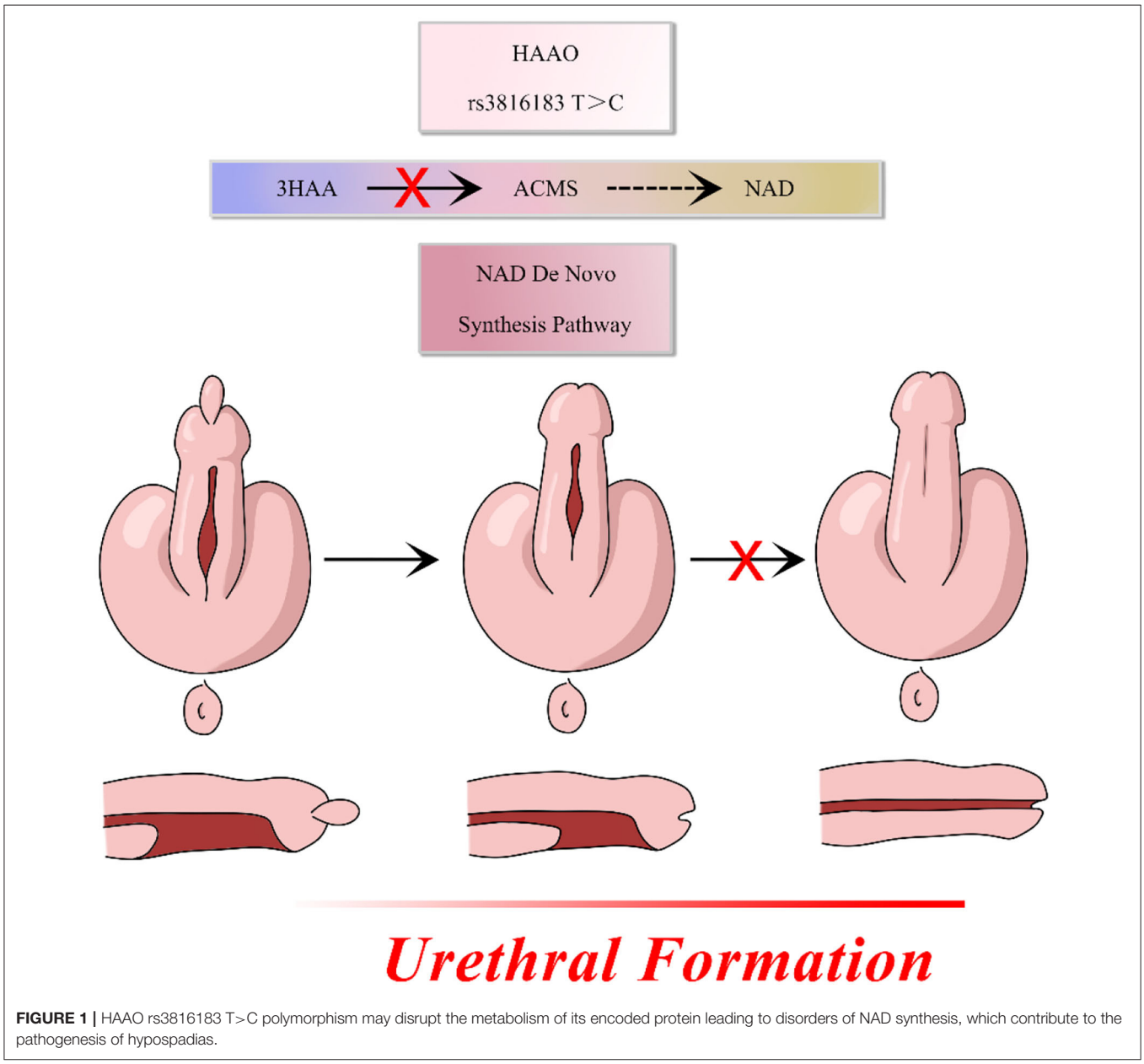


**FIGURE 1 |** HAAO rs3816183 T>C polymorphism may disrupt the metabolism of its encoded protein leading to disorders of NAD synthesis, which contribute to the pathogenesis of hypospadias.

**TABLE 2 |** Stratification analysis to evaluate the association between *HAAO* rs3816183 T>C polymorphism and hypospadias susceptibility (by subgroup).

| rs3816183 | A1 | AF of cases | | AF of controls | Cases vs. controls | | Posterior vs. controls | | Anterior/middle vs. controls | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Posterior | Anterior/middle | | *p* | *OR (CI95)* | *p* | *OR (CI95)* | *p* | OR (CI95) |
| | T | 0.24 | 0.29 | 0.23 | 0.054 | 1.20 (1.00–1.47) | 0.81 | 1.03 (0.80–1.32) | **0.017** | 1.31 (1.05–1.64) |

*A1, effect allele; AF, allele frequency of effect allele. Significant p values (<0.05) are in bold.*

and patients with posterior hypospadias (OR = 1.03, 95% CI = 0.80–1.32, $p = 0.81$).

## DISCUSSION

Hypospadias is a complex, congenital, external genitalia malformation. Genetic factors are important causative reason in the development of hypospadias (11, 12). Kojima et al. replicated rs3816183 of *HAAO* polymorphism with hypospadias and found that rs3816183 [T] was significantly increased the hypospadias susceptibility toward both posterior and anterior/middle hypospadias (16). However, the *HAAO* rs3816183 polymorphism was only significantly associated with an increased susceptibility toward anterior/middle hypospadias susceptibility in the present study. Therefore, our study demonstrated that *HAAO* rs3816183 polymorphism is not equally associated with hypospadias risk in different populations.

The *HAAO* gene, which is widely distributed in various organs (22–24), encodes a protein that catalyzes the synthesis of quinolinic acid (QUIN) from 3-hydroxyanthranilic acid. Huang et al. showed that hypermethylation of the HAAO gene predicts disease-free survival in patients with endometrioid endometrial cancer (25). Martin et al. reported that hypercholesterolemia and atherosclerosis may be treated and prevented by targeting the *HAAO* gene (26). Previous studies have demonstrated that the *HAAO* gene is associated with cancer biomarkers and degenerative diseases. The relationship between the *HAAO* gene and developmental disorders has also been reported. *HAAO* has also been correlated with congenital malformations and miscarriage and, when combined with environmental factors, may impair embryo outcomes (27). Pathogenesis of hypospadias has been attributed to the incomplete fusion of the urethra in a portion of the penis and the expression of *HAAO* in male mouse genital tubercle. Moreover, genetic variants of *HAAO* may specifically impede the migration and proliferation of normal urethral cells. We hypothesized that the *HAAO* rs3816183 T>C polymorphism may disrupt the metabolism of its encoded protein leading to disorders of NAD synthesis, which contribute to the pathogenesis of hypospadias (**Figure 1**). Similar genetic studies have suggested that rs3816183[T] *HAAO* polymorphisms may result in increased hypospadias susceptibility (16). However, in our study, the association between rs3816183 T>C *HAAO* polymorphism and hypospadias susceptibility was observed in anterior/middle group but not in posterior hypospadias patients. This discrepancy could be attributed to the sample size and ethnic differences in patients. In addition, causes of hypospadias may be genetic, maternal, environmental, or a combination of all of these factors. Posterior hypospadias have been reported to be associated with maternal factors, such as oligohydramnios, premature birth, and hypertension, suggesting that the underlying placental insufficiency may be an important contributing factor (28). Environmental factors, such as phthalates, have been associated with a toxic effect on the male reproductive system and the development of hypospadias (29). The fact that there may be many complex causes for hypospadias and that the environmental and maternal factors were not accounted for in our study could be the reason that the *HAAO* rs3816183 variants was found to be associated only with anterior/middle hypospadias.

This is the largest Asian case–control study to investigate the association of *HAAO* polymorphism rs3816183 T>C with hypospadias susceptibility. Our results demonstrated that the SNPs rs3816183[T] in *HAAO* may be associated with increased anterior/middle hypospadias but not posterior hypospadias (**Table 2**), suggesting that *HAAO* may influence distal part of penile urethral formation.

However, there were some limitations to this study. First, environmental factors, such as difference in diet and geographic locations, were not analyzed. Second, in-depth exploration of *HAAO* rs3816183T>C and hypospadias sensitivity mechanisms is required. This may have potential implications for hypospadias prevention. Finally, multiple center studies are warranted to confirm our findings.

## CONCLUSION

The *HAAO* rs3816183[T] is associated with increased risk to anterior/middle hypospadias in Southern Han Chinese population. Our findings support the hypothesis that the mechanism underlying the variations in the *HAAO* gene may contribute to the pathogenesis of hypospadias and thus requires in-depth research.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Standards of the Institutional Review Board of Guangzhou Women and Children's Medical Center (NO. 39401). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

## AUTHOR CONTRIBUTIONS

FD designed experiment. YL, WF, KF, XZ, WJ, NW, GL, and FD collected samples and conducted the study. YZ and XZ analyzed the data. YL and FD wrote the paper. All authors have read and approved the manuscript.

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BRIEF RESEARCH REPORT
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# Relationship Between Vitamin D Level and Platelet Parameters in Children With Viral Respiratory Infections

*Gavriela Feketea*[1,2,3], *Vasiliki Vlacha*[3,4], *Raluca Maria Pop*[5], *Ioana Corina Bocsan*[5*], *Luminita Aurelia Stanciu*[6], *Anca Dana Buzoianu*[5] *and Mihnea Zdrenghea*[1,7]

[1] *Department of Haematology, "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania,* [2] *Department of Paediatrics, Amaliada Hospital, Amaliada, Greece,* [3] *Department of Paediatrics, Karamandaneio Children's Hospital, Patras, Greece,* [4] *Department of Early Years Learning and Care, University of Ioannina, Ioannina, Greece,* [5] *Department of Pharmacology, Toxicology and Clinical Pharmacology, "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania,* [6] *National Heart and Lung Institute, Imperial College London, London, United Kingdom,* [7] *Department of Hematology, "Ion Chiricuta" Oncology Institute, Cluj-Napoca, Romania*

Apart from their classical roles, both platelets and vitamin D play important roles in inflammation and infectious diseases. This study evaluated the platelet response to viral respiratory tract infection in children aged 4–16 years, 32 with influenza, 27 with non-influenza viral infection tested by nasopharyngeal swab and 21 healthy children of the same age. Blood count, including platelet count (PLT), mean platelet volume (MPV) and other platelet indices, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and vitamin D (vit D) levels were compared. The influenza group showed lower PLT and platelet mass (PLT*MPV), and the non-influenza group showed significantly lower MPV, which was correlated with the vit D levels, but not CRP or ESR, and the value vit D*MPV was significantly lower in this group. These results revealed that platelet activation in viral respiratory tract infections in children, as measured by MPV, is related to the vit D level, with differences between influenza and non-influenza infection.

**Conclusions:** Viral respiratory tract infection in children can diminish the platelet size most likely by suppressing the platelet activation. This response is associated with low levels of vit D. Whether the vit D status is associated with the virus-platelet immune/inflammatory process needs further investigation.

**Keywords: vitamin D, platelets, MPV, influenza, respiratory infections, children**

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***Correspondence:**
*Ioana Corina Bocsan*
*bocsan.corina@umfcluj.ro*

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## INTRODUCTION

Acute respiratory tract infections (RTIs) are the most common infections worldwide (1). In a study conducted in previously healthy children by Taylor et al., the most common cause of RTIs was respiratory viruses, mainly rhinoviruses (42.2%), followed by influenza virus (15.8%) (2). The clinical symptoms involve the upper and lower respiratory tract, ranging from mild to severe, with a diagnosis including bronchiolitis, bronchitis, pneumonia, etc.

The incidence of RTIs related to influenza virus is higher among children aged <3 years, reaching 179/1,000 (3). One study analyzing hospitalization in children contracting influenza,

reported the rate to be higher among infants under 6 months, with 15% of them needing admission to a pediatric intensive care unit (PICU) (4).

The complete blood count (CBC) is the most frequently used laboratory test during an infection process (5). The changes in the white blood cell (WBC) in response to infection are most commonly analyzed (6), but the platelet response to infection is diverse, with regard to both platelet count (PLT) and other platelet parameters, notably the mean platelet volume (MPV).

The primary function of platelets is haemostatic, but recently, their role in inflammation and immunogenicity has also been evaluated. They show the ability to recruit leukocytes and release proinflammatory and anti-inflammatory factors (7). Several disorders are known to be associated with platelet activation, including acute lung injury (8), inflammatory bowel disease (IBD) (9), rheumatoid arthritis (RA) and sepsis (10). The role of platelets in viral infections has also been documented (11). Specifically, platelet interaction has been described with adenovirus (12), dengue (13, 14), hepatitis C (15), and Epstein-Barr virus (EBV) (16).

The main role of vitamin D (vit D) is in bone homeostasis, but in recent years its role in infections (17), inflammation and immune response (18) has been documented. Vit D acts as a stimulant of innate defense (19), and as an immunomodulator in adaptive immunity. Low levels of vit D are associated with a variety of viral infectious diseases (20, 21). In patients with HIV-1 with low levels of vit D, a decrease in inflammation was observed when vit D supplementation restored the levels to normal (22). Vit D deficiency increases the susceptibility to enveloped viral infections, including respiratory syncytial virus (RSV) (23). With respect to influenza, vit D has been shown to enhance the immunogenicity of influenza vaccine in elderly subjects (24, 25), although other studies failed to confirm this finding (26).

In this study we investigated the platelet changes during influenza and non-influenza RTIs in children, and the possible role of vit D in the process.

## MATERIALS AND METHODS

The study population consisted of Caucasian children, aged 4–16 years presenting with symptoms of RTI in the emergency department (ED) of a regional hospital in Greece during a 6-month period (September 2019–February 2020). The exclusion criteria were comorbidities, need for hospital admission, vit D supplement during the preceding 3 months, a diagnosis of bacterial infection, and administration of medication other than antipyretics. A control group was selected from children of the same age attending the pediatric outpatient clinic of the same hospital for a well-child visit, during the same time period. Similar exclusion criteria were applied for the control subjects. Children with even minor infections on the day of examination were excluded. The study was approved by the hospital ethics committee. Written, informed consent was provided by parents'care givers of all the children in the study.

Testing for Influenza A and B by a nasopharyngeal swab was performed on each child on arrival in the ED. The demographic characteristics of each child, and the duration and height of fever and the presence of cough were documented. A sample of venous blood was drawn for analysis of (CBC), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and the level of vit D. The CBC was performed in a Unicel DxH 600 Coulter Cellular Analysis System by Beckman Coulter. The blood samples for CBC were obtained by ethylenediaminetetraacetic acid (EDTA) and were analyzed within 1 h after collection in order to prevent platelet swelling.

The influenza viruses were identified by a rapid immunochromatographic test for the qualitative detection of influenza antigens in nasopharyngeal swab. Vit D (25(OH)D) was measured by ELISA method, using 25OH Vitamin D Total ELISA Kit (DIAsource Immuno Assays, Louvain-la-Neuve—Belgium). All the determinations were done according to the manufacturers' instructions.

### Statistical Analysis

SPSS 25 was used for statistical analyses. Normal distribution for continuous variables was assessed with the Kolmogorov–Smirnov normality test. Continuous variables were expressed as mean ± standard deviation (SD) or median (Percentile 25–75), and they were compared using an unpaired Student's *t*-test or the non-parametric Mann-Whitney test. Categorical variables were presented as counts and percentages, which were compared using $\chi^2$-statistics or Fisher's exact test. Statistical significance was defined as $P < 0.05$.

## RESULTS

A total of 80 children were included in the study. The patients with RTI were divided into 2 groups: those diagnosed with influenza (32 children), and those ones with a negative influenza test (27 children). The healthy control group comprised 21 children.

### Demographic Characteristics and Clinical Symptoms

The demographic characteristics and the symptoms of the study children are shown in **Table 1**. The age distribution of the patients was similar in all groups. Females were predominant in the group of children testing positive for influenza (53.1%), while the male patients predominated in the non-influenza and control groups (66.7%, respectively, 52.4%). With regard to the clinical symptoms, the duration of fever was similar in the influenza and non-influenza groups, but the maximum body temperature was significantly higher in the influenza group (39.79 ± 0.316 vs. 39.13 ± 0.52, $p < 0.01$), although both groups recorded a maximum temperature above 39°C. The duration of fever was lower in children with influenza compared to non-influenza group, but the difference was not statistically significant. The presence of cough was similar in both groups, around two thirds.

**TABLE 1 |** Demographic and clinical characteristics of children attending the emergency department with respiratory symptoms and healthy control subjects.

| | Influenza group $N = 32$ | Non influenza $N = 27$ | Control subjects $N = 21$ | Statistics* |
|---|---|---|---|---|
| **Gender no (%)** | | | | |
| Male | 15 (46.9%) | 18 (66.7%) | 11 (52.4%) | |
| Female | 17 (53.1%) | 9 (33.3%) | 10 (47.6%) | |
| Age (Median, percentiles, 95% CI) | 11 (5–16)<br>95% CI (9.78–12.02) | 10 (5–16)<br>95% CI (9.35–12.02) | 10 (8–14)<br>95% CI (9.68–11.21) | $P^{1,2} = 0.98$<br>$P^{1,3} = 0.522$<br>$P^{2,3} = 0.578$ |
| Days of fever<br>(Mean ± SD, 95% CI) | 1.25 ± 0.567<br>95% CI (1.04–1.45) | 1.59 ± 0.888<br>95% CI (1.24–1.94) | | 0.078 |
| Fever max, (°C)<br>(Mean ± SD, 95% CI, 95% CI) | 39.79 (±0.316)<br>95% CI (39.67–39.90) | 39.13 (±0.52)<br>95% CI (38.92–39.33) | | <0.001 |
| Presence of cough,<br>number of patients (%) | 20 (62.5%) | 21 (77.8%) | | 0.262 |

*$P^{1,3}$: influenza vs. controls, $P^{2,3}$: non-influenza vs. controls, $P^{1,2}$ influenza vs. non-influenza.

## Laboratory Findings

The laboratory values are reported in **Table 2**. The WBC and the hemoglobin level (Hb) were similar in the three groups. The ESR and CRP showed no difference between the groups of children with RTI (influenza vs. non-influenza).

The platelet count (PLT) showed significant differences between groups. The children diagnosed with influenza had lower PLT ($233 \times 10^3$/ml vs. $306 \times 10^3$/ml), although thrombocytopenia with PLT $<150 \times 10^3$ cells/ml was observed in only one patient, in the non-influenza group. The platelet indices were examined further among the groups. **Figure 1** shows the graphic representation of mean PLT, mean platelet volume (MPV) and platelet distribution width (PDW).

The MPV was significantly higher in the influenza group than in the non-influenza group (8.43 vs. 7.89, $p = 0.005$) (**Figure 1B**). The PDW showed no statistical difference between the groups (**Figure 1C**). The sensitivity and specificity of the MPV using a cut-off value of 8 fL was 62.96% [95%CI (42.4–80.6)] and 78.12% [95%CI (42.4–80.6)], respectively, in predicting influenza RTI. The ROC curve of MPV is shown in **Figure 2**.

The platelet mass (MPV*PLT) showed significant differences between the groups, being greatest in the control group and least in the influenza group: influenza vs. control [2,020.55 (1,394–3,053) vs. 2,360 (1,710–3,948), $p = 0.018$], and influenza vs. non-influenza [2,020.55 (1,394–3,053) vs. 2,088 (1,341.9–3,766.8), $p = 0.048$].

With regard to vit D levels, the non-influenza group showed significantly lower levels of vit D compared to the control subjects [21.49 (15.47–36.2) vs. 26 (16.25–79.6), $p = 0.013$]. The median level of vit D was slightly lower in the influenza group than in control group [23.97 (13.88–52.69) vs. 26 (16.25–79.6)], but the difference did not reach the level of statistical significance ($p = 0.108$). Additionally, several complex parameters resulting from the multiplication of the vit D level with MPV, PLT and PDW, respectively, are lower in the non-influence than in the control group and this difference is statistically significant.

# DISCUSSION

The aim of this study was to evaluate the platelet response to viral RTI in children by identifying the differences in PLT and platelet indices among children suffering from influenza and other viral RTI, compared with healthy control subjects, and exploring the impact of vit D on platelet parameters. Moreover, we intended to evaluate the role of platelet indices, specifically of MPV as a potential factor to differentiate influenza by other viral RTI. The early etiological diagnosis of RTI may be difficult in certain location. The PLT indices can offer useful information and they are already determined and available through complete blood count (CBC).

None of the children with influenza developed thrombocytopenia, while only one from the non-influenza group had PLT $123 \times 10^3$ cells/ml. Vit D deficiency, with vit D levels < 20 mg/dl, was observed in 7/32 children in the influenza group, 7/27 in the non-influenza group and 3/21 in the control group. None of the children had very low levels of vit D of < 10 mg/dl.

The two groups with RTI had similar clinical characteristics, regarding the duration of the illness, measured by the days of fever, although those in the influenza group developed higher fever, and the presence of cough. The inflammation indices CRP and ESR were similar in the two groups with RTI, and higher in both than in the control group.

The groups with RTI showed similar PLT, but the PLT in the influenza group was significantly lower than in the control group. Also, in this study, we found higher MPV in children with influenza RTI than in those with other RTI, suggesting that the PLTs are activated. MPV acted as a positive acute phase reactant which reacted differently comparing with other acute phase markers (WBC, CRP, ERS) that are not different in the two groups ($p = 0.7$, 0.96, 0.98, respectively). A higher MPV occurs as a result of increased platelet activity and thus of more intense inflammation as a result of a certain infection (27).

The mechanism of low PLT can be explained by studying further the platelet parameters, MPV and PDW. The MPV in the

**TABLE 2 |** The laboratory values of children attending the emergency department with respiratory symptoms and healthy control subjects.

| Laboratory values (median and range) | Influenza group $N = 32$ | Non-influenza group $N = 27$ | Control subjects $N = 21$ | Statistics** |
|---|---|---|---|---|
| ESR mm/h | 17 (5–55)<br>95% CI (14.47–23.77) | 15 (3–85)<br>95% CI (12.94–26.10) | 12 (3–35)<br>95% CI (9.12–16.50) | $P^{1,3} = 0.044$<br>$P^{2,3} = 0.115$<br>$P^{1,2} = 0.98$ |
| CRP (mg/dl) | 0.8 (0.4–1.4)<br>95% CI (0.74–1.5) | 0.4 (0.2–1.1)<br>95% CI (0.47–1.6) | 0.15 (0.07–0.4)<br>95% CI (0.13–0.4) | $P^{1,3} < 0.001$<br>$P^{2,3} = 0.01$<br>$P^{1,2} = 0.964$ |
| VitD (mg/dl) | 23.97 (13.88–52.69)<br>95% CI (21.85–28.52) | 21.49 (15.47–36.2)<br>95% CI (20.91–24.91) | 26 (16.25–79.6)<br>95% CI (23.95–36.77) | $P^{1,3} = 0.108$<br>$P^{2,3} = 0.013$<br>$P^{1,2} = 0.48$ |
| WBC cell/ml | 5,650 (4,750–7,250)<br>95% CI (5,197.48–6,865.02) | 5,600 (4,150–7,100)<br>95% CI (5,058.84–7,474.49) | 6,600 (5,450–8,150)<br>95% CI (6,296.06–8,380.13) | $P^{1,3} = 0.07$<br>$P^{2,3} = 0.250$<br>$P^{1,2} = 0.701$ |
| PLT $\times 10^3$/ml | 233 (161–367)<br>95% CI (226.37–265.51) | 258 (129–438)<br>95% CI (238.70–296.11) | 306 (174–430)<br>95% CI (268.67–338.95) | $P^{1,3} = 0.005$<br>$P^{2,3} = 0.1117$<br>$P^{1,2} = 0.290$ |
| PCT, % | 0.20 (0.14–0.30)<br>95% CI (0.19–0.22) | 0.21 (0.13–0.37)<br>95% CI (0.19–0.23) | 0.23 (0.17–0.39)<br>95% CI (0.22–0.28) | $P^{1,3} = 0.011$<br>$P^{2,3} = 0.025$<br>$P^{1,2} = 0.951$ |
| MPV, fl | 8.4 (6.5–9.9)<br>95% CI (8.16–8.71) | 7.8 (6.5–10.5)<br>95% CI (7.55–8.22) | 8.4 (5.9–10.7)<br>95% CI (7.77–8.90) | $P^{1,3} = 0.610$<br>$P^{2,3} = 0.102$<br>$P^{1,2} = 0.005$ |
| PDW % | 16.65 (15.6–18.7)<br>95% CI (16.49–16.96) | 16.3 (15.8–17.7)<br>95% CI (16.29–16.69) | 16.3 (15.1–18)<br>95% CI (15.95–16.71) | $P^{1,3} = 0.063$<br>$P^{2,3} = 0.452$<br>$P^{1,2} = 0.112$ |
| MPV*PLT | 2,020.55 (1,394–3,053)<br>95% CI (0.03–0.04) | 2,088 (1,341.9–3,766.8)<br>95% CI (0.03–0.04) | 2,360 (1,710–3,948)<br>95% CI (0.03–0.04) | $P^{1,3} = 0.018$<br>$P^{2,3} = 0.377$<br>$P^{1,2} = 0.048$ |
| VitD*MPV | 199.46 (103.09–433.48)<br>95% CI (184.35–238.07) | 168.26 (117.63–380.08)<br>95% CI (161.07–200.72) | 242.48 (133.86–684.73)<br>95% CI (196.70–307.12) | $P^{1,3} = 0.102$<br>$P^{2,3} = 0.004$<br>$P^{1,2} = 0.048$ |
| VitD*PLT | 5,543.29 (3,011.96–16,544.66)<br>95% CI (5,222.05–7,285.27) | 6,128.76 (3,210.56–9,720.51)<br>95% CI (5,363.44–6,612.34) | 7,729.05 (3,453.9–22,373.22)<br>95% CI (7,124.52–11,535.08) | $P^{1,3} = 0.006$<br>$P^{2,3} = 0.003$<br>$P^{1,2} = 0.840$ |
| VitD*PDW | 398.21 (238.73–862.05)<br>95% CI (365.16–479.09) | 355.16 (252.20–640.70)<br>95% CI (343.56–412.96) | 433.98 (269.75–1,257.99)<br>95% CI (392.07–593.46) | $P^{1,3} = 0.102$<br>$P^{2,3} = 0.010$<br>$P^{1,2} = 0.315$ |

***$P^{1,3}$: influenza vs. control, $P^{2,3}$: non-influenza vs. control, $P^{1,2}$ influenza vs. non-influenza.*

*ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; PLT, platelet count; PCT, Plateletcrit; MPV, mean platelet volume; PDW, platelet distribution width; Vit D, Vitamin D. *denote the multiplication operation.*

influenza group showed no difference from the control group, while the PDW was higher, but not to a statistically significant degree. This means that in response to influenza, the platelets maintain their size, but have increased variability, compared to the control subjects. In the non-influenza RTI group, the PLT showed no difference, but the platelet size was significantly lower compared to the control group, but with stable PDW, i.e., no significant size variability.

To explain these findings, we must review the platelet response to inflammation/infection. The platelets are cytoplasmic fragments of megakaryocytes. The MPV has been extensively evaluated, both in healthy subjects and in several medical conditions (28). The regulation of platelet production and maturity is a result of the effects of several hormones and immunological factors on the megakaryocytes. The aim is to maintain the platelet mass stable by inverse changes PLT and MPV, with the final goal being to preserve an adequate haemostatic potential (29). Thrombopoietin, as the major regulator, is positively correlated with PLT but not with MPV (30). In several pathological conditions the platelets become activated (31). This process can be related to increased production of thrombopoietin in some cases, but not in others. Various inflammatory conditions causing an increase in thrombopoiesis, with an increase in the number and the size of platelets. The platelets migrate to the area of inflammation where they are consumed, leading to thrombocytopenia. Activation of platelets is associated with increased MPV (32), which has been observed in many inflammatory conditions, including RA and Mediterranean fever (28). However, the MPV may be decreased with activity of the disease in ankylosing spondylitis RA (33), and a drop in MPV has been seen in some cases of active inflammation (34). Karadag-Oncel et al. suggested that MPV

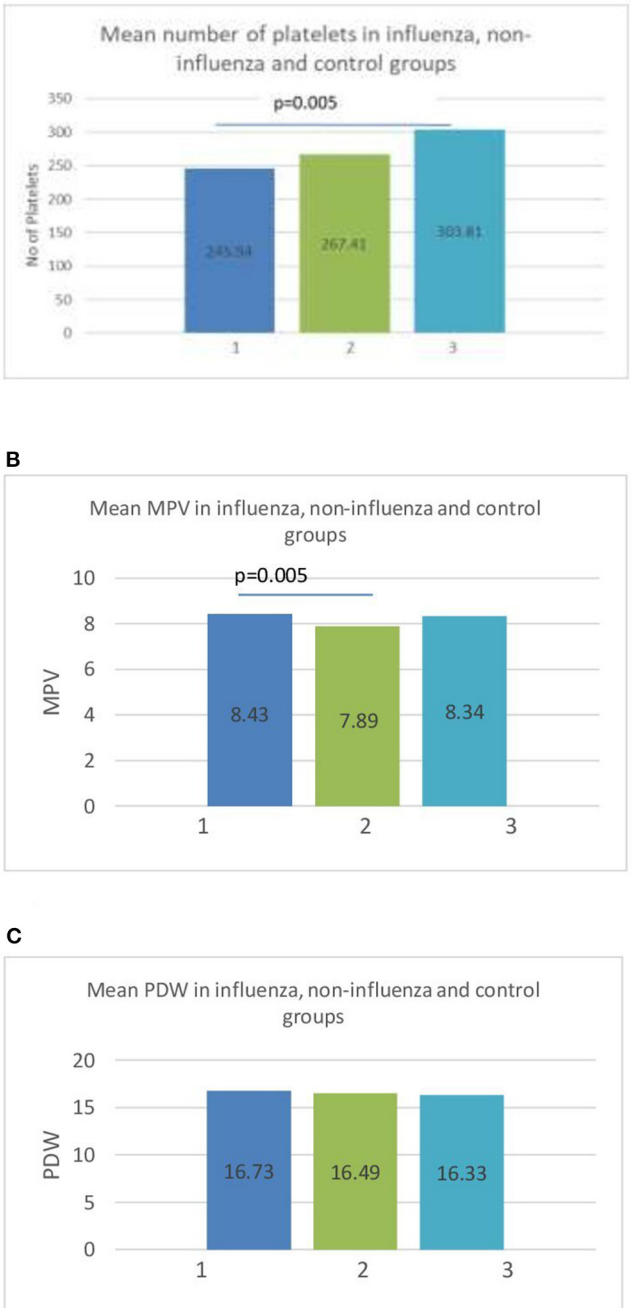


**FIGURE 1 | (A)** Mean number of platelets (PLT) in influenza (1), non-influenza (2), and control (3) groups. **(B)** Mean value of mean platelet volume (MPV) in influenza (1), non-influenza (2), and control (3) groups. **(C)** Mean platelet distribution width (PDW) in influenza (1), non-influenza (2) and control (3) groups.

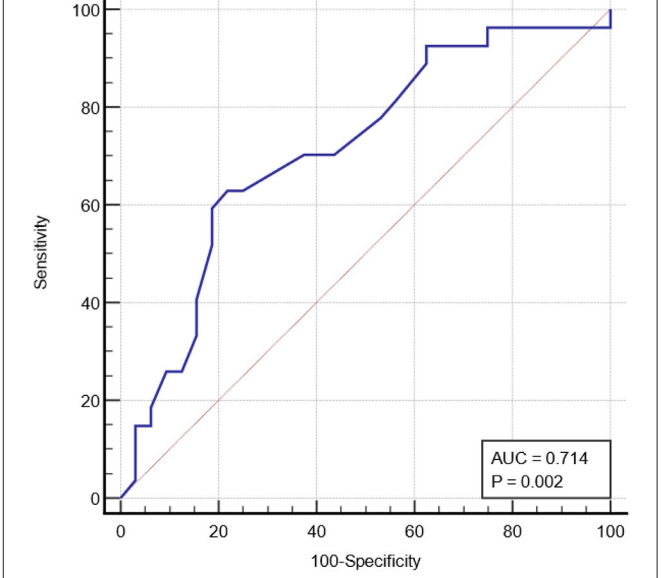


**FIGURE 2 |** Receiver operating characteristic curve ROC of mean platelet volume (MPV) for influenza respiratory tract infection.

may be a useful predictor for diagnosed community-acquired pneumonia, but not in disease severity, that is to the decision for hospitalization (35).

With regard to viral infections, several different mechanisms of PLT modulation have been proposed (36), and the final outcome is increase in platelet reactivity, and activation (37). Direct interaction of viral particles with platelets has been observed, and the viruses can interact not only with circulating platelets but also with megakaryocytes. In addition, the virus-antibody complex can also activate platelets. As a result of inflammation, the platelets can migrate to, and be consumed in, the infection sites.

The defense mechanism mediated by platelets is very complex, involving activation and recruitment of leukocytes. The platelet-leucocyte activation results in vascular inflammation. Finally, platelets are also implicated in the resolution of inflammation (38).

In the case of influenza, the platelets play an important role in the host immune defense, and the inflammation process, and live viruses have been identified inside the platelets (39). It has been suggested that the initial defense against influenza is mediated by platelet-neutrophil cross-communication (40). Platelets play a major role in influenza inflammation, as it has been shown in the lungs of influenza infected mice (41). The platelet activating factor receptor plays a role in recruiting neutrophils and is associated with the final the morbidity and mortality (42). In addition, in influenza infection, the platelets are recruited by the endothelial cells at the sites of inflammation, causing lung tissue damage (43, 44). Statistical differences in PLT have been reported between influenza and COVID-19 (45).

In the current study, the children with non-influenza RTI had low MPV, but the PLT remained stable, suggesting diminished platelet activation. The platelets are not activated and consequently they are not consumed at the sites of inflammation.

This may be a direct effect of the viral strains on the platelet response, or due to alterations in the innate host immunity.

With regard to the children with influenza, their mean PLT was lower than that of the control group, but with no differences in MPV. Their mean PDW was minimally elevated, but the difference did not reach statistical significance. These findings suggest platelet activation and consumption at the inflammatory sites. A dysregulation of platelet mass was observed, as PLT*MPV was lower than in the control subjects, due to decreased PLT.

The differences in platelet indices between the two groups with RTI, influenza and non-influenza, cannot be explained by the degree of inflammation as this was documented to be similar by the clinical symptoms and the levels of the inflammatory indices ESR and CRP. The only notable difference between the two groups was the vit D level, which was significantly lower in the non-influenza group compared to the control group.

The immunomodulatory effect of vit D has been described in several illnesses (18, 46, 47). In addition, the role of vit D has been demonstrated in respiratory diseases (48), and specifically in influenza (49). Regarding the association of PLT and other platelet parameters with vit D levels, several studies have reported the absence of any relationship under healthy conditions (50–52), but in several pathological processes the results are contradictory. Many reports have shown an inverse relationship between vit D levels and MPV. In female patients with chronic diseases, vit D levels <20 mg/dl an inverse linear relationship with MPV has been demonstrated (53). The same relationship was described at even lower vit D levels, <10 mg/dl (31, 54), in pregnant women with gestational diabetes mellitus (DM) (55) and in patients with fibromyalgia (56), although in the latter study no differences in MPV from the control subjects were noted with vit D levels 20–30 mg/dl. Our study showed that low vit D levels are associated with low MPV in children with non-influenza RTI. These results are in accordance with a study in patients with thyroid cancer, which showed low levels of MPV to be associated with lower vit D levels (57). It appears that an altered cause-effect relationship between vit D levels and MPV may develop under different circumstances. Silvagno et al. study's results suggest that the platelet activation might be modulated by a mitochondrial non-genomic activity of vit D receptor (VDR) (58). Lower vit D levels lead to a decrease in VDR expression and therefore to a decrease in PLT activation. Also, a possible mechanism could be that the lower vit D may prevent the innate inflammatory/immune response from affecting the platelet activation- destruction process. Furthermore, the deficient state of vit D in the event of viral infection may alter the virus-platelet or the inflammation-platelet interaction by affecting the metabolism of the platelets themselves. In health adults, Park et al. showed that PLT and MPV are inversely associated with vit D levels (53). Based on our findings, we consider that the interactive mechanism of vit D-platelet-inflammation/immune response is quite complex, and that the final outcome of platelet activation depends on other additional factors.

The low vit D levels could be a result of insufficient dietary intake of vit D compared to increased demand of vit D as a consequence of the infection in the non-influenza patients. It is of note that our series consists of patients who were brought to the ED with symptoms severe enough to alarm their parents. It is not clear whether the lower vit D levels led to an increase in symptom severity, resulting in the ED visit, or if the severity of the symptoms was due to the virulence of the microorganisms. In addition, the vit D status could be a cofounder to viral susceptibility.

The main strength of the paper is the demonstration of an influence of influenza virus on platelet reaction and the establishment of a direct relationship virus-platelet immune process. This study was limited to a specific age range, 4–16 years, and younger children and infants were not included. In addition, none of the children were diagnosed with severe vit D deficiency. The children were examined within the first 2 days of the beginning of illness. It would be interesting to evaluate the platelet parameters during the course of their disease. Further studies are needed to confirm our findings, and to extend the evaluation to the clinical outcome of those patients with low vit D levels and platelet inactivation.

The major limitation of this study was the small sample size of children. Our study was designed to be conducted over 2 flu seasons, 2019–2020 and 2020–2021. Unfortunately, the appearance of the pandemic, did not give us the opportunity to continue the study. Shortly after WHO declared the SARS-CoV-2 pandemic, the cases of influenza showed a sudden decrease, and the "flu season" ended earlier than usual. Next year, due to the introduction of the measures to limit SARS-CoV-2 transmission, a decrease in respiratory viral infections has been observed in children.

This study has identified alterations of platelet indices in patients with influenza and other respiratory viral illnesses in association with vit D levels. Measures of molecules secreted by activated platelets and platelets functional studies should be performed to verify the relationship of platelet parameters with the platelet activation.

## CONCLUSIONS

In conclusion, the present study showed that viral RTI in children can diminish the platelet size probably by suppressing the platelet activation. Also, we found that MPV acted as a positive acute phase reactant in children with influenza RTI, and MPV levels were significantly elevated in these children. This response is associated with low levels of vit D, which probably alters the virus-platelet-immune/inflammatory process.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Scientific Commission of General Hospital of

Amaliada, Greece. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

## AUTHOR CONTRIBUTIONS

GF, VV, IB, and MZ: conceptualization. GF, RP, IB, and MZ: methodology. GF, VV, and IB: analysis and writing—original draft preparation. GF, RP, and IB: investigation. GF, VV, IB, LS, and MZ: writing—review and editing. LS, AB, and MZ: supervision. GF and MZ: project administration. All authors read and agreed to the published version of the manuscript.

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# Predictor of Syncopal Recurrence in Children With Vasovagal Syncope Treated With Metoprolol

*Chunyan Tao[1,2†], Bowen Xu[1†], Ying Liao[1], Xueying Li[3], Hongfang Jin[1,4*] and Junbao Du[1,4*]*

[1] Department of Pediatrics, Peking University First Hospital, Beijing, China, [2] Department of Genetics and Endocrinology, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangzhou, China, [3] Department of Statistics, Peking University First Hospital, Beijing, China, [4] Key Laboratory of Molecular Cardiovascular Sciences, Ministry of China, Beijing, China

**Objective:** To explore the predictors for syncopal recurrence in a pediatric population with vasovagal syncope (VVS) treated with metoprolol.

**Study Design:** This study was conducted retrospectively among children suffering from VVS with or without syncopal recurrence. Data on the detailed medical history and auxiliary examinations were obtained from the electronic medical records. The risk factors for syncopal recurrence were studied by cox regression analyses and the corresponding best cutoff values were determined using receiver operating characteristic analysis. Kaplan–Meier curves were plotted to determine the trends of the syncopal recurrence-free survival rate.

**Results:** Forty-two consecutive VVS children were enrolled in the study. At the end of a median follow-up duration of 9.0 (4.8, 19.1) months, 12 patients (29%) experienced ≥1 syncopal episode. Cox regression analyses revealed that the number of previous syncopal episodes before treatment was a risk factor for syncopal recurrence (hazard ratio = 1.027, 95% confidence interval 1.009 – 1.045, $P = 0.003$). Moreover, 4 previous syncopal episodes were certified as the best cutoff value, and the Kaplan–Meier curves showed that the syncopal recurrence-free survival rate over time in patients with > 4 previous syncopal episodes was significantly lower than that in patients with ≤4 episodes ($P = 0.019$ at the log-rank test).

**Conclusion:** In a pediatric population with VVS while on the treatment of metoprolol, the number of previous syncopal episodes before treatment played a significant role in predicting syncopal recurrence.

**Keywords: vasovagal syncope, children, syncopal recurrence, metoprolol, predictor**

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*Cheng Wang, Central South University, China*

***Correspondence:**
*Hongfang Jin*
*jinhongfang51@126.com*
*Junbao Du*
*junbaodu1@126.com*

*† These authors have contributed equally to this work and share first authorship*

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## INTRODUCTION

Vasovagal syncope (VVS) is the most common subtype of syncope in the young. Furthermore, it is speculated that VVS constitutes more than 50% causes of syncopal events (1). Up to 15% of children are reported to experience at least one syncopal event before the end of adolescence, and the syncope recurrence rate is approximately 25–35% for a one-year period and 33–51% for a five-year period (2, 3). VVS is believed to be benign; however, recurrent episodes notably decrease the

quality of life and increase the risk of severe injuries (4–6). Metoprolol, a β-adrenoceptor blocker, is considered a reasonable therapy on the basis of the commonly recognized mechanism of excessive sympathetic activity (7, 8). However, based on our experience, treatment with metoprolol could not ensure no recurrence of syncope in a substantial number of VVS cases (9). Indeed, syncopal attack greatly restricts daily life activities such as school and physical activities in those patients. Therefore, identifying the predictors of the recurrence of syncope is necessary for the clinical management and prevention. Thus, this research was designed to identify the predictors of syncopal recurrence in VVS of children with or without syncopal recurrence while on the metoprolol treatment.

## STUDY SUBJECTS AND METHODS

### Study Subjects

This study authorized by the Ethics Committee of Peking University First Hospital was performed retrospectively. Patients younger than 18 years accompanied by their parents or other relatives visited the Pediatric Syncope Unit, Department of Pediatrics, Peking University First Hospital, China, from November 2011 to July 2019. For further diagnosis, they were assigned to be hospitalized. They were finally diagnosed with VVS with the exclusion of cardiogenic, neurologic or metabolic causes and psychogenic pseudo-syncope. The exact diagnostic criteria for VVS in childhood were previously described (10, 11). The detailed histories (medical history, family history and allergic history) and the results of auxiliary examinations [i.e., QT dispersion (QTd) and QTc dispersion (QTcd)] from an electrocardiogram and head-up tilt test (HUTT) results were obtained from the electronic medical records. We also analyzed the hemodynamic response data during the HUTT.

### Head-Up Tilt Test

Patients who refrained from any medication influencing autonomic nervous function for ≥5 half-life times and fasted for ≥4 h were assigned to complete the HUTT in the morning. The testing room was warm, dimly lit and quiet, and the necessary resuscitation equipment was available with the informed consent from the legal guardian/next of kin of the subjects. After emptying their bladders, the subjects were asked to lie quietly for 10–20 min on a tilt table (SHUT-100A, Standard, and ST-711, Juchi, China) which was controlled electronically. Then, they were passively tilted 60° for 45 min at most or the tilting process was discontinued once a positive response happened. The exact criteria for a positive response were that a subject was distressed by orthostatic intolerance symptoms like blurred vision, dizziness, sweating, headache or even syncope, accompanied by one of the following hemodynamic changes (12): (1) a significant hypotension, namely systolic blood pressure (BP) ≤80 mmHg, diastolic BP ≤50 mmHg or ≥25% decrease in mean BP; (2) an obvious bradycardia, namely heart rate (HR) <75 bpm, 65 bpm, and 60 bpm, respectively, in children at the age of 4–6 years, 6–8 years, and >8 years; (3) second or third-degree atrioventricular block and asystole for longer than 3 s; and (4) sinus arrest (12). On the basis of hemodynamic alterations during a positive response, the types of VVS were categorized as a vasodepressive pattern (an obvious decline in BP without notable reduction in HR), a cardioinhibitory pattern (a notable reduction in HR without obvious decline in BP), or a mixed pattern (a notable decrease in both BP and HR) (12). No medicine (e.g., isoproterenol or nitroglycerin) was administered during the test.

### QT Dispersion Measurement

QT dispersion, an index from electrocardiogram reflecting autonomic function, was defined as the maximum QT interval minus the minimum one and QTcd was defined as QTd corrected by HR using Bazett's equation (QTcd = QTd/$\sqrt{R-R}$, in which R-R means the RR interval in seconds) (13). Naturally, the QT interval meant the interval between the QRS complex onset and the end point of the T wave (14–16). The QTd and QTcd data were obtained from standard 12-lead resting electrocardiograms, which were recorded at 25 millimeters/second. The intervals of QT and RR were measured manually by one investigator using a caliper, and they were determined as the mean values of three consecutive beats in each lead. If the end point of the T wave was not identified reliably, the lead would be eliminated from the subsequent analysis.

### Treatment and Follow-Up

All enrolled patients received the regimen of metoprolol [0.5 mg/kg/d, for 3.0 (2.0, 4.0) months] and health education empirically once the diagnosis of VVS was established. From the ending of treatment, they were followed up for a median duration of 9.0 (4.8, 19.1) months by telephone or outpatient visits. The follow-up was performed by a professionally trained investigator and the recurrence of syncope or presyncope was recorded in the electronic medical records. The first recurrence of syncope was the endpoint of this study. Patients with recurrent syncopal episode(s) during follow-up were recorded as "patients with recurrence," otherwise recorded as "patients without recurrence." All the tasks were conducted by one professionally trained researcher.

### Statistical Analyses

SPSS, version 21.0 (IBM, Armonk, New York, United States), was applied for data analyses. The normality of continuous variables was examined with the Shapiro–Wilk test. The data with normal distribution are listed as the mean ± standard deviation, otherwise as the median (interquartile range) and they were compared using the Student's *t*-test or the Mann–Whitney *U*-test appropriately. Categorical variables are presented as numbers (percentages) and were compared using the chi-square test or the continuity correction. The data of patients with and without recurrent syncope were compared as described above. Univariate and multivariate Cox proportional hazards models were performed to assess the unadjusted and adjusted hazard ratios with 95% confidence intervals for some suspicious risk variables. The best cutoff values of continuous risk variables for syncopal recurrence were calculated by the receiver operating characteristic analysis. Kaplan–Meier curves for the cumulative rate free from recurrence were utilized to study the trends

of patients suffering recurrent events over time, and the log-rank test was applied to assess the trends correspondingly. Differences with two-tailed *P*-value <0.05 were supposed to be statistically significant.

# RESULTS

## Recurrent Syncopal Events During Follow-Up

A total of 46 pediatric patients with VVS while on the metoprolol treatment were initially recruited in this study. However, 4 of them were lost to follow-up. At admission, 687 syncopal episodes in total were reported in a median symptomatic duration of 14.5 (4.0, 39.0) months. The median frequency was 4.0 (1.3, 21.0) episodes/person/year (**Figure 1**). At the end of a median follow-up duration of 9.0 (4.8, 19.1) months, 12 (29%) of the 42 patients experienced at least one recurrent syncopal event, and the median interval from the first recurrent syncope was 2.5 (1.0, 4.0) months. The Kaplan–Meier curve in **Figure 2** presents the overall trend of syncopal recurrence in all included patients over time.

## Baseline Characteristics Between Patients With and Without Syncopal Recurrence

The baseline characteristics between patients with and without syncopal recurrence are listed in **Table 1**, and they were compared. Compared to patients without syncopal recurrence, those with recurrence had more previous syncopal episodes before treatment ($P$ = 0.013) and higher supine mean BP ($P$ = 0.016). No differences were found for the sex ratio, age at the first syncopal episode, age at the first visit of our department, duration of symptoms, triggers of syncope, prodromes, duration of unconsciousness, injury at syncope, history of allergy, family history of syncope, body mass index, other hemodynamics during the HUTT and the time to positive response, types of VVS, QTd, QTcd, treatment duration and follow-up duration ($P > 0.05$).

## Predictors of Syncopal Recurrence in Patients With Vasovagal Syncope

**Table 2** shows the possible associated factors for the recurrence of syncope. In univariate Cox regression analysis, age at the first visit of our department (hazard ratio = 1.313, 95% confidence interval 1.013–1.700, $P$ = 0.039), the number of previous syncopal episodes before treatment (hazard ratio = 1.027, 95% confidence interval 1.009–1.045, $P$ = 0.003) and supine mean BP (hazard ratio = 1.096, 95% confidence interval 1.022–1.177, $P$ = 0.011) were recognized as possible associated factors for syncopal recurrence. Furthermore, the number of previous syncopal episodes before treatment was identified as an independent risk factor (hazard ratio = 1.027, 95% confidence interval 1.009–1.045, $P$ = 0.003) when taken into the multivariate analysis, but other parameters that were included showed no predictive value for recurrence.

Then, we performed the receiver operating characteristic curve analysis to decide the best cutoff number of previous syncopal episodes before treatment for predicting syncopal recurrence (**Figure 3**). The area under the curve was 0.743 (95% confidence interval 0.574–0.912, $P$ = 0.015), and 4 previous syncopal episodes before treatment were determined as the best cutoff value for reaching the highest Youden index. Moreover, the Kaplan–Meier curves showed that the syncopal recurrence-free survival rate over time in patients with >4 previous syncopal episodes before treatment was much lower than that of patients with ≤4 episodes ($P$ = 0.019 at the log-rank test, **Figure 4**).

# DISCUSSION

To our best knowledge, this was the first study on the prediction of syncopal recurrence in children with VVS while on the

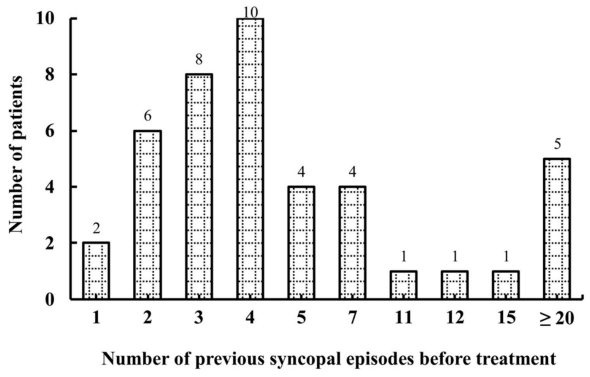
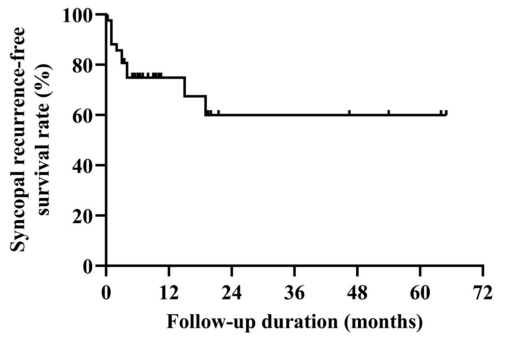



**FIGURE 1 |** Number of patients with different syncopal episodes before treatment. The *y*-axis represents the number of patients; the *x*-axis represents the number of previous syncopal episodes before treatment. The median frequency was 4.0 episodes/person/year.




| Follow-up duration (months) | 0 | 12 | 24 | 36 | 48 | 60 |
|---|---|---|---|---|---|---|
| Number of patients at risk | 42 | 11 | 5 | 5 | 4 | 3 |

**FIGURE 2 |** Trend of the syncopal recurrence-free survival rate in all eligible patients over time. The *y*-axis represents the survival rate for non-recurrence of syncope; the *x*-axis represents follow-up duration.

**TABLE 1 |** The baseline characteristics of the study population and comparisons of patients with and without syncopal recurrence.

| Parameters | All patients | Patients with syncopal recurrence | | t/Z/$\chi^2$ value | *P*-value |
|---|---|---|---|---|---|
| | | Yes | No | | |
| Number [n (%)] | 42 (100) | 12 (29) | 30 (71) | – | – |
| Female [n (%)] | 29 (69) | 8 (67) | 21 (70) | 0.000 | 1.000 |
| Age at first syncopal episode (years) | 9.6 ± 3.5 | 11.5 (10.3, 12.0) | 9.2 ± 3.5 | −1.359 | 0.174 |
| Age at first visit of our department (years) | 11.6 ± 2.5 | 12.6 ± 2.9 | 11.3 ± 2.3 | −1.565 | 0.126 |
| Duration of symptoms before treatment (months) | 14.5 (4.0, 39.0) | 22.4 ± 19.5 | 14.5 (3.0, 39.0) | −0.391 | 0.696 |
| Triggers of syncope [yes, n (%)] | 31 (74) | 8 (67) | 23 (77) | 0.077 | 0.781 |
| Prodromes [yes, n (%)] | 39 (93) | 12 (100) | 27 (90) | 0.224 | 0.636 |
| Duration of unconsciousness [<1 min, n (%)] | 13 (31) | 4 (33) | 9 (30) | 0.000 | 1.000 |
| Injury at syncope [yes, n (%)] | 6 (14) | 3 (25) | 3 (10) | 0.588 | 0.443 |
| Number of previous syncopal episodes (times) | 4 (3, 7) | 6 (3, 44) | 4 (2, 5) | −2.467 | 0.013 |
| History of allergy [yes, n (%)] | 13 (31) | 2 (17) | 11 (37) | 0.805 | 0.370 |
| Family history of syncope [yes, n (%)] | 7 (17) | 1 (8) | 6 (20) | 0.210 | 0.647 |
| Body mass index (kg/m$^2$) | 18.7 (16.9, 23.3) | 19.6 (17.7, 25.7) | 19.7 ± 4.3 | −1.114 | 0.265 |
| Supine heart rate during head-up tilt test (beats/minute) | 80 ± 12 | 79 ± 13 | 80 ± 12 | 0.218 | 0.829 |
| Supine mean blood pressure during head-up tilt test (mmHg) | 80 ± 8 | 85 ± 9 | 78 ± 7 | −2.506 | 0.016 |
| Positive heart rate during head-up tilt test (beats/minute) | 100 ± 33 | 105 ± 33 | 98 ± 33 | −0.670 | 0.507 |
| Positive mean blood pressure during head-up tilt test (mmHg) | 54 ± 9 | 51 ± 8 | 55 ± 9 | 1.426 | 0.162 |
| Time to positive response during head-up tilt test (minutes) | 27 (11, 34) | 26 ± 13 | 23 (10, 36) | −0.405 | 0.686 |
| Types of vasovagal syncope [vasodepressive type, n (%)] | 33 (79) | 10 (83) | 23 (77) | 0.004 | 0.953 |
| QT dispersion (milliseconds) | 25 (20, 31) | 25 (20, 36) | 25 (20, 30) | −0.183 | 0.855 |
| QTc dispersion (milliseconds) | 29 (22, 39) | 29 ± 9 | 29 (22, 34) | −0.223 | 0.824 |
| Treatment duration (months) | 3.0 (2.0, 4.0) | 3.5 ± 1.7 | 3.0 (2.0, 3.6) | −1.011 | 0.312 |
| Follow-up duration (months) | 9.0 (4.8, 19.1) | 15.0 (4.3, 19.0) | 8.0 (4.8, 19.1) | −0.599 | 0.549 |

treatment of metoprolol. We found that in the follow-up duration, 29% of the VVS patients suffered at least one recurrent syncopal episode. Based on Cox regression analyses, the number of previous syncopal episodes before treatment was confirmed as a predictor of the syncopal recurrence.

VVS is common in children, and the final goal of all treatments is to terminate the unpredictable recurrence of syncope. To date, no therapeutic approaches have been documented to completely control syncopal recurrence (17). Therefore, a great number of studies have been devoted to mapping prognosis and finding predictors for syncopal recurrence (18). Variable recurrence rates from 19% to 78% were reported during a mean follow-up duration of 1.5–6 years under different situations (nonpharmacological or pharmacological interventions). In this study, the recurrence rate of 29% during a median follow-up duration of 9.0 (4.8, 19.1) months was in accordance with the results of previous studies (19–23), but our study focused on the VVS cases while on the metoprolol intervention in patients of young ages.

In the literature, many studies have suggested the number of previous syncopal episodes before treatment as a common risk factor for syncopal recurrence (4, 19–23). The findings in the present study also confirmed this, with an increase of 2.7% in the probability of recurrence for each 1-time increase in the number of previous syncopal episodes. Moreover, in the present study, we, for the first time, identified the number of previous syncopal episodes, instead of the HUTT indexes, as a predictor for syncopal recurrence in VVS children treated with metoprolol and patients with over 4 previous syncopal episodes before treatment significantly impacted the recurrence survival over time. The Kaplan–Meier curves showed that the syncopal recurrence-free survival rate over time in patients with >4 previous syncopal episodes was significantly lower than that in patients with ≤4 episodes, suggesting that the severity of the illness impacted the prognosis of the patients. The severe cases likely had a relatively high syncopal recurrence rate at follow-up. The heterogeneity of the studied populations might explain why other cutoff values were also determined (4, 21). The number of previous syncopal episodes is capable of reflecting the severity of VVS to some extent. It shows the paroxysmal trait and provides a rational clue to predict recurrence or not.

Girls in childhood and adolescence suffer from VVS twice as often as boys (24). Furthermore, females are believed to experience recurrent syncope much more often than males (21, 22). A similar sex ratio (females accounting for 69%) to that from previous reports was found in our research, but no significant difference in recurrence proportion was observed in females and males.

The QT interval is a parameter derived from an electrocardiogram, which reflects the period of ventricular repolarization. QTd implies the electrical instability of myocardia and exhibits the degree of interlead spatial variability. Kula et al.

**TABLE 2 |** Risk factors for syncopal recurrence from Cox regression analyses.

| Parameters | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|
| | Hazard ratio (95% confidence interval) | *P*-value | Hazard ratio (95% confidence interval) | *P*-value |
| Female gender | 0.955 (0.286–3.191) | 0.940 | – | – |
| Age at first syncopal episode (years) | 1.163 (0.970–1.393) | 0.103 | – | – |
| Age at first visit of our department (years) | 1.313 (1.013–1.700) | 0.039 | – | – |
| Duration of symptoms before treatment (months) | 0.992 (0.972–1.013) | 0.448 | – | – |
| Triggers of syncope (yes) | 0.537 (0.160–1.809) | 0.316 | – | – |
| Prodromes (yes) | 24.861 (0.007–87551.961) | 0.441 | – | – |
| Duration of unconsciousness (<1 min) | 1.162 (0.348–3.877) | 0.808 | – | – |
| Injury at syncope (yes) | 1.972 (0.532–7.310) | 0.310 | – | – |
| Number of previous syncopal episodes (times) | 1.027 (1.009–1.045) | 0.003 | 1.027 (1.009–1.045) | 0.003 |
| History of allergy (yes) | 0.338 (0.073–1.565) | 0.165 | – | – |
| Family history of syncope (yes) | 0.508 (0.064–4.014) | 0.521 | – | – |
| Body mass index (kg/m$^2$) | 1.079 (0.983–1.183) | 0.108 | – | – |
| Supine heart rate during head-up tilt test (beats/minute) | 0.992 (0.943–1.045) | 0.770 | – | – |
| Supine mean blood pressure during head-up tilt test (mmHg) | 1.096 (1.022–1.177) | 0.011 | – | – |
| Heart rate at positive response of head-up tilt test (beats/minute) | 1.007 (0.989–1.026) | 0.433 | – | – |
| Mean blood pressure at positive response of head-up tilt test (mmHg) | 0.963 (0.905–1.024) | 0.229 | – | – |
| Time to positive response during head-up tilt test (minutes) | 1.032 (0.984–1.082) | 0.196 | – | – |
| Types of vasovagal syncope (vasodepressive type) | 1.607 (0.351–7.366) | 0.541 | – | – |
| QT dispersion (milliseconds) | 1.008 (0.953–1.066) | 0.789 | – | – |
| QTc dispersion (milliseconds) | 1.001 (0.951–1.054) | 0.959 | – | – |
| Treatment duration (months) | 1.156 (0.836–1.598) | 0.382 | – | – |

found that pediatric VVS patients with positive HUTT results had higher QTcd in the morning and late night than those with negative HUTT results and healthy controls (25). Xue et al. observed that pediatric VVS patients with positive HUTT results had higher supine QTd and QTcd values than healthy volunteers, but in VVS patients, both QTd and QTcd decreased significantly when transferring from supine to standing (26). Therefore, we analyzed QTd and QTcd in this study, but no differences were found between patients with and without recurrence, suggesting

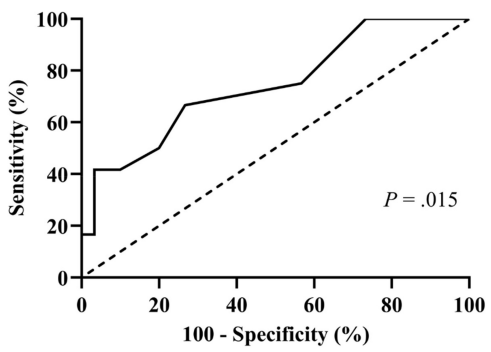



**FIGURE 3 |** Receiver operating characteristic curve for determining the best cutoff for the number of previous syncopal episodes in predicting syncopal recurrence. The *y*-axis represents the sensitivity to predict the recurrence of syncope; the *x*-axis represents the false-positive rate (100%–specificity%). The 45 reference line of the chart indicates that the sensitivity and the false-positive rate are equal. The area under the curve was 0.743 with a 95% confidence interval 0.574 to 0.912 ($P = 0.015$).

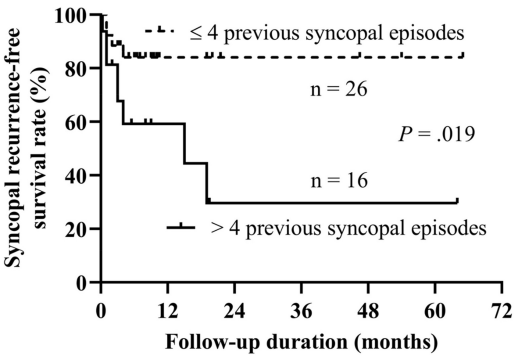



**FIGURE 4 |** Kaplan–Meier curve analysis of the syncopal recurrence-free survival rate between patients with >4 previous syncopal episodes and with ≤4 previous sycopal episodes. The *y*-axis represents the survival rate for non-recurrence of syncope; the *x*-axis represents follow-up duration. The recurrence-free survival rate of syncope in patients with >4 previous syncopal episodes before treatment was significantly lower than that of patients with ≤4 episodes ($P = 0.019$ at the log-rank test).

that electrical instability did not significantly impact the syncopal recurrence in VVS cases treated with beta-adrenoceptor blocker.

Inheritance in the pathophysiology of VVS should not be ignored because a relatively high ratio of positive family history was observed (27, 28). Its role is strengthened by relevant gene encoding results (29). Tanriverdi Yilmaz S et al. found a higher recurrence rate in patients with a positive family history (23). Although 17% of the patients in our study had syncope-affected families, those with syncopal recurrence did not show a higher probability of a positive family history.

Both syncope and allergic conditions are common at young ages. Du's team found that approximately 26% of included pediatric VVS patients were affected by allergic diseases, and there were some clinical differences between VVS patients with and without allergic diseases (30). However, the underlying mechanisms underlying the relationship between allergic condition and VVS have not been fully illuminated. Our results did not show any distinction in syncopal recurrence between patients with and without allergic diseases.

Actually, there were some limitations in this study. The recall bias would impact the results for that the study was carried out retrospectively. The relatively small number of participants limits the extrapolation to other subjects. In the future, the understanding of the predictors of the long-term prognosis of children with VVS merits further large sample-sized multi-center clinical studies.

Conclusively, the present study showed that the predictor for syncopal recurrence in VVS children treated with metoprolol was the number of previous syncopal episodes. Recognition of the predictors of the prognosis of children with VVS would be beneficial to patients, relatives and physicians.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Peking University First Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

## AUTHOR CONTRIBUTIONS

CT had primary responsibility for the protocol development, patient enrollment, data collection, preliminary data analysis, and wrote the draft. BX analyzed the data together and revised important content. YL assisted with the study design, data collection, data analysis, and draft editing. XL designed the data analysis process and reviewed and revised the manuscript. HJ gave important advice on study design, supervised the data collection, and reviewed the manuscript for important intellectual content. JD supervised the design and execution of the study, checked the data analysis, contributed to the writing of the manuscript, and had a final approval of the manuscript submitted. All authors read and approved the final manuscript and agreed to be accountable for all aspects of the work.

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# Salivary miRNA Expression in Children With Persistent Post-concussive Symptoms

*Katherine E. Miller[1†], James P. MacDonald[2,3†], Lindsay Sullivan[4,5], Lakshmi Prakruthi Rao Venkata[1], Junxin Shi[6], Keith Owen Yeates[7], Su Chen[8], Enas Alshaikh[4], H. Gerry Taylor[3,9], Amanda Hautmann[4], Nicole Asa[4,10], Daniel M. Cohen[3,11], Thomas L. Pommering[2,3], Elaine R. Mardis[1,3,12‡], Jingzhen Yang[3,4*‡] and the NCH Concussion Research Group[§]*

[1] The Steve and Cindy Rasmussen Institute for Genomic Medicine, Abigail Wexner Research Institute at Nationwide Children's Hospital, Columbus, OH, United States, [2] Division of Sports Medicine, Nationwide Children's Hospital, Columbus, OH, United States, [3] Department of Pediatrics, The Ohio State University College of Medicine, Columbus, OH, United States, [4] Center for Injury Research and Policy, Abigail Wexner Research Institute at Nationwide Children's Hospital, Columbus, OH, United States, [5] Discipline of Children's Studies, School of Education, National University of Ireland, Galway, Ireland, [6] Biostatistics Resource Core at Nationwide Children's Hospital, Columbus, OH, United States, [7] Department of Psychology, Alberta Children's Hospital Research Institute, and Hotchkiss Brain Institute, University of Calgary, Calgary, AB, Canada, [8] Department of Biostatistics, University of Nebraska Medical Center, Omaha, NE, United States, [9] Biobehavioral Health Center, The Abigail Wexner Research Institute at Nationwide Children's Hospital, Columbus, OH, United States, [10] Department of Epidemiology, University of Washington, Seattle, WA, United States, [11] Division of Emergency Medicine, Nationwide Children's Hospital, Columbus, OH, United States, [12] Department of Neurosurgery, The Ohio State University College of Medicine, Columbus, OH, United States

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**Edited by:**
Nis Borbye-Lorenzen,
Statens Serum Institute, Denmark

**Reviewed by:**
Vera Ignjatovic,
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Regan King,
University of Calgary, Canada
Alfred Born,
University of Copenhagen, Denmark

***Correspondence:**
Jingzhen Yang
Ginger.Yang@nationwidechildrens.org

[†] These authors have contributed equally to this work and share first authorship
[‡] These authors have contributed equally to this work and share senior authorship
[§] Members listed at end of report

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**Background:** Up to one-third of concussed children develop persistent post-concussive symptoms (PPCS). The identification of biomarkers such as salivary miRNAs that detect concussed children at increased risk of PPCS has received growing attention in recent years. However, whether and how salivary miRNA expression levels differ over time between concussed children with and without PPCS is unknown.

**Aim:** To identify salivary MicroRNAs (miRNAs) whose expression levels differ over time post-concussion in children with vs. without PPCS.

**Methods:** We conducted a prospective cohort study with saliva collection at up to three timepoints: (1) within one week of injury; (2) one to two weeks post-injury; and (3) 4-weeks post-injury. Participants were children (ages 11 to 17 years) with a physician-diagnosed concussion from a single hospital center. We collected participants' daily post-concussion symptom ratings throughout their enrollment using the Post-concussion Symptom Scale, and defined PPCS as a total symptom score of $\geq 5$ at 28 days post-concussion. We extracted salivary RNA from the saliva samples and measured expression levels of 827 salivary miRNAs. We then compared the longitudinal expression levels of salivary miRNAs in children with vs. without PPCS using linear models with repeated measures.

**Results:** A total of 135 saliva samples were collected from 60 children. Of the 827 miRNAs analyzed, 91 had expression levels above the calculated background threshold and were included in the differential gene expression analyses. Of these 91 miRNAs, 13 had expression levels that differed significantly across the three timepoints

post-concussion between children with and without PPCS (i.e., hsa-miR-95-3p, hsa-miR-301a-5p, hsa-miR-626, hsa-miR-548y, hsa-miR-203a-5p, hsa-miR-548e-5p, hsa-miR-585-3p, hsa-miR-378h, hsa-miR-1323, hsa-miR-183-5p, hsa-miR-200a-3p, hsa-miR-888-5p, hsa-miR-199a-3p+hsa-miR-199b-3p). Among these 13 miRNAs, one (i.e., hsa-miR-203a-5p) was also identified in a prior study, with significantly different expression levels between children with and without PPCS.

**Conclusion:** Our results from the longitudinal assessment of miRNAs indicate that the expression levels of 13 salivary miRNAs differ over time post-injury in concussed children with vs. without PPCS. Salivary miRNAs may be a promising biomarker for PPCS in children, although replication studies are needed.

**Keywords: concussion, miRNA expression, children, persistent post-concussive symptoms, saliva, biomarkers**

## INTRODUCTION

Concussion affects approximately two million children in the United States each year (1–3). Although most concussion symptoms resolve within one to three weeks, up to one-third of children with a concussion develop persistent post-concussive symptoms (PPCS), which can last months following injury (3–5). Children with PPCS are at increased risk of experiencing missed school days, depressed mood, loss of social activities, and lower quality of life compared to children without PPCS (6–8).

Predicting clinical recovery from concussion, including identifying patients at increased risk for PPCS, is challenging (9). Several potential risk factors for PPCS in children have been identified with varying strength of association, including time from injury to initial clinical visit, acute symptom severity, vision and vestibular system dysfunction, and a history of co-morbidities such as ADHD (9–12). Currently, predicting clinical recovery from concussion is largely dependent on patient-reported symptoms and clinician-elicited signs, with the most consistent predictor of prolonged recovery being the severity of a patient's acute/subacute symptoms (10). Although increased efforts have been made to determine causes and predictors of PPCS in children, objective biomarkers that identify children at increased risk for PPCS are lacking.

MicroRNAs (miRNAs), small non-coding RNA molecules that regulate gene expression, are emerging as promising biomarkers to monitor disease, aid treatment decisions, and stratify risk, including for children with traumatic brain injury (TBI) (13–20). Prior studies show that miRNAs may control cellular processes essential to neuronal injury and repair in both the primary and secondary pathophysiology involved in TBI (21–24). Furthermore, studies have demonstrated the feasibility of identifying changes in miRNA expression associated with brain injury in biofluids, including blood and saliva (19–21, 25, 26).

Recent investigations, though mostly focused on adult patients, have shown the utility of salivary miRNA expression levels as diagnostic or prognostic biomarkers for TBI (17–19, 21, 27). In a study that included patients aged 7–21 years, Johnson et al. demonstrated overexpression of five human salivary miRNAs associated with PPCS at four weeks post-injury (18). Fedorchak et al. found an algorithm of 16 non-coding RNAs, obtained within 14 days of injury among patients aged 8–24 years, predicted concussion symptoms that lasted more than 21 days with greater accuracy than computerized balance and cognitive test performance (19). Of note, both studies included patients over the age of 18. Whether and how miRNA expression levels change over time post-injury among children aged younger than 18 years with and without PPCS remains unknown. Further studies of pediatric populations are required to confirm whether salivary miRNAs can provide accurate, objective, easily obtainable, and non-invasive biomarkers for the screening and detection of PPCS risk in children during the acute phase post-concussion.

This study aimed to longitudinally measure the expression of a panel of human salivary miRNAs at up to three timepoints post-concussion (i.e., within one week of injury, one to two weeks post-injury, and four weeks post-injury) in children aged 11–17 years, and to identify salivary miRNAs whose expression levels differ over time post-concussion in children with vs. without PPCS. The findings of this study could reveal potential biomarkers that can help detect children who may be at greater risk for PPCS in the acute or post-acute phase post-concussion. Such biomarkers could inform early, individualized, and multifaceted concussion care for children following a concussive injury (7, 19).

## METHODS

### Study Design

This study involved a prospective cohort design with repeated saliva sample collection. We enrolled concussed children within 2 weeks of injury from the Emergency Department (ED) or hospital-based concussion clinics affiliated with a single children's hospital located in central Ohio (United States) and followed them until four weeks post-injury. We collected saliva

**Abbreviations:** ED, emergency department; FDR, false discovery rate; miRNA, microRNA; PPCS, persistent post-concussive symptoms; SD, standard deviation; TBI, traumatic brain injury.

samples from each participant at up to three timepoints: 1) within one week of injury (if enrollment occurred < 7 days post-injury), 2) one to two weeks post-injury, and 3) four weeks post-injury. Participants also rated their daily post-concussive symptoms throughout their participation (28). We collected study data between February 2019 and November 2019. The study received ethical approval from the Institutional Review Board at the authors' hospital (IRB 18–00088). This report follows STROBE (STrengthening the Reporting of OBservational studies in Epidemiology) reporting guidelines for observational studies.

## Study Participants

Study participants were children aged 11 to 17 years with a physician-confirmed concussion diagnosis. Concussion was defined as a mild TBI induced by a direct or indirect blow to the head, neck, face, or other part of the body, resulting in transient neurological deficits (28). We excluded children if their concussion met any of the following criteria: 1) required surgery, 2) had any associated injury that independently interfered with neuropsychological testing (e.g., injury that affected vision), 3) was intentionally caused (i.e., resulted from assault, abuse, or self-harm), and/or 4) was associated with illicit drug or alcohol use. We also excluded children who had periodontal disease, pre-existing inflammatory/autoimmune disease(s), a current infection, or were receiving steroids/immunosuppressants; these children were excluded due to the procedures employed to collect the saliva samples (29–31). We approached 76 eligible concussed children. Of these, 66 consented. We included 60 participating children in the final analysis, excluding two participants who were lost to follow-up, and four participants who withdrew from the study due to their busy schedules.

## Study Variables and Measures

### miRNA Expression Level

Normalized expression counts for all miRNAs on our panel were generated for each sample. The data from our saliva samples populated a database of salivary miRNA expression levels for downstream statistical evaluation and generation of a PPCS "signature" of miRNA expression. Expression levels of each of the 827 human salivary miRNAs were measured as a digital count and included in the statistical analyses as a continuous variable. Of the 827 miRNAs analyzed, 91 had expression levels above the calculated background threshold (as described in "miRNA expression assay" methods section) and were included in the results.

*Acute signs and symptoms of concussion and acute mental status* were assessed using the Standardized Assessment of Concussion (SAC), which was completed as part of routine clinical care (32). The SAC, a validated tool, includes measures of orientation, immediate memory, concentration, and delayed recall, summing to a total composite score of up to 30 points (32).

*Post-concussive symptoms* were assessed daily using the self-reported Post-concussion Symptom Scale, which is contained in the Sport Concussion Assessment Tool, 5th edition (28). The Post-concussion Symptom Scale is a validated Likert scale that measures 22 current concussion symptoms rated from 0 = no symptoms to 6 = severe symptoms. The Post-concussion Symptom Scale has established reliability ($\alpha = 0.93$), construct validity, and normative data (33). Similar to prior published studies (18), we defined PPCS as a total symptom score of $\geq$5 at 28 days post-concussion.

*Demographic and injury variables* included age, sex, race, whether the injury occurred during a sporting event, history of prior concussion, symptom score at injury, and date of symptom resolution.

## Data Collection

Once a concussion diagnosis was confirmed, trained clinical research coordinators (at the ED) or physicians (at the concussion clinics) communicated study information to potentially eligible participants and referred them to our research team. We then contacted the child and their parent/legal guardian ("parent") to confirm their interest and eligibility for

**TABLE 1 |** Demographic characteristics of study participants ($N = 60$ participants).

| | Total $N = 60$ | | PPCS = No $N = 42$ | | PPCS = Yes $N = 18$ | | P-value* |
|---|---|---|---|---|---|---|---|
| Male sex, No (%) | 32 | 53.3 | 23 | 54.8 | 9 | 50.0 | 0.735 |
| Age in years, mean (SD) | 14.4 | 1.8 | 14.3 | 1.8 | 14.9 | 1.7 | 0.235 |
| White, No (%) | 54 | 90.0 | 38 | 90.5 | 16 | 88.9 | 0.850 |
| Injured in sporting activity, No (%) | 53 | 88.3 | 37 | 88.1 | 16 | 88.9 | 0.658 |
| History of prior concussion, No (%) | 20 | 33.3 | 15 | 35.7 | 5 | 27.8 | 0.550 |
| Symptom score at injury, mean (SD) | 46.2 | 32.0 | 43.0 | 28.3 | 52.5 | 41.2 | 0.331 |
| Days from injury to enrollment, mean (SD) | 7.8 | 3.7 | 7.5 | 3.8 | 8.6 | 3.4 | 0.306 |
| Saliva samples collected at three timepoints: | | | | | | | |
| Within 1 week of injury, No (%) | 29 | 48.3 | 21 | 72.4** | 8 | 27.6** | |
| One to two weeks post-injury, No (%) | 47 | 78.3 | 33 | 70.2** | 14 | 29.8** | |
| four weeks post-injury, No (%) | 59 | 98.3 | 41 | 69.5** | 18 | 30.5** | |

*P-values were based on chi-square tests (categorical variables) or two-sample t-tests (continuous variables).*

***Row percent was presented for children with and without PPCS.*

**TABLE 2** | Thirteen individual miRNAs showing significant overexpression over time post-concussion in children with persistent post-concussive symptoms (PPCS) as compared to children without PPCS, Adjusted analysis.

| | PPCS (yes vs no) | | | Timepoints | | | Sex (female vs. male) | | | Age | | | Prior concussion (yes vs. no) | | | Symptom score at injury | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **miRNA** | **β** | **SE** | **P-value** | **β** | **SE** | **P-value** | **β** | **SE** | **P-value** | **β** | **SE** | **P-value** | **β** | **SE** | **P-value** | **β** | **SE** | **P-value** |
| hsa–miR–95–3p[$] | 2.15 | 0.70 | **0.00** | 0.01 | 0.02 | 0.60 | −0.02 | 0.07 | 0.77 | 0.01 | 0.03 | 0.64 | −0.08 | 0.08 | 0.33 | 0.00 | 0.00 | 0.66 |
| hsa–miR–301a–5p | 0.69 | 0.15 | **<0.0001** | 0.03 | 0.02 | 0.13 | 0.11 | 0.14 | 0.45 | −0.04 | 0.04 | 0.31 | 0.06 | 0.16 | 0.70 | 0.00 | 0.00 | 0.45 |
| hsa–miR–626* | 0.49 | 0.14 | **0.00** | 0.08 | 0.03 | **0.00** | 0.13 | 0.13 | 0.32 | −0.01 | 0.04 | 0.69 | 0.29 | 0.18 | 0.11 | 0.00 | 0.00 | 0.82 |
| hsa–miR–548y | 0.59 | 0.12 | **<0.0001** | 0.01 | 0.02 | 0.60 | 0.09 | 0.11 | 0.43 | −0.01 | 0.03 | 0.70 | 0.06 | 0.12 | 0.60 | 0.00 | 0.00 | 0.35 |
| hsa–miR–203a–5p | 0.62 | 0.13 | **<0.0001** | 0.03 | 0.02 | 0.25 | 0.11 | 0.12 | 0.35 | −0.06 | 0.04 | 0.11 | 0.10 | 0.14 | 0.48 | 0.00 | 0.00 | 0.27 |
| hsa–miR–548e–5p[$] | 3.03 | 1.19 | **0.01** | 0.02 | 0.02 | 0.40 | 0.13 | 0.13 | 0.32 | 0.00 | 0.05 | 0.96 | −0.03 | 0.14 | 0.86 | 0.00 | 0.00 | 0.58 |
| hsa–miR–585–3p | 0.59 | 0.14 | **<0.0001** | 0.03 | 0.02 | 0.22 | 0.13 | 0.13 | 0.30 | −0.04 | 0.04 | 0.34 | −0.03 | 0.14 | 0.85 | 0.00 | 0.00 | 0.94 |
| hsa–miR–378h | 0.41 | 0.13 | **0.00** | 0.00 | 0.02 | 0.95 | 0.17 | 0.12 | 0.15 | 0.00 | 0.03 | 0.91 | −0.01 | 0.13 | 0.95 | 0.00 | 0.00 | 0.84 |
| hsa–miR–1323 | 0.62 | 0.16 | **0.00** | 0.01 | 0.03 | 0.61 | 0.17 | 0.14 | 0.24 | −0.04 | 0.04 | 0.32 | 0.05 | 0.16 | 0.73 | 0.00 | 0.00 | 0.63 |
| hsa–miR–183–5p | 0.65 | 0.14 | **<0.0001** | 0.02 | 0.03 | 0.38 | 0.03 | 0.13 | 0.84 | −0.05 | 0.04 | 0.15 | −0.04 | 0.14 | 0.78 | 0.00 | 0.00 | 0.62 |
| hsa–miR–200a–3p[$] | 2.20 | 0.80 | **0.01** | 0.00 | 0.02 | 0.78 | −0.07 | 0.08 | 0.41 | 0.01 | 0.03 | 0.78 | −0.09 | 0.10 | 0.37 | 0.00 | 0.00 | 0.79 |
| hsa–miR–888–5p | 0.29 | 0.14 | **0.04** | −0.01 | 0.03 | 0.67 | 0.23 | 0.13 | 0.07 | 0.00 | 0.04 | 0.93 | −0.11 | 0.14 | 0.44 | 0.00 | 0.00 | 0.25 |
| hsa–miR–199a–3p+hsa–miR–199b–3p | 0.68 | 0.14 | **<0.0001** | 0.01 | 0.02 | 0.59 | −0.04 | 0.13 | 0.76 | −0.03 | 0.04 | 0.42 | 0.12 | 0.14 | 0.40 | 0.00 | 0.00 | 0.10 |

**Adjusted model included the interaction between prior concussion and timepoints.*
*[$]Adjusted model included the interaction between PPCS and age.*

the study and to schedule the first in-person assessment. After obtaining written informed consent/assent from a parent and the concussed child, respectively, we then collected unstimulated, non-fasting saliva using Oragene RNA RE-100 collection vials (DNA Genotek, Ottawa, ON, Canada) and completed other study assessments. We instructed participants to rinse their mouth with water prior to providing saliva and then to spit into the collection vials until the designated volume of saliva was reached (2 milliliters). We then stored the saliva samples at 4°C until they were processed for RNA isolation. We also instructed participants on how to complete online daily surveys, which were completed from the day of the initial research assessment until four weeks post-injury.

## RNA Extraction and miRNA Expression Assay

We used 2 mL of saliva to extract RNA using plasma/serum circulating and exosomal RNA slurry purification kits (Norgen Biotek, Thorold, ON, Canada). We used 300 ng of extracted RNA as input for expression of 827 different human miRNAs using the nCounter® human V3 miRNA assay kit (NanoString Technologies Inc., Seattle, WA, USA), following the manufacturer's protocol. A list of all targets plus control probes can be found in **Supplementary Table 1**. All hybridizations were 18 h in length, and all counts were obtained by scanning on MAX mode for 555 fields of view per sample. The resulting data were analyzed using nSolver analysis software (version 4.0). We generated normalized expression counts using two parameters, positive control normalization and housekeeping normalization, as suggested by the analytical software per the manufacturer's instructions. We calculated a background "threshold" using expression values of the spike-in negative control probes (i.e., non-mammalian probes). We used the mean of the negative control counts, plus two standard deviations as our threshold. For downstream analyses, we included miRNAs only if >25% of the samples in our cohort had expression above background threshold.

## miRNA Target Gene Prediction and Functional Annotation

We used miRWalk and miRTarBase databases to identify predicted gene targets of miRNAs (34, 35). We entered each miRNA name of interest into miRWalk, an online resource that generates both predicted and validated miRNA-binding sites by identifying complementary sequence regions within known human genes. We filtered the output to include binding sites within 3'UTR and 5'UTR, and coding sequences only if they were reported as validated interactions in the miRTarBase database. The resulting gene list was used for input into The Database for Annotation, Visualization, and Integrated Discovery (DAVID; version 6.8) to assign target genes of interest to biological and functional annotations and processes (36). We considered DAVID output significant if the reported Benjamini false discovery rate (FDR) corrected $p$-value was $\leq$0.10.

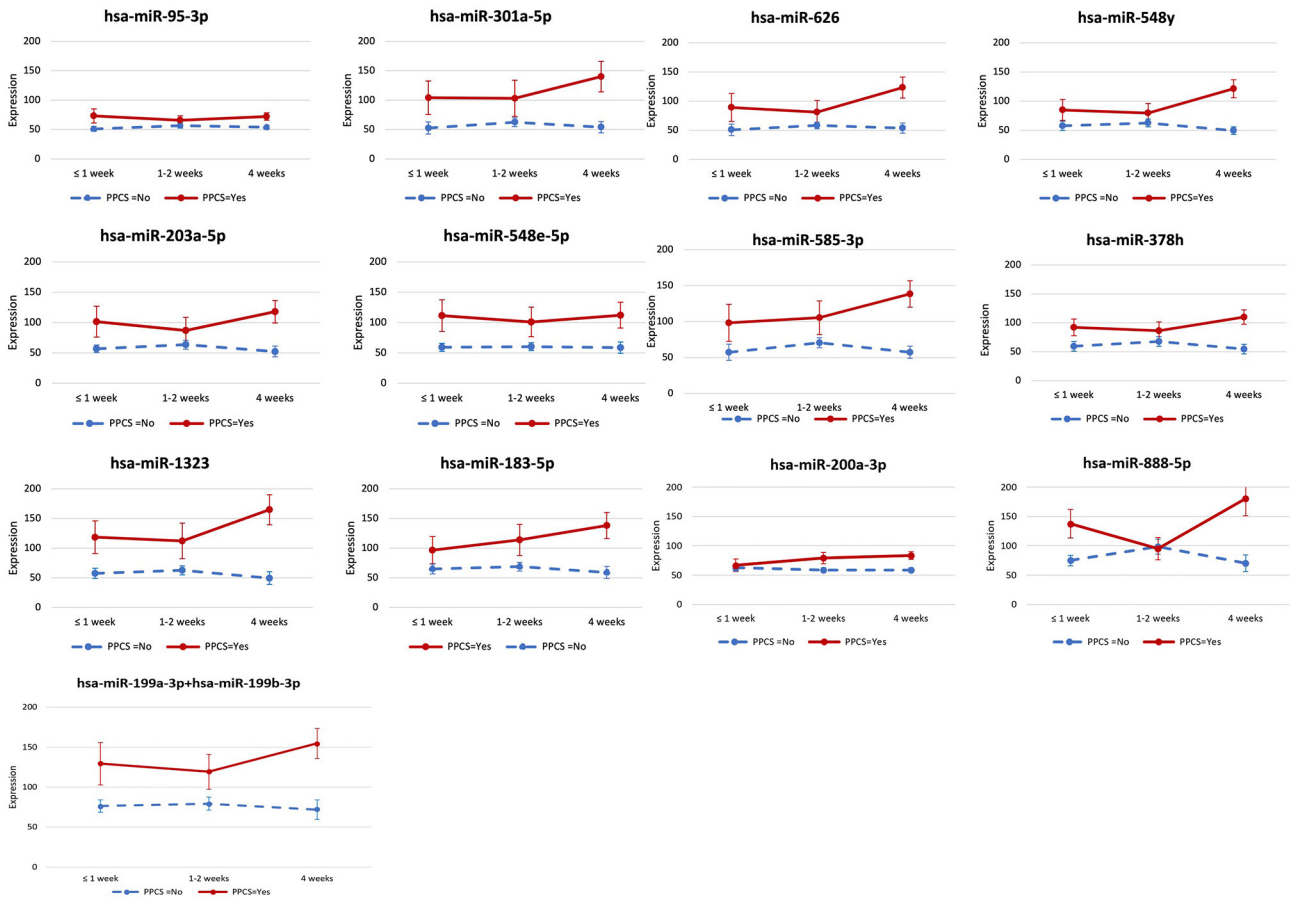


**FIGURE 1 |** miRNA expression levels between concussed children with and without persistent post-concussive symptoms (PPCS) across the three timepoints post-injury. We identified 13 miRNAs whose expression levels were significantly higher across the three timepoints post-injury in concussed children with PPCS compared to those without PPCS. In this graph, we show the longitudinal expression levels of each miRNA at the three timepoints in concussed children with PPCS (red) vs. concussed children without PPCS (blue) without adjusting for any covariates.

## Statistical Analysis

We conducted longitudinal analyses across the three timepoints (i.e., within 1 week of injury, one to two weeks post-injury, and four weeks post-injury) to test whether miRNA expression levels differed significantly between children with and without PPCS. We first calculated the mean and standard deviation (SD) of gene expression levels of the 91 miRNAs that were above the background threshold set for our study. Next, we analyzed the longitudinal salivary miRNA expression levels using linear models with repeated measures. Due to skewed distributions of the miRNA expressions, we log-transformed miRNA expression data prior to analysis. We first modeled the expression level for each of the 91 miRNAs with main effects of time and presence of PPCS as well as the interaction between time and presence of PPCS. If the interaction term was not statistically significant, we then removed it and re-ran the longitudinal models with only the main effects of time and presence of PPCS included in the model. The FDR method was used to adjust for multiple comparisons. For each miRNA that was significantly associated with the presence of PPCS, we also tested whether their associations remained significant while adjusting for time, sex, age, history of prior concussion, and symptom score at injury by repeating similar linear models. Additionally, we assessed pair-wise interaction one at a time in each model. For models showing statistically significant interactions, we presented the results from the models including the interaction term. Finally, we characterized the miRNAs expressed in our study, and compared them to those reported in previous studies.

# RESULTS

## Study Participants and Saliva Samples

Of the 60 participants, 32 (53.3%) were male, 55 (91.7%) were White, and 22 (36.7%) had a history of prior concussion. The mean age of participants was 14.4 (SD = 1.8) years (**Table 1**). Most concussions ($n$ = 53, 88.3%) occurred during a sporting event, including 15 (25%) in football and 13 (21.7%) in soccer. The mean symptom score at time of injury was 46.2 (SD = 32.0). Eighteen (30.0%) participants reported persistent post-concussive symptoms at 28 days post-injury. A total of 135 saliva

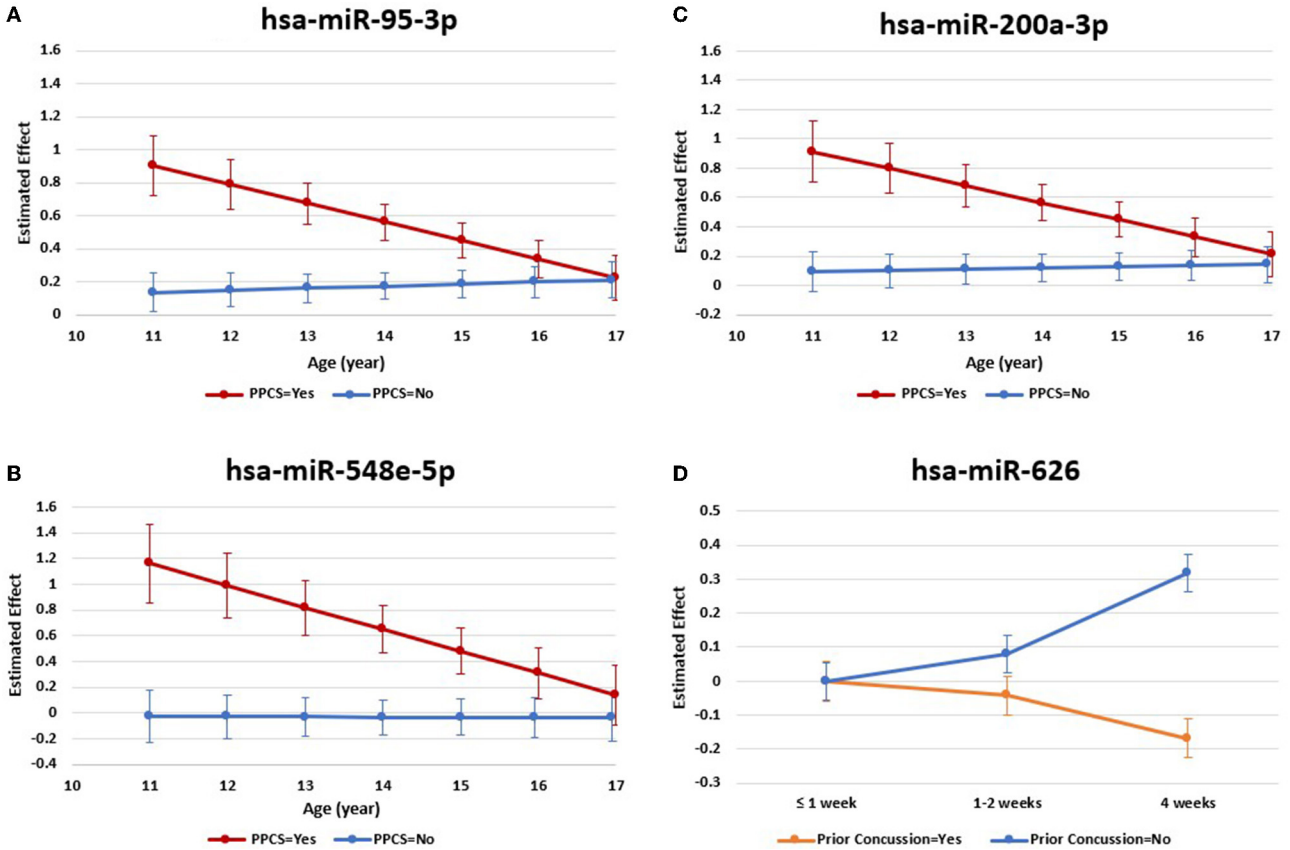


**FIGURE 2 |** miRNA expression levels with significant interactions post-injury. We found a statistically significant interaction between age and presence of PPCS in the expression levels of three miRNAs [**(A)** hsa-miR-95-3p; **(B)** hsa-miR-548e-5p; **(C)** hsa-miR-200a-3p]. Results also revealed a statistically significant interaction between time and history of prior concussion in the expression levels of one miRNA (**D** hsa-miR-626). These results were based on adjusted models in longitudinal analyses.

samples were included in our analyses. These included 29 unique samples from 29 children collected within one week of injury (27.6% of samples from children with PPCS), 47 unique samples from 47 children collected one to two weeks post-injury (29.8% of samples from children with PPCS), and 59 unique samples from 59 children collected four weeks post-injury (30.5% of samples from children with PPCS).

## Salivary miRNA Expression in Children With and Without PPCS

A total of 91 miRNAs were detected at expression levels above background levels and were included in the current analysis (Table 2 in the **Supplementary Material**). No statistically significant interactions were found between time and presence of PPCS for the 91 miRNAs, after adjusting for multiple comparisons (**Table 2**). Thirteen miRNAs had expression levels that differed significantly between children with and without PPCS across the three timepoints (hsa-miR-95-3p, hsa-miR-301a-5p, hsa-miR-626, hsa-miR-548y, hsa-miR-203a-5p, hsa-miR-548e-5p, hsa-miR-585-3p, hsa-miR-378h, hsa-miR-1323, hsa-miR-183-5p, hsa-miR-200a-3p, hsa-miR-888-5p, hsa-miR-199a-3p+hsa-miR-199b-3p), after adjusting for multiple comparisons (**Table 2**). The differences in the 13 miRNA expression levels between children with and without PPCS remained significant after adjusting for time, sex, age, history of prior concussion, and symptom score at injury (**Table 2**).

The expression levels of the 13 miRNAs that were differentially expressed in the two groups were consistently higher in children with PPCS than those without PPCS across the three timepoints, except for one miRNA (hsa-miR-888-5p), which had a similar expression level in both groups at one to two weeks post-injury (**Figure 1**). The group differences in expression levels of the 13 miRNAs appeared to be larger at four weeks post-injury; however, the interaction between PPCS and time was not statistically significant.

We found a statistically significant interaction between age and presence of PPCS in the expression levels of three of the 13 miRNAs (hsa-miR-95-3p, hsa-miR-548e-5p, hsa-miR-200a-3p). As shown in **Figures 2A–C**, the relationships between expression levels of these three miRNAs and presence of PPCS

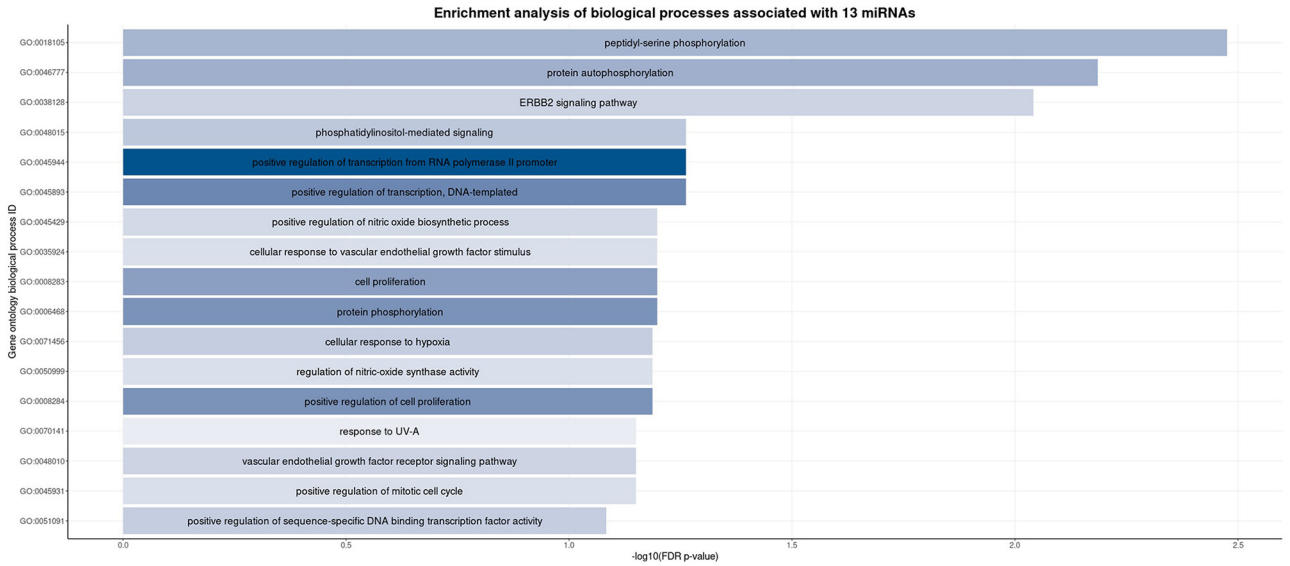


**FIGURE 3 |** Enrichment analysis of the biological processes associated with our miRNA gene target list at three different timepoints post-injury. We used the DAVID database to upload a list of target genes based on the miRNAs found to be significantly associated with PPCS in our cohort. This figure presents the biological processes found to be significantly associated (false discovery rate $p$-value $< 0.10$). Each bar represents a biological process ID, whose gene ontology ID and corresponding description are listed. The width of each bar represents the –log10(FDR $p$-value) while the color of each bar indicates the count (number) of our target genes associated with that particular biological process.

differed by age. Specifically, as age increased, the expression levels of the miRNAs decreased among children with PPCS while the expression levels remained relatively stable among children without PPCS. We also found a statistically significant interaction between time and history of prior concussion for one of the 13 miRNAs (hsa-miR-626) (**Figure 2D**), with expression levels decreasing across timepoints in children with a history of prior concussion but increasing across timepoints in children without a history of prior concussion.

## miRNA Target Gene Prediction and Biological Annotation

The number of validated, unique target genes for all miRNAs of interest ranged from 1 to 144 (Table 3 in the **Supplementary Material**). We uploaded the aggregated list of miRNA target genes to the DAVID functional annotation webtool to identify any biological process(es) for which the gene list was enriched (**Figure 3**). These results indicated that many of the target genes are involved in phosphorylation, transcriptional regulation, and cell signaling pathways.

## miRNA Expressed in the Current Study as Compared to Prior Published Studies

Three miRNAs found to be associated with PPCS in prior studies were available in our dataset (after quality control) for replication (i.e., hsa-miR-203a-5p, hsa-miR-148-3p, hsa-miR-1246) (**Table 3**). Of these three miRNAs, one (i.e., has-miR-203a-5p) showed significant association with PPCS in the current study and a prior study.[19]

# DISCUSSION

This study, to the best of our knowledge, is the first to longitudinally assess a panel of human salivary miRNA expression levels over time in a sample consisting exclusively of children after concussion (37). Of the 91 miRNAs expressed above background levels, 13 were significantly upregulated post-concussion in children with PPCS as compared to those without PPCS. Expression levels of these 13 miRNAs were higher in children with PPCS than those without PPCS across the three timepoints. Our findings add to a growing body of knowledge regarding changes in salivary miRNA expression levels among children post-concussion (17–19, 21, 22). These findings also add to previous work demonstrating the potential for miRNAs to discriminate between concussed children with and without PPCS (19). Identifying potential objective prognostic biomarkers of PPCS may help clinicians detect children at greater risk of PPCS during the acute post-injury phase (18, 19), and thereby facilitate early, individualized treatment plans for concussed children (7–12). The non-invasive nature of sampling for salivary miRNA makes this especially appealing when caring for children.

Three of the 91 miRNAs expressed in a prior study of PPCS by Fedorchak et al. (19) were also expressed in the current study. However, only one (hsa-miR-203a-5p) of these three miRNAs demonstrated significant overexpression across the three timepoints post-concussion in children with PPCS. These findings reflect the inconsistency among previous studies regarding miRNA expression levels such that salivary miRNAs may be overexpressed in children with PPCS in one study but not in other studies (18, 19). Such inconsistencies in study findings

**TABLE 3 |** Previously identified potential salivary miRNA biomarkers associated with persistent post–concussive symptoms (PPCS).

| | Association in other studies | Associated with PPCS in our cohort? | P–value in current study |
|---|---|---|---|
| **Previously identified salivary miRNA that are expressed in current study** | | | |
| hsa–miR–203a–5p | PPCS (19) | Yes | **0.00319** |
| hsa–miR–148–3p | PPCS (19) | No | hsa–miR–148a–3p: 0.28106<br>hsa–miR–148b–3p: 0.52139 |
| hsa–miR–1246 | PPCS (19) | No | 0.85564 |
| **Previously identified salivary miRNA that are not expressed or not measured in current study** | | | |
| hsa–miR–320c–1 | PPCS (18) | No | NA |
| hsa–miR–133a–5p | PPCS (18) | No | NA |
| hsa–miR–769–5p | PPCS (18) | No | NA |
| hsa–let–7a–3p | PPCS (18) | Not measured | NA |
| hsa–miR–100–5p | PPCS (19) | No | NA |
| hsa–miR–148a–5p | PPCS (19) | Not measured | NA |
| hsa–miR–423–5p | PPCS (19) | No | NA |
| hsa–miR–92b–3p | PPCS (19) | No | NA |
| hsa–miR–1307–3p | PPCS (18) | No | NA |
| **Salivary miRNA significantly expressed in current study that were not previously identified** | | | |
| hsa–miR–548y | NA | Yes | **0.00015** |
| hsa–miR–585–3p | NA | Yes | **0.00015** |
| hsa–miR–378h | NA | Yes | **0.00015** |
| hsa–miR–1323 | NA | Yes | **0.00015** |
| hsa–miR–183–5p | NA | Yes | **0.00015** |
| hsa–miR–199a–3p+hsa–miR–199b–3p | NA | Yes | **0.00015** |
| hsa–miR–301a–5p | NA | Yes | **0.00148** |
| hsa–miR–626 | NA | Yes | **0.00244** |
| hsa–miR–888–5p | NA | Yes | **0.00518** |
| hsa–miR–548e–5p | NA | Yes | **0.00696** |
| hsa–miR–200a–3p | NA | Yes | **0.00789** |
| hsa–miR–95–3p | NA | Yes | **0.01010** |

may be due, in part, to differences in the study populations, sample size, number and time of data collection, definition of PPCS, or analytic approaches employed. For example, Johnson et al. (18) only collected one salivary sample per participant, while Fedorchak's (19) and our study collected multiple salivary samples from each participant. Further, the definition of PPCS used in Johnson's (18) and our study (i.e., symptom presentation at $\geq$28 days post-injury) differed from the definition used by Fedorchak et al. (19) (i.e., symptom presentation at $\geq$ 21 days post-injury. In addition, it is important to consider matching the age of patients in future studies as we found a statistically significant interaction between age and presence of PPCS in the expression levels of three miRNAs. While there are currently no databases which catalog salivary miRNA expression levels in pediatric patients without disease, there is evidence of age-related effects on expression levels of miRNA in both saliva (38, 39) and serum (40) samples. As research on biomarkers for the prognosis of concussion is still in its infancy, further verification of these findings in a larger, more diverse cohort of concussed children and with standardized data collection tools and uniform analytic approaches is needed. Further, since biological changes do not provide information about the clinical manifestation of concussion (7, 8, 13), future studies should validate our findings with established, standardized clinical measures (e.g., balance and cognitive testing) or risk for PPCS (e.g., 5P Risk Score) to ensure the clinical utility of these biomarkers (7, 19). Our findings, along with others (13–19, 41), highlight the need for more research, with larger samples, to determine whether salivary miRNAs can accurately predict and detect PPCS in concussed children. If confirmed in other studies, these findings could inform the design of clinical assays (e.g., saliva collection) for use as an objective, non-invasive, and easily administrated test for children with concussion.

This study contributes to scientific knowledge on miRNA target gene prediction and biological annotation. The miRNAs that accurately discriminated between children with and without PPCS over time post-injury target genes that are significantly enriched for many biological processes, including protein phosphorylation, cell signaling pathways, and transcriptional regulation. The different distributions of the identified salivary miRNAs in children with vs. without PPCS may signal a differential physiological response to the concussive injury or its

subsequent repair. However, more research is needed to better understand the functional effects of miRNAs on their target genes, specifically within the context of concussion.

We found no statistically significant associations between symptom score at injury and the expression levels of the 13 miRNAs that were differentially expressed in concussed children with vs. without PPCS. This result may suggest that the 13 miRNAs that were overexpressed in the current study may serve as prognostic biomarkers for PPCS independent of acute symptom severity (13, 17–19, 26). Additional studies with more participants and multiple timepoints are needed to confidently identify all miRNAs that demonstrate an altered expression level following concussion as well as those associated with an increased risk for PPCS. Moreover, future studies are needed to further our understanding of the biological mechanisms of salivary miRNAs in children with concussion.

This study is not without limitations. First, the study had a relatively small sample size, which was relatively lacking in diversity, consisting almost exclusively of White participants from a single hospital center. Second, saliva samples were not available from all participants at all three timepoints, although our analytic approach accounted for this limitation. Third, we did not have knowledge about the resting expression levels of salivary miRNAs in age-matched children without concussion. Fourth, regarding functional annotation, while several databases curate both validated and predicted binding sites of miRNAs, these databases are not comprehensive, and no single centralized database houses all known cataloged gene targets (32, 33, 42–45). This is an evolving field, as we have noted. Fifth, while we restricted our target gene search to only those reported as validated by *in vitro* assays, some miRNAs are better studied than others, especially if they are implicated in more commonly studied diseases like cancer. Finally, participants were from hospital-based ED and concussion clinics, which potentially may treat more severe concussive injuries; thus, our findings may not readily generalize to concussed children seen in primary care clinics or other clinical settings, nor to children who are 10 years of age or younger. Despite these limitations, our study contributes to an emerging body of knowledge on potential biomarkers for the prognosis of pediatric concussion by longitudinally assessing miRNA expression levels over time post-concussion in children aged 11–17 years old and by exploring the physiological processes of the significantly upregulated miRNAs.

In conclusion, this study demonstrated that 13 salivary miRNAs were significantly upregulated post-concussion in children with PPCS as compared to their counterparts, suggesting that salivary miRNAs may serve as an objective prognostic biomarker for PPCS. Additional research is needed to determine the ability of salivary miRNA biomarkers to enhance the early prediction of PPCS risk in concussed children, including studies that verify our findings in larger, more diverse cohorts and with standardized clinical assessment tools. Although research on biomarkers for the prognosis of concussion is still in its infancy, salivary biomarkers hold promise for assisting in the early identification and treatment of children at increased risk for PPCS.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board at Abigail Wexner Research Institute at Nationwide Children's Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

## NCH CONCUSSION RESEARCH GROUP

Steven C. Cuff, MD; Drew Duerson, MD; Anastasia N. Fischer, MD; Jonathan T. Napolitano, MD; Reno Ravindran, MD; Richard E. Rodenberg Jr., MD; Amy E. Valasek, MD; Division of Sports Medicine, Nationwide Children's Hospital and Department of Pediatrics, The Ohio State University, College of Medicine, Columbus, Ohio, USA

## AUTHOR CONTRIBUTIONS

JY, EM, KM, and LS contributed to concept and design. JM, LS, AH, NA, DC, and TP collected study data. KM, LV, JY, LS, KY, HT, SC, JM, and EM contributed to analysis and interpretation of data. SC, EA, and JS conducted statistical analysis. KM, JY, LS, and JM drafted the initial manuscript. All authors contributed to critical revision of the manuscript for important intellectual content and approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2022.890420/full#supplementary-material

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SYSTEMATIC REVIEW
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# Metabolomic Biomarkers to Predict and Diagnose Cystic Fibrosis Pulmonary Exacerbations: A Systematic Review

*Anna-Lisa V. Nguyen*[1,2], *Dominic Haas*[2], *Mégane Bouchard*[3] *and Bradley S. Quon*[1,4*]

[1] *Centre for Heart Lung Innovation, University of British Columbia, Vancouver, BC, Canada,* [2] *Faculty of Health Sciences, McMaster University, Hamilton, ON, Canada,* [3] *Department of Epidemiology, Biostatistics, and Occupational Health, McGill University, Montréal, QC, Canada,* [4] *Faculty of Medicine, University of British Columbia, Vancouver, BC, Canada*

**Introduction:** Metabolomics is an emerging area of research and has the potential to identify clinical biomarkers for predicting or diagnosing cystic fibrosis (CF) pulmonary exacerbations (PEx).

**Objective:** To identify clinically promising metabolites across different sample sources that can be used to predict or diagnose PEx in CF.

**Evidence Review:** Searches for original literature were completed through EMBASE, MEDLINE, and all databases on the Web of Science with no restrictions on language or publication date. Gray literature was collected through Google Scholar. Additional studies were obtained by contacting authors and searching reference lists of candidate papers. The patient population included individuals with CF. Studies involving patients who underwent lung transplantation were excluded. The outcome was the prediction or diagnosis of pulmonary exacerbations from metabolites directly measured from biological samples. Search results were downloaded and imported into Covidence and duplicates were removed automatically. Any remaining duplicates were manually tagged and excluded. Two independent reviewers screened each abstract for eligibility and repeated this process for full texts. Risk of bias was conducted using QUADAS-2 by two independent reviewers. A third author resolved any remaining conflicts.

**Results:** A combined 3974 relevant abstracts were identified and 115 full texts were assessed for eligibility. The final 25 studies underwent data extraction for study design, patient demographics, studied metabolites, concentration values, and diagnostic accuracy values. Included studies differed considerably in methodologies, sample specimen types (exhaled breath condensate [EBC], sputum, saliva, plasma, urine), and disease states. We identified 19 unique metabolites that were measured by two or more studies of which 2 have the potential to predict PEx (EBC 4-hydroxycyclohexylcarboxylic acid [4-HCHC] and lactic acid) and 6 to diagnose PEx (EBC 4-HCHC and lactic acid, sputum lactic acid and nitrate, and plasma arginine and methionine).

**Conclusion and Relevance:** This systematic review has identified promising metabolites for further study in CF. Certain metabolites may provide clinical potential in predicting or diagnosing PEx, but further validation studies are required. With better

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*Samin Hamidi, Tabriz University of Medical Sciences, Iran*

***Correspondence:**
*Bradley S. Quon*
*Bradley.Quon@hli.ubc.ca*

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tools to aid in the earlier identification of PEx, clinicians can implement preventative measures to mitigate airway damage.

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**Keywords: metabolomics, biomarkers, systematic review, respirology, cystic fibrosis**

# INTRODUCTION

Cystic fibrosis (CF) is one of the most common inherited life-shortening conditions that causes chronic progressive lung disease. The disease course is characterized by episodes of acute or subacute clinical worsening referred to as pulmonary exacerbations (PEx) due to increased airway infection and inflammation. PEx symptoms typically include increased productive cough and systemic symptoms such as fatigue, loss of appetite, and weight loss (1). The clinical presentation with PEx can be subtle, especially early in its course, resulting in missed opportunities to intervene with antibiotics to preserve lung function. By using biomarkers to predict imminent exacerbation risk or facilitate earlier diagnosis, patient outcomes can be improved.

Within the "-omics" field, most of the focus has been on examining sputum and blood proteomic and transcriptomic inflammatory markers in CF patients to diagnose PEx (2, 3). However, metabolomic studies are also emerging and involve a range of biospecimens. While most studies have focused on differences in metabolites between CF and non-CF subjects, several studies have also focused on PEx. The present systematic review has synthesized data from the available literature to determine if there are promising metabolites across the various biospecimens to be evaluated further as clinical biomarkers in the prediction and diagnosis of PEx.

# MATERIALS AND METHODS

## Study Eligibility

To define the review question, the PICO framework for diagnostic tests was implemented (4). Our population of interest was patients who had a confirmed diagnosis of CF. The investigated test of interest was the analysis of metabolites as a predictive or diagnostic biomarker. A comparator test was not applicable for this review. The outcome was the prediction or diagnosis of a PEx based on various research criteria, such as Fuchs, Rosenfeld, or physician decision to begin antibiotic treatment (5, 6).

Studies that collected biospecimens for the purpose of analyzing metabolites were included in this review. We broadly included studies that used metabolites to predict or diagnose PEx in CF patients. Studies that only examined metabolite changes throughout PEx treatment were excluded. Furthermore, studies were excluded if they solely focused on laboratory samples (e.g., *in vitro* studies). Studies were also excluded if they used genomic, proteomic, or microbiome approaches without addressing metabolites. A detailed summary of study characteristics is presented in **Table 1**.

## Search Strategy

The search strategy and protocol were registered through PROSPERO [CRD42021269309]. Our study followed the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) guidelines for the search strategy, text screening, and data extraction (32). A health sciences librarian advised on the search strategy throughout the piloting process. The primary author (AVN) conducted the last search on August 26, 2021. An example of the search terms used and a completed search can be found in **Supplementary Table 1** (Appendix). Alerts for newly published literature were sent to the primary author. Searches for original literature were completed through MEDLINE *via* Ovid, EMBASE *via* Ovid, and all databases on Web of Science with no restrictions on language, publication date, or publication status. The search strategy was adapted for each database. Gray literature was collected through Google Scholar. The authors decided to arbitrarily limit citations on Google Scholar to the first 200 results based on recommendations by Haddaway et al. (33). Case reports and case series were excluded from the review.

## Data Collection Process

A data collection form was created and modified based on recommendations from the PRISMA guidelines and included study details such as study design, patient demographics, metabolite processing methodology, statistical analysis methodology, sample source, identifiable metabolite nomenclature, metabolite concentration data, diagnostic accuracy data, association to inflammation, and risk of bias using Quality Assessment of Diagnostic Accuracy Studies (QUADAS-2) (34). The data extraction form was piloted on five studies and adjusted accordingly. Two independent reviewers (AVN and DH) extracted data from the eligible full-texts and proceeded to reach consensus through discussion and input from a third reviewer (BSQ).

## Synthesis of Results

Due to the heterogeneity of the included studies, a meta-analysis was not feasible. Details of the included studies are demonstrated in **Table 1**, including study designs and patient characteristics. A tabulated summary of studied metabolites that were classified based on their chemical taxonomy is presented in **Table 2**. The biospecimen types and analytical platforms used to measure the metabolites are detailed in **Table 3**. Furthermore, we included information on whether these unique metabolites were used to predict or diagnose PEx, the sample source, the direction of change in concentrations between disease states, and any statistical significance in comparisons between disease states. Metabolites that appeared in two or more studies are summarized in **Table 4**. Metabolite ratios and broad pathways implicated in

**TABLE 1 |** Study design details for the included studies.

| Author | Country | Single or multi-center | Paired or independent groups | HC vs. SCF | # Total participants (HC, SCF, PEx) | Adult, pediatric, or both | Age Mean (SD) Median [IQR] | PEx $FEV_1$ (%) Mean (SD) Median [IQR] | PEx diagnostic criteria |
|---|---|---|---|---|---|---|---|---|---|
| Alvarez et al. (7) | United States | Single | Independent | HC | 52 (28, 0, 24) | Adult | 28.5 (7.5) | 45.0 (31.0) | P |
| Barr et al. (8) | UK | Multi-center | Paired | SCF | 89 (0, 29, 60) | Adult | 29.3 (10.4) | 47.2 (16.9) | R |
| Cantin et al. (9) | Canada | Single | Paired and separate (subgroups of larger cohort were paired) | Both | 75 (47, 16, 12) | Adult | 15.1 (8.1) | — | R |
| Felton et al. (10) | United States | Single | Paired | SCF | 27 (0, 0, 27) | Pediatric | 10.0 (—) | 81.7 (19.4) | R |
| Ghorbani et al. (11) | Canada | Single | Independent | SCF | 20 (0, 11, 9) | Pediatric | 13.4 (2.7) | 52.9 (11.9) | — |
| Grasemann et al. (12) | Germany | Single | Independent | Both | 92 (53, 18, 21) | Both | 19.6 (8.6) | 35.9 (16.0) | R |
| Grasemann et al. (13) | Canada | Single | Independent | HC | 20 (10, 0, 10) | Both | 23.2 (4.2) | 32.4 (10.6) | P |
| Grasemann et al. (14) | Canada | Single | Independent | Both | 45 (11, 16, 18) | Pediatric | 13.7 (—) | 52.5 (—) | P |
| Grasemann et al. (15) | Canada | Single | Independent | Both | 40 (10, 10, 20) | Both | 14.8 (2.9) | 53.1 (—) | — |
| Hanusch et al. (16) | Germany | Single | Independent | Both | 148 (78, 24, 46) | Pediatric | 11.7 [8–14] | 81.7 (18.3) | S |
| Ho et al. (17) | UK | Single | Independent | Both | 46 (0, 36, 10) | Adult | 26.8 (0.3) | — | S |
| Lagrange-Puget et al. (18) | France | Multi-center | Paired | Both | 312 (53, 312, 312)[a] | Both | 16.0 (—) | — | P |
| Linnane et al. (19) | Ireland | Single | Independent | Both | 47 (9, 13, 25) | Adult | 24.7 (4.6) | 34.0 (10.0) | — |
| Lucca et al. (20) | Italy | Single | Independent | SCF | 50 (16, 21, 13) | Pediatric | 14.2 (3.1) | 80.8 (10.2) | P |
| McGrath et al. (21) | UK | Single | Independent | Both | 24 (12, 0, 12) | Adult | 25.0 (—) | 1.6 (0.3)*† | S |
| Montuschi et al. (22) | Italy | Single | Independent | Both | 84 (31, 29, 24) | Both | 14.7 (0.8) | 75.7 (3.4)* | S |
| Quinn et al. (23) | United States | Single | Paired | SCF | 6 (0, 6, 6)[a] | Adult | — | — | R |
| Raghuvanshi et al. (24) | United States | Single | Paired | SCF | 6 (0, 6, 6)[a] | Adult | 32.7 (7.8) | — | S |
| Topcu et al. (25) | Turkey | Single | Independent | Both | 45 (17, 9, 19) | Pediatric | 11.3 (3.0) | 61.0 (26.9) | S |
| Twomey et al. (26) | UK | Single | Independent | SCF | 80 (5, 0, 75) | Adult | 28.3 (—) | — | R |
| vanHorck et al. (27) | Netherlands | Multi-center | Paired | SCF | 49 (0, 11, 38) | Pediatric | 10.3 (3.6) | — | R |
| Vazquez et al. (28) | United States | Single | Independent | SCF | 28 (—) | — | — | — | — |
| Wojewodka et al. (29) | Canada | Single | Paired | Both | 52 (0, 15, 37) | Both | 32.8 (1.8) | 56.8 (4.6)* | S |
| Zang et al. (30) | United States | Single | Paired | SCF | 26 (0, 17, 9) | Both | 27.0 (8.0) | — | S, P |
| Zang et al. (31) | United States | Single | Paired | SCF | 138 (0, 97, 41) | Both | 26.8 (—) | — | R |

[a] *Paired samples were used in both states.*

*HC, Healthy controls; SCF, Stable CF; P, Physician-defined criteria; R, Researched diagnostic criteria (e.g., Fuchs, Rosenfeld, etc.); S, Study-specific criteria; SD, Standard deviation; IQR, Interquartile range; vs, versus.*

** = Reported in standard error of the mean (SEM).*

*† = Reported in liters (L).*

PEx that were identified within studies were not included in our summary. A summary of the risk of bias using QUADAS-2 can be accessed in **Supplementary Table 2** (**Appendix**).

# RESULTS

## Study Selection

Initially, 9055 search results were uploaded onto Covidence for screening. A combined 3974 relevant abstracts were identified after duplicates were automatically removed. Authors were contacted for full-texts or additional information. 115 full texts were assessed for eligibility prior to data extraction. Of these 115, one was added through an updated search alert. The final 25 studies underwent full data extraction and risk of bias assessment. Three studies were deemed to be predictive and 22 studies were diagnostic. The study selection process is illustrated in **Figure 1** through a PRISMA diagram.

**TABLE 2 |** Metabolites evaluated in the included studies.

| Class | Biomarker(s) |
|---|---|
| Benzoic acids and substituted derivatives | Hippurate (7) |
| Carboxylic acids and derivatives | Acetic acid/acetate (22, 31), ADMA (asymmetric dimethylarginine) (14, 16), alanine (13), arginine (7, 13, 16), asparagine (13), citrulline (13, 16), cysteine (13), desmosine, glutamic acid/glutamate (7, 13), glutamine (7), glutathione (18), glycine (13), histidine (7, 13), isoleucine (13), kynurenine (28), leucine (13), lysine (7, 13), methionine (7, 13), ornithine (13), phenylalanine (7, 13), proline (7, 13), prolylhydroxyproline (31), pyroglutamic acid/oxoproline (7, 30, 31), SDMA (symmetric dimethylarginine) (14), serine (13), sulphydryls (21), threonine (7, 13, 28), tryptophan (7, 13), tyrosine (7, 13), valine (13) |
| Diazines | Dihydrothymine (31) |
| Dihydrofurans | Ascorbic acid/ascorbate (21) |
| Fatty acyls | 3-methylglutaconic acid (31), 2-methylglutaconic acid (31), 2-hexenedioic acid (31), 3-hexenedioic acid (31), nonanedioic acid/azelaic acid (31), sebacic acid (31), palmitate (26), rhamnolipids (24) |
| Glycerophosphocholines | Diacylglycerophosphocholine lipid PC (18:0/3:1) (23) |
| Hydroxy acids and derivatives | Lactic acid/lactate (26, 30, 31) |
| Imidazopyrimidines | Hypoxanthine (7), uric acid (7) |
| Keto acids and derivatives | Levulinic acid (31), pyruvate (26) |
| Lactones | γ-butyrolactone/oxolan-3-one (31) |
| Non-metal oxoanionic compounds | Nitrate (12, 16), nitrite (12, 16) |
| Organonitrogen compounds | Carnitine (7), putrescine (15, 26), spermidine (15), spermine (15) |
| Organooxygen compounds | 4-hydroxycyclohexylcarboxylic acid (30, 31), 4-hydroxycyclohexylacetic acid (31), acetone (22), docosahexaenoic acid (DHA) (29), glucose (7), lipid peroxides (18), malondialdehyde (MDA) (18, 21), sialic acid (9) |
| Other non-metal organides | Nitric oxide (NO) (17, 19) |
| Prenol lipids | α-carotene (18, 21), β-carotene (18, 21), α-tocopherol (21), γ-tocopherol (21), lycopene (18, 21), retinal (21), retinol/vitamin A (18), vitamin E (18), ubiquinol 10 (21), zeaxanthine (18) |
| Quinolines and derivatives | 4-hydroxy-2-heptyl quinolone (24), 4-hydroxy-2-nonylquinolone (NHQ) (24) |

## Study Designs

Details of eligible studies are shown in **Table 1**. 13/25 (52%) studies were conducted in North America and the remainder were completed in Europe. 13/25 (52%) of the studies had <50 total participants. 14/25 (56%) studies followed the same patients during stable and PEx visits (paired samples). Two studies only used non-CF healthy controls instead of stable CF patients. The remaining studies collected data in both non-CF healthy individuals and stable CF patients. 8/25 (32%) of the eligible studies defined PEx diagnosis using previously published criteria (e.g., Fuchs, Rosenfeld, etc.) while the remaining studies used a variety of criteria such as physician-defined criteria, the decision to use intravenous antibiotics, or specific symptoms or tests. Most of the studies (22/25, 88%) in this present review examined metabolites for the diagnosis of PEx, as detailed in **Table 1**. Diagnostic validity concepts such as sensitivity and specificity of metabolites were only analyzed in three eligible studies.

## Biospecimen Types and Metabolite Methodologies

Biospecimen types and methods of metabolite identification and analysis varied among studies (**Table 3**). Out of the 25 included studies, 11/25 (44%) analyzed sputum, 10/25 (40%) plasma and/or serum, 5/25 (20%) exhaled breath condensate (EBC), and 2/25 (8%) urine samples. Within certain studies, multiple analytical platforms were used for various metabolites. For at least one included metabolite within a study, 4/25 (16%) used liquid chromatography with tandem mass spectrometry (LC-MS-MS), 3/25 (12%) used LC-MS, 4/25 (16%) used gas chromatography in some capacity with either MS or another apparatus, 6/25 (24%) used high-performance liquid chromatography (HPLC), and 4/25 (16%) used ultra-performance liquid chromatography (UPLC). Other analytical platforms were variable. A list of metabolites from the eligible studies categorized according to class as outlined by the Human Metabolome Database (HMDB) (35) is detailed in **Table 2**. Only named metabolites were included in the table, whereas compounds with only molecular formulas were not.

## Patient Characteristics From Included Studies

Patient characteristics of the included studies are detailed in **Table 3**. Seven studies focused on pediatric patients (<18 years old), nine studies included solely adult patients, and 8 studies included both populations studies included both populations. Few of the studies reported metabolites found in adults and pediatric patients separately. $FEV_1$ values for both stable CF and PEx visits were not reported in every study. In **Table 3**, the $FEV_1$ values represent only the PEx visits, which ranged from 32 to 82% across all studies.

## Risk of Bias Assessment

Using QUADAS-2, the risk of bias of eligible studies was determined by two independent reviewers (AVN and DH). Summary of the risk of bias and applicability concerns can be found in **Supplementary Table 2** (**Appendix**). 10/25 (40%)

**TABLE 3 |** Metabolite sample sources and analytical methodologies.

| Author | Sample source(s) | Analytical platform(s) used | Fasted status |
|---|---|---|---|
| Alvarez et al. (7) | Plasma | LC-MS | No |
| Barr et al. (8) | Sputum, blood, urine | LC-MS/MS | — |
| Cantin et al. (9) | Plasma | Colorimetric assay | — |
| Felton et al. (10) | Sputum | DNA quantification | — |
| Ghorbani et al. (11) | Sputum | GC | — |
| Grasemann et al. (12) | Sputum, saliva | Colorimetric assay | — |
| Grasemann et al. (13) | Plasma | ELISA, ion exchange chromatography | Yes |
| Grasemann et al. (14) | Sputum | LC-MS | — |
| Grasemann et al. (15) | Sputum | HPLC, LC-MS/MS | — |
| Hanusch et al. (16) | Plasma, urine, sputum | GC-MS, GC-MS/MS, HPLC | No |
| Ho et al. (17) | EBC | Chemiluminescence analysis | — |
| Lagrange-Puget et al. (18) | Plasma | HPLC, HPLC-UV | — |
| Linnane et al. (19) | Sputum | Chemiluminescence analysis | — |
| Lucca et al. (20) | EBC | UPLC-ESI-MS/MS | — |
| McGrath et al. (21) | Plasma | HPLC | — |
| Montuschi et al. (22) | EBC | H-NMR, TOCSY | Yes |
| Quinn et al. (23) | Sputum | LC-MS/MS | — |
| Raghuvanshi et al. (24) | Sputum | UPLC | — |
| Topcu et al. (25) | Plasma | LC-MS | — |
| Twomey et al. (26) | Sputum | LC-MS/MS, HPLC | — |
| van Horck et al. (27) | EBC | GC-MS | — |
| Vazquez et al. (28) | Plasma | HPLC | — |
| Wojewodka et al. (29) | Plasma | GC-MS, ELISA | — |
| Zang et al. (30) | EBC | LC-MS, UPLC-MS | — |
| Zang et al. (31) | EBC | CCS, MS/MS, UPLC-MS | — |

*EBC, Exhaled breath condensate; CCS, Collision cross-section; ELISA, Enzyme-linked immunosorbent assay; GC, Gas chromatography; H-NMR, Proton nuclear magnetic resonance; LC, Liquid chromatography; MS, Mass spectroscopy; HPLC, High performance liquid chromatography; UPLC, Ultra performance liquid chromatography; TOCSY, Total correlation spectroscopy spectra.*

of the studies had two or more domains within the risk of bias assessment rated as either "High risk" or "Unclear" and most studies demonstrated high risk of bias for the index test as the index test assessor, typically whoever was retrieving or analyzing the sample, was not blinded. Most included studies (18/25; 72%) did not pose any applicability concerns in any of the domains. Three studies (23–25) were deemed as high risk for applicability in the patient selection domain and only one (24) was deemed as high risk within the index test applicability domain. Since most of the studies were not explicitly designed to be diagnostic accuracy studies, some QUADAS-2 signaling questions were not applicable. In this case, the reviewers indicated the prompt as "Unclear." Depending on the answers of the other domain-specific signaling questions, the reviewers would determine if there was a risk of bias despite the "Unclear" response.

## Biomarkers for Predicting and Diagnosing PEx

We identified 19 unique metabolites that were measured by two or more studies (**Table 4**). Metabolites were deemed to have potential for either prediction or diagnosis of PEx if: (1) the changes had consistent directionality in at least two studies within the same sample type (regardless of statistical significance) and at least one of the two studies compared PEx to stable CF samples as opposed to healthy controls as the latter comparison is more likely to be confounded by other factors; or (2) there was statistical significance within at least one study when comparing PEx to stable CF samples.

## Biomarkers to Predict PEx

Just three studies evaluated metabolites to predict PEx in CF and two were performed by the same research group (30, 31). In both studies, EBC lactic acid levels were higher in pre-PEx compared to stable CF samples. Zang et al. (30) also found statistical significantly higher levels of EBC pyroglutamic acid in pre-PEx compared to paired stable samples. Zang et al. (31) evaluated other metabolites from EBC including arginine, lysine, proline, glutamic acid, and 4-HCHC, but these were not found to be significantly different in pre-PEx compared to paired stable CF samples.

## Biomarkers to Diagnose PEx

Several metabolites from plasma and airway samples (sputum, EBC) were deemed to have potential as biomarkers to diagnose PEx. Plasma methionine was found to be lower in PEx compared to stable CF samples in two studies (7, 13), with one comparison being statistically significant (7). Three studies (7, 13, 16) found

**TABLE 4 |** Metabolites analyzed in two or more studies from various biospecimens to predict or diagnose PEx.

| | | | | PEx metabolite levels relative to healthy controls or stable CF | |
|---|---|---|---|---|---|
| **Metabolites** | **Sample source** | **Study** | **Predictive or Diagnostic** | **PEx vs. Healthy controls** | **PEx vs. Stable CF** |
| Arginine | EBC | Zang et al. (31) | Predictive | | Higher (paired)[a] |
| | Sputum | Hanusch et al. (16) | Diagnostic | | Higher |
| | Plasma | Hanusch et al. (16) | Diagnostic | | Lower |
| | Plasma | Alvarez et al. (7) | Diagnostic | Lower | |
| | Plasma | Grasemann et al. (13) | Diagnostic | Lower* | |
| ADMA (extracellular) | Sputum | Grasemann et al. (14) | Diagnostic | Higher | Higher |
| ADMA (intracellular) | Sputum | Grasemann et al. (14) | Diagnostic | Higher | Higher |
| ADMA | Sputum | Hanusch et al. (16) | Diagnostic | | No change |
| | Plasma | Hanusch et al. (16) | Diagnostic | | No change |
| | Urine | Hanusch et al. (16) | Diagnostic | | No change |
| Carnitine | EBC | Zang et al. (31) | Diagnostic | | Higher (paired) |
| | Plasma | Alvarez et al. (7) | Diagnostic | Higher* | |
| Citrulline | Plasma | Grasemann et al. (13) | Diagnostic | Higher | |
| | Plasma | Hanusch et al. (16) | Diagnostic | | Lower |
| Glutamate/glutamic acid | EBC | Zang et al. (31) | Predictive | | Higher (paired)[a] |
| | Plasma | Grasemann et al. (13) | Diagnostic | Higher** | |
| | Plasma | Alvarez et al. (7) | Diagnostic | No change | |
| Histidine | Plasma | Alvarez et al. (7) | Diagnostic | Lower** | |
| | Plasma | Grasemann et al. (13) | Diagnostic | Lower | |
| 4-hydroxycyclohexylcarboxylic acid (4-HCHC) | EBC | Zang et al. (31) | Predictive | | Higher (paired)[a] |
| | EBC | Zang et al. (30) | Diagnostic | | Lower (paired) |
| | EBC | Zang et al. (31) | Diagnostic | | Higher (paired)** |
| Lactic acid/lactate | EBC | Zang et al. (30) | Predictive | | Higher (paired)*[a] |
| | EBC | Zang et al. (31) | Predictive | | Higher (paired)[a] |
| | EBC | Zang et al. (31) | Diagnostic | | Higher (paired)** |
| | Sputum | Twomey et al. (26) | Diagnostic | | Higher[†] |
| Lysine | EBC | Zang et al. (31) | Predictive | | Higher (paired)[a] |
| | Plasma | Alvarez et al. (7) | Diagnostic | Lower* | |
| | Plasma | Grasemann et al. (13) | Diagnostic | No change | |
| Malondialdehyde | Plasma | Lagrange-Puget et al. (18) | Diagnostic | | Lower (paired)[†] |
| | Plasma | McGrath et al. (21) | Diagnostic | Higher | |
| Methionine | Plasma | Alvarez et al. (7) | Diagnostic | | Lower* |
| | Plasma | Grasemann et al. (13) | Diagnostic | | Lower |
| Nitric oxide (NO) | EBC | Linnane et al. (19) | Diagnostic | Lower* | No change |
| | EBC | Ho et al. (17) | Diagnostic | No change | 7/10 same or lower than stable CF; 3/10 higher |
| Nitrate | Sputum | Hanusch et al. (16) | Diagnostic | | Lower |
| | Sputum | Grasemann et al. (12) | Diagnostic | Higher | Lower |
| | Saliva | Grasemann et al. (12) | Diagnostic | Higher | Higher |
| | Plasma | Hanusch et al. (16) | Diagnostic | | No change |
| | Urine | Hanusch et al. (16) | Diagnostic | | No change |
| Nitrite | Sputum | Grasemann et al. (12) | Diagnostic | | Lower |
| | Sputum | Hanusch et al. (16) | Diagnostic | | Higher |
| | Saliva | Grasemann et al. (12) | Diagnostic | Higher | Lower |

*(Continued)*

**TABLE 4 |** Continued

| | | | | PEx metabolite levels relative to healthy controls or stable CF | |
|---|---|---|---|---|---|
| **Metabolites** | **Sample source** | **Study** | **Predictive or Diagnostic** | **PEx vs. Healthy controls** | **PEx vs. Stable CF** |
| | Plasma | Hanusch et al. (16) | Diagnostic | | No change |
| | Urine | Hanusch et al. (16) | Diagnostic | | No change |
| Phenylalanine | Plasma | Alvarez et al. (7) | Diagnostic | Lower | |
| | Plasma | Grasemann et al. (13) | Diagnostic | Lower | |
| Proline | EBC | Zang et al. (31) | Predictive | | Lower (paired)[a] |
| | Plasma | Alvarez et al. (7) | Diagnostic | No change | |
| | Plasma | Grasemann et al. (13) | Diagnostic | No change | |
| Putrescine | Sputum | Grasemann et al. (15) | Diagnostic | Higher* | Higher |
| | Sputum | Twomey et al. (26) | Diagnostic | Higher | Higher[†] |
| Pyroglutamic acid, oxoproline | EBC | Zang et al. (30) | Predictive | | Higher (paired)*[a] |
| | EBC | Zang et al. (31) | Diagnostic | | Higher (paired) |
| | Plasma | Alvarez et al. (7) | Diagnostic | Lower* | |
| Tryptophan | EBC | Zang et al. (31) | Diagnostic | | Lower |
| | Plasma | Grasemann et al. (13) | Diagnostic | Lower** | |
| | Plasma | Alvarez et al. (7) | Diagnostic | Lower* | |

[a] *Examined using pre-PEx samples.*
**p-value ≦ 0.05.*
***p-value ≦ 0.01.*
[†] *p-value ≦ 0.001.*
*vs, versus; ADMA, asymmetric dimethylarginine; EBC, exhaled breath condensate.*

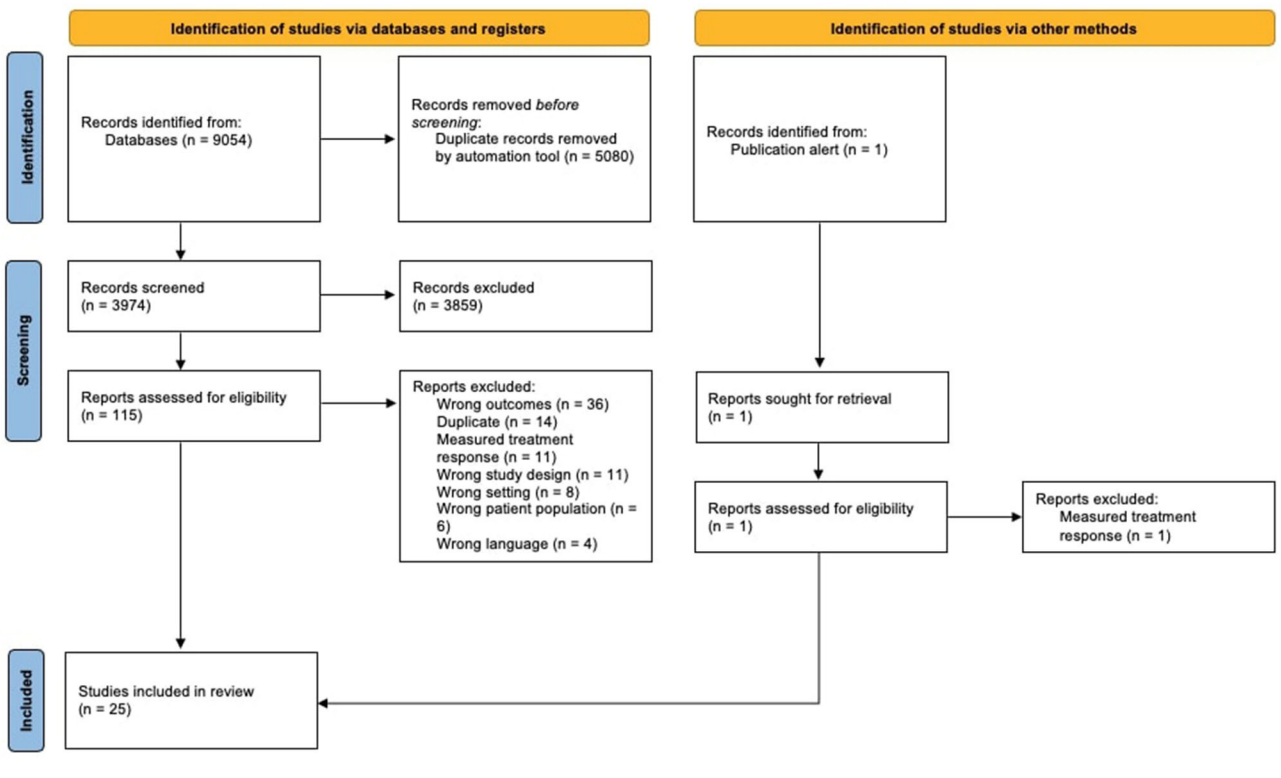


**FIGURE 1 |** PRISMA (Preferred Reporting Items for Systematic Review and Meta-Analysis) flow diagram detailing study selection process.

lower plasma arginine levels in PEx compared to healthy control or stable CF samples, but only the comparison to healthy controls was statistically significant (13). Plasma histidine, phenylalanine, and tryptophan levels were lower in PEx compared to healthy control samples in two separate studies (7, 13) but these were not compared to stable CF samples. Plasma carnitine (7, 31) and glutamate (7, 13) were significantly higher and plasma lysine and pyroglutamic acid levels were significantly lower in PEx compared to healthy control samples in one study (7) each but once again these were not compared to stable CF samples.

In terms of EBC, 4-HCHC and lactic acid levels were significantly higher in PEx compared to paired stable CF samples and both were evaluated in the same study (31). EBC NO levels were significantly lower in PEx compared to healthy control samples in one study (19) but was not compared to stable CF samples. For sputum, putrescine was consistently higher in PEx compared to healthly control and stable CF samples (15, 26) but only the comparison to healthy control samples was statistically significant. Sputum nitrate was consistently lower in PEx compared to stable CF samples in two studies, but the differences were not statistically significant (12, 16).

# DISCUSSION

By applying metabolomic biomarkers to track disease activity, there is the potential to diagnose PEx earlier in its course. Thus, PEx management can be enacted earlier to prevent long-term and irreversible lung damage. While individual studies have evaluated metabolites to either predict or diagnose PEx in CF, this systematic review fills a gap in the literature by synthesizing all of the published studies to date to identify promising metabolites in need for further study.

## Heterogeneity and Bias in Study Designs

Despite some metabolites being evaluated in two or more studies, heterogeneity in study designs posed a challenge in synthesizing the results. While some studies used paired samples from individual patients during different disease states (i.e., stable vs. PEx), other studies used independent groups of healthy or CF patients to serve as stable controls. Use of independent groups is more vulnerable to confounding by factors other than disease state that might be driving the group differences in metabolite profiles.

Other considerations must also be made when studying the volatile nature of metabolites. One concern is the lack of a fasting protocol in most studies. The associated vitamins, minerals and macronutrients contained in food can impact the accuracy of test samples, especially when these same metabolites were the primary analyte of interest (36). The inconsistency across studies may impact the concentration of metabolites. Another consideration is the timeframe between sample collection and treatment. Antibiotics are routinely used for PEx treatment and may impact the metabolome during the initial treatment period. Thus, studies should ensure that samples are taken prior to any intervention and with uniform fasting protocols.

Another aspect to be considered is the reporting of blinding during index and reference testing. While it may be difficult to blind research coordinators collecting samples from CF or non-CF patients, those who process and analyze the samples should be blinded to the patient's disease state. One randomized pilot study did blind researchers during sample anaylsis (7).

Furthermore, existing studies have been conducted using a wide variety of sample sources and analytical methods to identify and quantify the metabolites. While there are advantages in collecting samples such as urine and blood due to ease of collection across most age groups and translation into clinical practice (37), sensitivity and specificity are potential concerns when monitoring lung disease status as detected metabolites could arise from other organs. Due to the wide array of metabolites and dynamic range of concentrations, no single analytical tool is capable of measuring all metabolites which is reflected in the diversity of platforms examined. As such, several of the included studies used two or more complementary analytical platforms. The most widely used analytical approaches were LC- and GC-MS, as well as ultra-performance LC (UPLC) and high-performance LC (HPLC), the latter separation techniques sometimes used in combination with MS to improve sensitivity and analyte resolution (38, 39). An advantage of LC-MS is that it does not require chemical pre-processing and can detect a broad range of metabolites which makes it ideal for untargeted discovery analysis. While GC-MS is more quantitative with excellent separation reproducibility and lower running costs than LC-MS, it is limited to the measurement of thermally stable and volatile compounds (40). Sample preparation and metabolite extraction differences across these platforms can pose a challenge in making comparisons across studies (41). As such, future studies must continue to explore the differences and similarities in metabolites across the various biological compartments and factor in the analytical platform used.

## Synthesis of Metabolite Results

Metabolites from the nitric oxide pathway have been the most extensively studied to date. Based on available data, it remains unclear if exhaled NO is lower during PEx compared to stable state as the studies performed to date have been relatively small and the findings have been inconsistent (17, 19). Metabolites of NO, including nitrate and nitrite, do not appear to be consistently higher or lower during PEx when sampled from saliva, plasma or urine but nitrates were lower in sputum in two studies but the differences were not statistically significant when compared to stable CF subjects (12, 16). Arginine, an amino acid that acts as a substrate to form NO, was not significantly higher in sputum during PEx (16) but another study demonstrated its potential to predict PEx when elevated in EBC (31). Contrary to the increase in airway sampling, plasma arginine was lower in CF PEx compared to healthy controls (13) but was not significantly lower than stable CF samples (16).

Carboxylic acids and derivatives have also been investigated in the context of PEx. EBC pyroglutamic acid levels were higher in pre-PEx compared to stable CF samples demonstrating the potential to predict PEx (30, 31). Plasma methionine levels were lower in PEx compared to stable CF samples and has the potential to diagnose PEx (7, 13). Plasma histidine, lysine, phenylalanine, pyroglutamic acid, and tryptophan levels were found to be lower

and glutamic acid levels higher in CF PEx compared to healthy control samples but levels were not compared to stable CF (7, 13). This may reflect intrinsic differences between CF and healthy controls as opposed to differences related to PEx specifically as malnutrition in CF can lead to changes in the plasma amino acid profile compared to healthy subjects (42).

Within the organonitrogen compound class, sputum putrescine shows promise as being a diagnostic biomarker of PEx as levels were found to be higher in PEx compared to both healthy control and stable CF samples in two separate studies although the comparisons were not statistically significant due to the small sample sizes of the studies (15, 26). The potential role of ornithine-derived putrescine in PEx is unclear but it may play a role in smooth muscle regulation and anti-inflammatory processes (43). Within the organooxygen class, 4-hydroxycyclohexylcarboxylic acid (4-HCHC) from EBC was higher in PEx compared to stable CF samples. In terms of its potential role in CF pathophysiology, 4-HCHC is a rare organic acid involved in gut microbial and mammalian metabolism and its appearance in EBC is suggestive of gut-lung crosstalk with increased inflammation in both compartments (44).

Lactic acid, or lactate, has demonstrated both predictive and diagnostic potential in our review. When analyzed predictively, two studies from the same research group have found EBC lactic acid to be higher during pre-PEx compared to stable CF samples. Diagnostically, EBC and sputum lactic acid levels were higher in PEx compared to stable CF samples (26, 31). Lactic acid is a fermentation metabolite that might originate from anaerobic bacteria within the hypoxic airway environment. Anaerobic bacteria have been implicated in the pathophysiology of PEx (45).

### Limitations

Within this current review, there are several limitations that must be considered. Firstly, studies with statistically significant findings are more likely to be published which poses concern for publication bias similar to most systematic reviews. Furthermore, most conference abstracts were excluded during full-text review due to a lack of patient details, data presentation, and inability to assess risk of bias. Secondly, due to the heterogeneity of the study designs and objectives, we were unable to synthesize the results in a formal meta-analysis. Thus, we were limited to providing a tabulated summary of the studied metabolites and their respective directionality of change and the statistical significance of the change.

### Future Directions

With the evolving landscape of metabolomic research in CF, future studies must consider better standardization in study design, timing and method of metabolite collection and analysis, and reporting. Future studies that aim to test the diagnostic or predictive value of metabolite biomarkers in PEx should also ensure that data is collected during clearly defined disease states. The distinction between stable CF, pre-PEx, PEx, and pre-treatment and post-treatment states can be challenging but should be clearly outlined in future study designs. As well, consistency in fasting protocols across all sample types may be warranted.

Moreover, based on this review's search results, there were only two studies from the same research group that examined metabolites to help predict PEx (30, 31). This result highlights the need for more studies to consider prediction alongside diagnosis when characterizing biomarkers. While predicting PEx is understandably a more challenging clinical setting to apply biomarkers, this is the setting that will provide the most clinical utility as demonstrating differences in metabolites in settings in which there is a clear difference in clinical presentation (stable vs. PEx) is less useful but a reasonable starting point. Furthermore, comparison of metabolite levels to healthy control samples is less useful as differences may reflect differences between CF disease vs. healthy as opposed to disease activity related to PEx whereby a comparison to stable CF samples is much more relevant. Furthermore, metabolomic biomarkers for disease monitoring must also be re-evaluated in the context of CFTR modulator use as promising biomarkers, such as FeNO, can be influenced by CFTR modulators (46).

Lastly, it is important to consider how these studies can be translated into clinical practice in the long-term. For any promising metabolites to be used, they must undergo rigorous testing and validation as mandated by the Institute of Medicine to determine its appropriateness in clinical practice (47). Within this systematic review, we were unable to identify any metabolomic biomarkers that have undergone this process.

## CONCLUSION

Overall, this systematic review creates a foundation for future metabolomic biomarker research in PEx. While there are promising metabolites for predicting and diagnosing PEx that have been identified in multiple studies, further validation and exploration is needed. These studies should aim to standardize study design, metabolite collection, analysis, and reporting before clinical validation can be considered.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

## AUTHOR CONTRIBUTIONS

AVN and BQ conceptualized the research question, designed the study protocol, contributed to study screening, drafted the manuscript, revised for scientific content, and provided critical revisions. AVN, MB, and DH contributed to study screening and data extraction. AVN and DH completed risk of bias assessment. AVN, BQ, MB, and DH contributed to table design and formatting. BQ supervised the study and provided support. All authors reviewed and approved the final draft.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2022.896439/full#supplementary-material

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# Maternal Levels of Cytokines in Early Pregnancy and Risk of Autism Spectrum Disorders in Offspring

*Martin Brynge*[1], *Renee M. Gardner*[1], *Hugo Sjöqvist*[1], *Brian K. Lee*[1,2,3], *Christina Dalman*[1,4†] *and Håkan Karlsson*[5*†]

[1] Department of Global Public Health, Karolinska Institutet, Stockholm, Sweden, [2] Department of Epidemiology and Biostatistics, Drexel University School of Public Health, Philadelphia, PA, United States, [3] A.J. Drexel Autism Institute, Philadelphia, PA, United States, [4] Centre for Epidemiology and Community Medicine, Region Stockholm, Stockholm, Sweden, [5] Department of Neuroscience, Karolinska Institutet, Stockholm, Sweden

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**Edited by:**
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***Correspondence:**
Håkan Karlsson
hakan.karlsson.2@ki.se

[†] These authors have contributed equally to this work and share senior authorship

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Previous studies indicate a role of immune disturbances during early development in the etiology of autism spectrum disorders (ASD). Any potential disturbances during fetal development are best addressed by prospective evaluation of maternal markers of inflammation. Previous studies have investigated maternal cytokines, a group of powerful effectors of the immune system, with inconsistent results. In this study, we aimed to clarify the relationship between maternal cytokines and ASD by evaluating levels of 17 cytokines in first trimester maternal serum samples, from 318 mothers to ASD-cases and 429 mothers to ASD-unaffected controls, nested within the register-based Stockholm Youth Cohort. Overall, we observed no consistent associations between levels of maternal cytokines and ASD. While we observed a number of individual associations, the patterns varied across the diagnostic sub-groups. Levels above the 90th percentile of IL-1β (OR = 2.31, 95% CI 1.16–4.60), IL-7 (OR = 2.28, 95% CI 1.20–4.33), IL-13 (OR = 2.42, 95% CI 1.29–4.55), and MCP-1 (OR = 2.09, 95% CI 1.03–4.24) were associated with increased odds of ASD with co-occurring intellectual disability (ID), whereas GMCSF (OR = 2.06, 95% CI 1.03–4.11) and TNF-α (OR = 2.31, 95% CI 1.18–4.50) were associated with increased odds of ASD with ADHD but none survived correction for multiple comparisons. Also, none of the measured maternal cytokines were associated with ASD without co-occurring ID or ADHD. Implementing a data-driven approach using machine learning (Random Forest's Variable Importance measurement), we found no evidence to suggest that adding these cytokines and other markers of maternal immunity, to register-based maternal factors (e.g., psychiatric history) improves prediction of ASD. In summary, we found no robust evidence of an association between maternal immune markers during early pregnancy and ASD.

**Keywords: autism, cytokines, pregnancy, ADHD, intellectual disability**

## INTRODUCTION

Autism Spectrum Disorder (ASD) is a heterogeneous group of neurodevelopmental disorders characterized by social impairments, restricted and repetitive patterns of behavior, and atypical sensory responses (1). The diagnosis is usually made in childhood, indicating the overall importance of causal factors acting during early development (2). The liability for ASD is dominated by genetic variation at the population level, although evidence also supports significant influences from environmental factors (3). The relative contribution of genetic and environmental factors to the etiology of ASD may vary according to commonly co-occurring diagnoses such as attention deficit/hyperactivity disorder (ADHD) and intellectual disability (ID) (4).

Several factors related to the prenatal environment have been linked to ASD in observational studies (5). A number of these environmental exposures during pregnancy, such as maternal infections (6), air pollution (7), and high BMI (8), are associated with altered systemic levels of cytokines and immune function (9–11). The maternal immune system has several functions during pregnancy that are important for normal fetal development (12). It has been hypothesized that perturbations of immune signaling pathways during pregnancy alter normal trajectories of pregnancy and fetal development, increasing the risk of ASD (13). Indeed, experimental murine models strongly suggest that activation of the maternal immune system during gestation can cause behavioral abnormalities in the offspring, including autism-related phenotypes, though the validity of such animal models remains to be established (14, 15).

Cytokines are a group of powerful regulators of the immune system, secreted mainly by immune cells such as tissue resident macrophages and lymphocytes, but also by other cell types, including endothelial cells and adipocytes. Cytokines play important regulatory roles at different stages of pregnancy, e.g., embryo implantation and placental development (12, 16), and maternal serum concentrations may vary considerably over the course of pregnancy (17). Nearly all known cytokines, including the pro-inflammatory IL-6, IL-1β and TNF-α, are also produced by the placenta (16). Various pregnancy complications, including gestational diabetes and maternal infections, are associated with dysregulated release of placental cytokines (18). A change in placental cytokine secretion can be caused by the actions of maternal cytokines on placental cells, and placental immune activation may thus transfer effects of a maternal immune disturbance to the fetus (19, 20). Moreover, some cytokines have the ability to reach the fetal circulation via direct transfer across the placental barrier (21). Thus, a maternal immune perturbation may be communicated across the placenta even without the direct transmission of the underlying causative agent, e.g., an infection. An altered cytokine profile in the fetal compartment may interfere with neurodevelopmental processes, such as neuronal migration and differentiation, glial cell activation, and synaptic pruning which all rely on immune signaling molecules, to affect the normal developmental trajectory of the fetal brain (22).

Several previous studies have investigated maternal cytokines during pregnancy related to offspring risk of ASD, all use samples collected in mid-pregnancy (23–26). The results are inconsistent, with significant findings failing to replicate across studies, for example, both elevated and decreased levels of IL-4 have been reported among mothers to ASD-cases (23, 26). However, there are substantial differences related to design and methodology, and only one of the studies used clinically evaluated cases in a large population-based sample. No studies to date have measured cytokines at a time-point other than the second or third trimesters.

Cytokines and other immune markers measured in early life may be useful as biomarkers for ASD, with potential implications for early detection and development of novel preventive strategies (27–29). In this study, we investigate the relationship between maternal immune status and ASD by measuring 17 cytokines in archived first trimester maternal serum samples, using different analytical strategies. First, we estimate the associations between individual cytokines and ASD. Second, we conduct a principal component analysis (PCA) to integrate the collective information from the entire range of immune markers measured, combining data on the cytokines with previous measurements of maternal acute phase proteins (APP) (30). We use the derived principal components to investigate if the ASD-cases and controls can be separated based on their immune marker profiles. Finally, we employ a random forest variable importance analysis to investigate if information on maternal immune markers in the first trimester, collectively, can improve the prediction of ASD, when added to other known predictors of ASD, such as maternal age, psychiatric illness and sex of the child.

## MATERIALS AND METHODS

### Study Population

This study is nested within the Stockholm Youth Cohort (SYC), a population-based cohort including all individuals born in Sweden and resident in Stockholm County for ≥4 years (31) and covering all pathways to psychiatric care and habilitation services within Stockholm County for the purposes of outcome ascertainment.

The study design and collection of biological samples have been described in detail previously (30). Briefly, our source population consists of children within SYC born 1996–2000 ($n$ = 98,597). We collected archived Neonatal Dried Blood Spots (NDBS) from a national biobank at Karolinska University Hospital, Solna, from nearly all children with a diagnosis of ASD ($n$ = 1,407) and a random sample of ASD-unaffected control individuals ($n$ = 1,847) (32). For a subset of the children with available NDBS, we also collected corresponding archived serum samples from mothers to ASD cases ($n$ = 430) and mothers to controls ($n$ = 549). Ethics approval was obtained by the Stockholm regional review board (DNR 2010/1185-31/5). Individual consent was not required for this anonymized register-based study.

**TABLE 1 |** Characteristics of individuals diagnosed with ASD and unaffected individuals in the study sample[a].

| | Unaffected ($n = 429$) | ASD ($n = 318$) | $p$-value[b] | ASD only ($n = 100$) | ASD with ID ($n = 101$) | ASD with ADHD ($n = 117$) | $p$-value[c] |
|---|---|---|---|---|---|---|---|
| **Sex** | | | | | | | |
| Female | 204 (47.6%) | 69 (21.7%) | < 0.001 | 19 (19.0%) | 27 (26.7%) | 23 (19.7%) | <0.001 |
| Male | 225 (52.4%) | 249 (78.3%) | | 81 (81.0%) | 74 (73.3%) | 94 (80.3%) | |
| **Birth order** | | | | | | | |
| 1st born | 187 (43.6%) | 164 (51.6%) | 0.090 | 57 (57.0%) | 40 (39.6%) | 67 (57.3%) | 0.031 |
| 2nd born | 167 (38.9%) | 109 (34.3%) | | 30 (30.0%) | 45 (44.6%) | 34 (29.1%) | |
| 3rd or higher | 75 (17.5%) | 45 (14.2%) | | 13 (13.0%) | 16 (15.8%) | 16 (13.7%) | |
| **Maternal age (years)** | | | | | | | |
| <25 | 43 (10.0%) | 38 (11.9%) | 0.041 | 8 (8.0%) | 12 (11.9%) | 18 (15.4%) | 0.054 |
| 25–29 | 108 (25.2%) | 101 (31.8%) | | 29 (29.0%) | 27 (26.7%) | 45 (38.5%) | |
| 30–34 | 178 (41.5%) | 101 (31.8%) | | 34 (34.0%) | 37 (36.6%) | 30 (25.6%) | |
| 35–39 | 89 (20.7%) | 64 (20.1%) | | 24 (24.0%) | 19 (18.8%) | 21 (17.9%) | |
| [3] 40 | 11 (2.6%) | 14 (4.4%) | | 5 (5.0%) | 6 (5.9%) | 3 (2.6%) | |
| **Maternal psychiatric history** | | | | | | | |
| No | 289 (67.4%) | 162 (50.9%) | <0.001 | 50 (50.0%) | 60 (59.4%) | 52 (44.4%) | <0.001 |
| Yes | 140 (32.6%) | 156 (49.1%) | | 50 (50.0%) | 41 (40.6%) | 65 (55.6%) | |
| **Maternal BMI** | | | | | | | |
| Underweight | 8 (1.9%) | 7 (2.2%) | 0.057 | 2 (2.0%) | 3 (3.0%) | 2 (1.7%) | 0.005 |
| Normal | 213 (49.7%) | 126 (39.6%) | | 49 (49.0%) | 43 (42.6%) | 34 (29.1%) | |
| Overweight | 59 (13.8%) | 54 (17.0%) | | 11 (11.0%) | 18 (17.8%) | 25 (21.4%) | |
| Obese | 16 (3.7%) | 21 (6.6%) | | 2 (2.0%) | 6 (5.9%) | 13 (11.1%) | |
| Missing | 133 (31.0%) | 110 (34.6%) | | 36 (36.0%) | 31 (30.7%) | 43 (36.8%) | |
| **Maternal region of birth** | | | | | | | |
| Africa | 18 (4.2%) | 16 (5.0%) | 0.89 | 3 (3.0%) | 12 (11.9%) | 1 (0.9%) | <0.001 |
| Asia | 34 (7.9%) | 27 (8.5%) | | 8 (8.0%) | 18 (17.8%) | 1 (0.9%) | |
| Nordic | 349 (81.4%) | 250 (78.6%) | | 83 (83.0%) | 61 (60.4%) | 106 (90.6%) | |
| Other | 15 (3.5%) | 12 (3.8%) | | 4 (4.0%) | 4 (4.0%) | 4 (3.4%) | |
| Other Europe | 13 (3.0%) | 13 (4.1%) | | 2 (2.0%) | 6 (5.9%) | 5 (4.3%) | |
| **Family income quintile** | | | | | | | |
| 1st (lowest) | 41 (9.6%) | 39 (12.3%) | 0.006 | 8 (8.0%) | 22 (21.8%) | 9 (7.7%) | <0.001 |
| 2nd | 75 (17.5%) | 81 (25.5%) | | 20 (20.0%) | 28 (27.7%) | 33 (28.2%) | |
| 3rd | 90 (21.0%) | 64 (20.1%) | | 22 (22.0%) | 17 (16.8%) | 25 (21.4%) | |
| 4th | 100 (23.3%) | 75 (23.6%) | | 18 (18.0%) | 23 (22.8%) | 34 (29.1%) | |
| 5th | 123 (28.7%) | 59 (18.6%) | | 32 (32.0%) | 11 (10.9%) | 16 (13.7%) | |
| **Maternal education at birth** | | | | | | | |
| <9 years | 55 (12.8%) | 42 (13.2%) | 0.22 | 10 (10.0%) | 13 (12.9%) | 19 (16.2%) | 0.14 |
| 9–12 years | 179 (41.7%) | 151 (47.5%) | | 41 (41.0%) | 52 (51.5%) | 58 (49.6%) | |
| >12 years | 194 (45.2%) | 124 (39.0%) | | 49 (49.0%) | 35 (34.7%) | 40 (34.2%) | |
| Missing | 1 (0.2%) | 1 (0.3%) | | | 1 (1.0%) | | |
| **Gestational week at serum sample** | | | | | | | |
| <10 weeks | 203 (47.3%) | 160 (50.3%) | 0.42 | 52 (52.0%) | 48 (47.5%) | 60 (51.3%) | 0.77 |
| 10–13 weeks | 226 (52.7%) | 158 (49.7%) | | 48 (48.0%) | 53 (52.5%) | 57 (48.7%) | |
| **Serum sampling quarter** | | | | | | | |
| 1 January–31 March | 126 (29.4%) | 98 (30.8%) | 0.33 | 30 (30.0%) | 35 (34.7%) | 33 (28.2%) | 0.54 |
| 1 April–30 June | 101 (23.5%) | 90 (28.3%) | | 34 (34.0%) | 24 (23.8%) | 32 (27.4%) | |
| 1 July–30 September | 96 (22.4%) | 64 (20.1%) | | 17 (17.0%) | 23 (22.8%) | 24 (20.5%) | |
| 1 October–31 December | 106 (24.7%) | 66 (20.8%) | | 19 (19.0%) | 19 (18.8%) | 28 (23.9%) | |

*(Continued)*

**TABLE 1** | Continued

| | Unaffected ($n = 429$) | ASD ($n = 318$) | $p$-value[b] | ASD only ($n = 100$) | ASD with ID ($n = 101$) | ASD with ADHD ($n = 117$) | $p$-value[c] |
|---|---|---|---|---|---|---|---|
| **Smoking at first antenatal visit** | | | | | | | |
| No | 297 (69.2%) | 220 (69.2%) | 1.00 | 67 (67.0%) | 74 (73.3%) | 79 (67.5%) | 0.31 |
| Yes | 27 (6.3%) | 20 (6.3%) | | 3 (3.0%) | 5 (5.0%) | 12 (10.3%) | |
| Missing | 105 (24.5%) | 78 (24.5%) | | 30 (30.0%) | 22 (21.8%) | 26 (22.2%) | |

[a] *The characteristics of the same population has been reported previously (30), https://creativecommons.org/licenses/by/4.0/.*

[b] *Pearson's chi-squared test was used for categorical variables, comparing the frequency distributions among unaffected individuals to the distributions among all ASD-affected individuals. Kruskal-Wallis tests were used for continuous variables, as the distributions of the APP concentrations were strongly skewed.*

[c] *Pearson's chi-squared test was used for categorical variables, comparing the frequency distributions among unaffected individuals to the distribution among the stratified ASD outcome groups. Kruskal-Wallis tests were used for continuous variables, as the distributions of the APP concentrations were strongly skewed.*

*ASD, autism spectrum disorders; ADHD, attention-deficit/hyperactivity disorder; ID, intellectual disability; BMI, body mass index; IQR, interquartile range.*

## Case Ascertainment

National and regional registers were used for the ascertainment of ASD, ID and ADHD, covering all pathways to care and diagnosis in Stockholm County. The case-finding procedure has been described in detail previously (31, 32). ASD was stratified by co-occurrence of ID and ADHD: ASD only (ASD without ID or ADHD), ASD with ID, and ASD with ADHD. The ASD with ID group also included individuals with co-morbid ID and ADHD.

## Laboratory Analysis

Archived maternal serum samples initially drawn as part of an antenatal screening program for maternal infections, were collected from regional biobanks at Karolinska University Hospital, Solna and Huddinge. Samples are usually, but not always, drawn at the first antenatal visit, near the end of the first trimester [median = 10.9 gestational weeks, interquartile range (IQR) 9.3–12.7]. In order to compare samples from the same stage of development, the samples were restricted to those drawn within the first trimester, resulting in a final study population of 318 ASD and 429 control mothers for the primary statistical analyses (30). After thawing on ice, samples were diluted 1:4 and analyzed for Interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, Granulocyte Colony-Stimulating Factor (GCSF), Granulocyte Monocyte Colony-Stimulating Factor (GMCSF), Interferon (IFN)-γ, Monocyte Chemoattractant Protein 1 (MCP-1), Macrophage Inflammatory Protein 1b (MIP-1β), Tumor Necrosis Factor-α (TNF-α) using the Bio-Plex Pro Human Cytokine 17-plex Assay (Bio-Rad, Hercules, CA, USA). Samples were applied randomly to 13 multiplex 96-well assay plates and analyzed using a premixed, multiplex panel on the Bio-Plex 200 System (Bio-Rad). There were two types of imputed values that resulted from samples with concentrations near or beyond the limits of quantitation (see **Supplementary Table 1**). Concentrations near the lower limit of quantitation (LLOQ) are assigned an imputed value by the BioPlex Manager software but are also marked as potentially uncertain estimates. In these cases, we used the value estimated by the software in our analyses but classified the values as imputed. Concentrations below the LLOQ (with no value estimated by the software) were assigned a value of LLOQ/$\sqrt{2}$, whereas values above the upper limit of quantitation (ULOQ) were assigned a value of ULOQ×1.1.

## Covariates

Based on previous associations with ASD, and a plausible relationship with cytokines, we considered the following covariates as potential confounders: maternal age (33), psychiatric history (34, 35), BMI (8), region of birth (36), education (34), and smoking at first antenatal visit (37), sex of fetus (31), birth order, family income (38), and gestational week and season at serum sample (39). Covariate data were extracted from the Medical Birth Register, the National Patient Register and the Integrated Database for Labor Market Research.

## Statistical Analysis

Data management and analyses were performed using Stata (v14.1) with external package xbrcspline (40), and R Statistical Software (v4.1.0), with external packages "randomForest," "ggplot2" and "factoextra" (41–44).

Due to skewed distributions, the concentrations of cytokines were log2-transformed (**Supplementary Figures 1A,B**). Plate specific standardized z-scores were created (**Supplementary Figure 1C**) to control for assay plate technical variation.

In our previous study of maternal APP in these same samples, the exposure variables were categorized into tertiles (30). In the present study, 11 of the total 17 cytokines had tertile cut-points within the non-detectable range. Since this strategy would exclude most of our analytes, we instead considered a dichotomous categorization based on the 90th percentile (≥90th percentile; <90th percentile), in order to make use of all data, and to better capture variation at the high end of the distribution. Similar analytical strategies have been used in previous studies of cytokines and ASD in archived samples (28, 45).

We tested the associations of covariates and cytokines by using a univariate linear regression model estimating mean cytokine z-scores over the categories of covariates, followed by a joint Wald-test of the overall of association between the cytokine and the covariate. Covariates were included in the models if they were even weakly associated ($p < 0.2$) with any of the outcomes and at least one of the cytokines among controls. For the cytokines

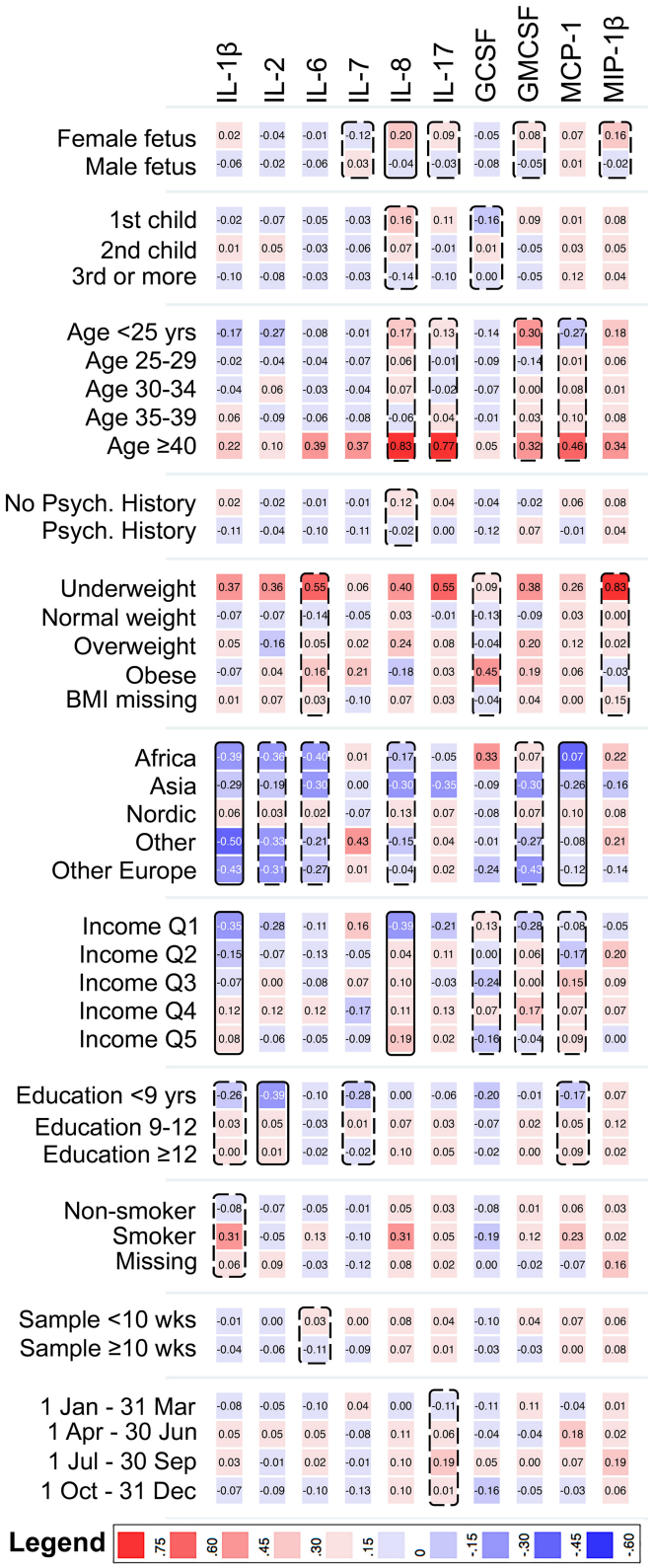


**FIGURE 1 |** Heat map showing the mean z-score of cytokines with <70% imputed values, by categories of the covariates, among mothers to 429 unaffected individuals in the cohort. Solid boxes indicate that the cytokine is associated with the covariate at $p < 0.05$. Dashed boxes indicate that the cytokine is associated with the covariate at $p < 0.20$. IL-1β, Interleukin-1β; IL-2, Interleukin-2; IL-6, Interleukin-6; IL-7, Interleukin-7; IL-8, Interleukin-8; IL-17, Interleukin-17; GCSF, Granulocyte Colony-Stimulating Factor; GMCSF, Granulocyte Monocyte Colony-Stimulating Factor; MCP-1, Monocyte Chemoattractant Protein 1; MIP-1β, Macrophage Inflammatory Protein 1β.

**TABLE 2 |** Serum sample characteristics of mothers to individuals diagnosed with ASD and mothers to unaffected individuals in the study sample.

| | Unaffected ($n = 429$) | ASD ($n = 318$) | $p$-value[a] | ASD only ($n = 100$) | ASD with ID ($n = 101$) | ASD with ADHD ($n = 117$) | $p$-value[b] |
|---|---|---|---|---|---|---|---|
| **Cytokines with <70% imputed values [median concentration (IQR)]** | | | | | | | |
| IL-1β (pg/ml) | 2.3 (0.8, 5.8) | 2.6 (1.0, 6.2) | 0.27 | 2.5 (0.9, 5.9) | 2.1 (1.2, 7.3) | 2.9 (0.9, 6.8) | 0.72 |
| IL-2 (pg/ml) | 5.4 (3.4, 9.9) | 6.0 (3.6, 10.7) | 0.20 | 6.1 (3.7, 10.3) | 5.8 (3.8, 9.6) | 6.6 (3.5, 11.3) | 0.48 |
| IL-6 (pg/ml) | 5.0 (2.6, 14.1) | 5.7 (2.6, 14.6) | 0.54 | 4.7 (2.0, 10.4) | 6.2 (2.6, 18.4) | 6.0 (2.8, 17.9) | 0.24 |
| IL-7 (pg/ml) | 7.3 (4.3, 11.6) | 7.9 (4.5, 12.9) | 0.15 | 8.4 (5.0, 13.4) | 8.0 (4.3, 15.2) | 7.9 (4.9, 12.3) | 0.46 |
| IL-8 (pg/ml) | 222.9 (25.6, 1152.0) | 164.1 (26.3, 917.3) | 0.32 | 157.9 (33.6, 697.9) | 240.8 (25.3, 1140.4) | 121.9 (25.0, 556.6) | 0.49 |
| IL-17 (pg/ml) | 18.0 (11.5, 26.9) | 17.2 (11.2, 26.9) | 0.96 | 19.8 (13.6, 29.4) | 16.4 (11.2, 30.1) | 15.8 (9.5, 25.4) | 0.21 |
| GCSF (pg/ml) | 8.3 (4.8, 12.0) | 8.7 (5.2, 12.8) | 0.30 | 8.7 (5.3, 12.9) | 7.8 (5.0, 12.4) | 9.3 (6.3, 13.4) | 0.37 |
| GMCSF (pg/ml) | 17.8 (6.0, 34.4) | 16.6 (4.9, 39.9) | 0.79 | 14.6 (3.5, 37.4) | 22.4 (6.0, 43.0) | 16.2 (6.3, 38.8) | 0.26 |
| MCP-1 (pg/ml) | 53.7 (29.2, 91.5) | 53.1 (30.1, 105.6) | 0.71 | 51.7 (28.9, 88.8) | 55.0 (32.8, 114.6) | 55.4 (31.6, 100.1) | 0.78 |
| MIP-1β (pg/ml) | 146.8 (103.9, 208.7) | 145.8 (101.6, 202.8) | 0.66 | 150.4 (97.0, 220.8) | 140.8 (103.2, 201.1) | 139.1 (101.6, 188.1) | 0.73 |
| **Cytokines with >70% imputed values [samples at or above the 90th percentile, *n* (%)]** | | | | | | | |
| IL-4 | 44 (10.3%) | 39 (12.3%) | 0.39 | 9 (9.0%) | 14 (13.9%) | 16 (13.7%) | 0.51 |
| IL-5 | 44 (10.3%) | 35 (11.0%) | 0.74 | 9 (9.0%) | 16 (15.8%) | 10 (8.5%) | 0.29 |
| IL-10 | 43 (10.0%) | 47 (14.8%) | 0.048 | 13 (13.0%) | 18 (17.8%) | 16 (13.7%) | 0.16 |
| IL-12 | 43 (10.0%) | 46 (14.5%) | 0.064 | 13 (13.0%) | 14 (13.9%) | 19 (16.2%) | 0.26 |
| IL-13 | 43 (10.0%) | 45 (14.2%) | 0.084 | 12 (12.0%) | 22 (21.8%) | 11 (9.4%) | 0.009 |
| IFN-γ | 43 (10.0%) | 39 (12.3%) | 0.33 | 8 (8.0%) | 12 (11.9%) | 19 (16.2%) | 0.19 |
| TNF-α | 43 (10.0%) | 45 (14.2%) | 0.084 | 11 (11.0%) | 13 (12.9%) | 21 (17.9%) | 0.13 |

[a] *Pearson's chi-squared test was used for categorical variables, comparing the frequency distributions among unaffected individuals to the distributions among all ASD-affected individuals. Kruskal-Wallis tests were used to compare concentrations among unaffected individuals to concentrations among all ASD-unaffected individuals.*

[b] *Pearson's chi-squared test was used for categorical variables, comparing the frequency distributions among unaffected individuals to the distribution among the stratified ASD outcome groups. Kruskal-Wallis tests were used to compare concentrations among unaffected individuals to concentrations among the stratified ASD outcome groups.*

*ASD, autism spectrum disorders; ADHD, attention-deficit/hyperactivity disorder; ID, intellectual disability; IQR, interquartile range; IL-1β, Interleukin-1β; IL-2, Interleukin-2; IL-4, Interleukin-4; IL-5, Interleukin-5; IL-6, Interleukin-6; IL-7, Interleukin-7; IL-8, Interleukin-8; IL-10, Interleukin-10; IL-12, Interleukin-12; IL-13, Interleukin-13; IL-17, Interleukin-17; GCSF, Granulocyte Colony-Stimulating Factor; GMCSF, Granulocyte Monocyte Colony-Stimulating Factor; IFN-γ, Interferon-γ MCP-1, Monocyte Chemoattractant Protein 1; MIP-1β, Macrophage Inflammatory Protein 1β; TNF-α, Tumor Necrosis Factor-α.*

with a low proportion of samples falling within the limits of detection and thus a high proportion (>70%) of imputed values (see "Laboratory Analysis" above, **Supplementary Table 1**), we instead evaluated associations with covariates by applying a chi-2 test using the dichotomous cytokine variables (based on the 90th percentile).

In the categorical analyses, we used logistic regression models to estimate the odds of ASD associated with dichotomized levels of each cytokine (based on the 90th percentile), with the lowest category (≥90th percentile) as the referent level. The analyses were repeated after stratifying on the co-occurrence of ID and ADHD. After dichotomizing the sample at the 90th percentile based on the distribution of the measured cytokines, we calculated that we would be able to detect an odds ratio of 1.92 at 80% power given the size of our study at an α level of 0.05. Because numerous statistical comparisons were made, we considered two approaches to correct for multiple comparisons. For the Bonferroni correction, the specified α ($p = 0.05$) is divided by the number of independent tests conducted. We calculated a Bonferroni-corrected $p$-value considering either 17 tests (if one considers the 17 cytokines and the main outcome of any ASD diagnosis; $p < 0.0029$) or 68 tests (if one considers the 17 cytokines across the main outcome and the three mutually exclusive outcomes; $p < 0.0007$). Bonferroni correction is considered to be highly conservative (46), and it is arguable that the tests in this case (of correlated cytokine values across related outcomes) are truly independent. Because of these issues, we also considered the Benjamini-Hochberg procedure to control the False Discovery Rate (FDR). We set the FDR, defined as the proportion of all "positive" findings (where the null hypothesis was rejected, $p < 0.05$) which are false positives (Type I errors in which the null hypothesis was incorrectly rejected), to levels of 5, 10, and 25%, respectively, and considered 68 tests.

In the continuous analyses, we used restricted cubic spline models with three knots, followed by a Wald-test of all spline variables to test for an association between the cytokine and the outcome of ASD. Only analytes with < 70% imputed values were included in these analyses.

**TABLE 3 |** The adjusted relationship between cytokines and odds of ASD, stratified by co-occurrence of ID and ADHD, when comparing mothers of 318 ASD-cases to mothers of 429 unaffected individuals selected from the cohort.

| | Any ASD [OR (LCI, UCI)] | *p*-value | ASD only [OR (LCI, UCI)] | *p*-value | ASD with ID [OR (LCI, UCI)] | *p*-value | ASD with ADHD [OR (LCI, UCI)] | *p*-value |
|---|---|---|---|---|---|---|---|---|
| IL-1β | **1.71 (1.05, 2.77)** | 0.030 | 1.51 (0.73, 3.14) | 0.271 | **2.31 (1.16, 4.60)** | **0.017** | 1.53 (0.76, 3.05) | 0.230 |
| IL-2 | 1.23 (0.74, 2.04) | 0.421 | 1.01 (0.46, 2.20) | 0.986 | 1.09 (0.50, 2.39) | 0.830 | 1.35 (0.67, 2.72) | 0.408 |
| IL-4 | 1.16 (0.70, 1.91) | 0.571 | 0.86 (0.38, 1.92) | 0.712 | 1.36 (0.67, 2.77) | 0.396 | 1.09 (0.54, 2.23) | 0.805 |
| IL-5 | 1.04 (0.63, 1.74) | 0.873 | 0.78 (0.35, 1.74) | 0.547 | 1.87 (0.94, 3.69) | 0.073 | 0.59 (0.26, 1.35) | 0.212 |
| IL-6 | **1.68 (1.03, 2.75)** | **0.037** | 1.32 (0.62, 2.83) | 0.475 | 1.85 (0.92, 3.69) | 0.082 | 1.77 (0.89, 3.52) | 0.102 |
| IL-7 | 1.23 (0.75, 2.02) | 0.420 | 0.75 (0.34, 1.69) | 0.490 | **2.28 (1.20, 4.33)** | **0.012** | 0.64 (0.28, 1.44) | 0.281 |
| IL-8 | 1.19 (0.69, 2.04) | 0.533 | 0.89 (0.37, 2.18) | 0.806 | 0.97 (0.40, 2.38) | 0.955 | 1.69 (0.82, 3.47) | 0.157 |
| IL-10 | **1.74 (1.08, 2.82)** | **0.024** | 1.65 (0.80, 3.41) | 0.175 | **2.23 (1.14, 4.34)** | **0.018** | 1.46 (0.72, 2.97) | 0.297 |
| IL-12 | 1.57 (0.97, 2.56) | 0.068 | 1.84 (0.89, 3.80) | 0.101 | 1.40 (0.68, 2.88) | 0.354 | 1.64 (0.83, 3.24) | 0.154 |
| IL-13 | 1.39 (0.86, 2.26) | 0.178 | 1.29 (0.62, 2.67) | 0.502 | **2.42 (1.29, 4.55)** | **0.006** | 0.70 (0.31, 1.57) | 0.383 |
| IL-17 | 1.14 (0.68, 1.90) | 0.626 | 1.31 (0.61, 2.81) | 0.483 | 1.32 (0.64, 2.75) | 0.453 | 0.86 (0.39, 1.92) | 0.719 |
| GCSF | 1.11 (0.67, 1.84) | 0.695 | 0.97 (0.44, 2.14) | 0.947 | 0.96 (0.44, 2.10) | 0.913 | 1.35 (0.66, 2.76) | 0.405 |
| GMCSF | 1.39 (0.84, 2.30) | 0.195 | 0.82 (0.35, 1.92) | 0.647 | 1.62 (0.78, 3.37) | 0.200 | **2.06 (1.03, 4.11)** | **0.041** |
| IFN-γ | 1.31 (0.79, 2.16) | 0.291 | 0.84 (0.36, 1.96) | 0.689 | 1.27 (0.61, 2.62) | 0.520 | 1.37 (0.68, 2.76) | 0.371 |
| MCP-1 | 1.39 (0.83, 2.34) | 0.208 | 0.78 (0.32, 1.90) | 0.584 | **2.09 (1.03, 4.24)** | **0.041** | 1.40 (0.65, 3.00) | 0.384 |
| MIP-1β | 1.21 (0.72, 2.05) | 0.467 | 1.73 (0.83, 3.61) | 0.146 | 0.80 (0.34, 1.87) | 0.604 | 1.34 (0.63, 2.86) | 0.448 |
| TNF-α | **1.79 (1.1, 2.91)** | **0.020** | 1.54 (0.71, 3.31) | 0.271 | 1.87 (0.91, 3.85) | 0.090 | **2.31 (1.18, 4.50)** | **0.014** |

*Dichotomous variables were created for each cytokine, using the distribution of z-scores among unaffected individuals to set the cut-offs, using values below the 90th percentile as the referent category. Models were adjusted for sex, maternal BMI, maternal psychiatric history, maternal region of origin, maternal age and family income quintile. p-values are shown for a Wald test with a null hypothesis that the cytokine categorical term was equal to zero, as a test of whether each cytokine was associated with the outcome. The bold values indicate the value of $p < 0.05$.*

*IL-1β, Interleukin-1β; IL-2, Interleukin-2; IL-4, Interleukin-4; IL-5, Interleukin-5; IL-6, Interleukin-6; IL-7, Interleukin-7; IL-8, Interleukin-8; IL-10, Interleukin-10; IL-12, Interleukin-12; IL-13, Interleukin-13; IL-17, Interleukin-17; GCSF, Granulocyte Colony-Stimulating Factor; GMCSF, Granulocyte Monocyte Colony-Stimulating Factor; IFN-γ, Interferon-γ MCP-1, Monocyte Chemoattractant Protein 1; MIP-1β, Macrophage Inflammatory Protein 1β; TNF-α, Tumor Necrosis Factor-α.*

The relationship between the maternal immune status and ASD is likely more complex than what can be captured by investigating one-by-one associations with individual cytokines. However, using the cytokines collectively in the same regression model is problematic, due to their high correlation/dependency and the risk of model overfitting. To achieve a more integrated view of the maternal immune status and its relationship with children's risk of ASD, and to simultaneously deal with the multiple correlated analytes, we used Principal Component Analysis (PCA) to re-construct the covariation between all the biomarkers into independent components. To explore the full range of available immune markers, we combined the cytokines measurements with the previously reported first trimester maternal APP (30), measured in the same cohort of pregnant women in the same serum samples. We used the extracted principal components (selecting those that explained >5% of variation) to examine any potential relation with ASD and the stratified outcomes, using cubic spline models with 4 knots to account for potentially non-linear relationships.

Finally, we used the Random Forest Classifier to assess the predictive performance of the biomarker measurements in comparison to other covariates and to compute the variable importance on the full sample size ($n = 747$). We used the Mean Decrease Accuracy measurement for the variable importance, which employs an algorithm to replace a variable with a randomly generated number and examine the decrease in the new prediction accuracy. To assess the predictive performance of the respective models, we randomly divided the data into a training set (2/3 of the sample) and a test set (1/3 of the sample) and ascertained the overall accuracy. We explored the performance of different sub-sets of our data by using as predictors: (a) only the non-biomarker (i.e., register-based) covariates used in the fully adjusted regression models (sex of fetus; family income quintile; maternal education, BMI, psychiatric history, region of origin, and age), (b) only the six largest PCA-components, (c) the six largest PCA-components together with the non-biomarker covariates. Because random forest models can in principle deal with a very large number of correlated covariates and therefore does not require dimension reduction techniques (e.g., extraction of principle components from PCA), we alternatively fit random forest models including as predictors: (d) the original cytokine and APP z-score variables only, and (e) the original cytokine and APP z-score variables together with the register-based covariates. We repeated these steps 1,000 times with randomized training and test sets and estimated the mean accuracy together with the 2.5th and 97.5th percentile value (i.e., 95% pseudo-bootstrap confidence interval).

## Sensitivity Analysis

In the main analyses, the samples were restricted to those drawn in the first trimester of pregnancy, in order to reduce variation in cytokines related to gestational age and improve

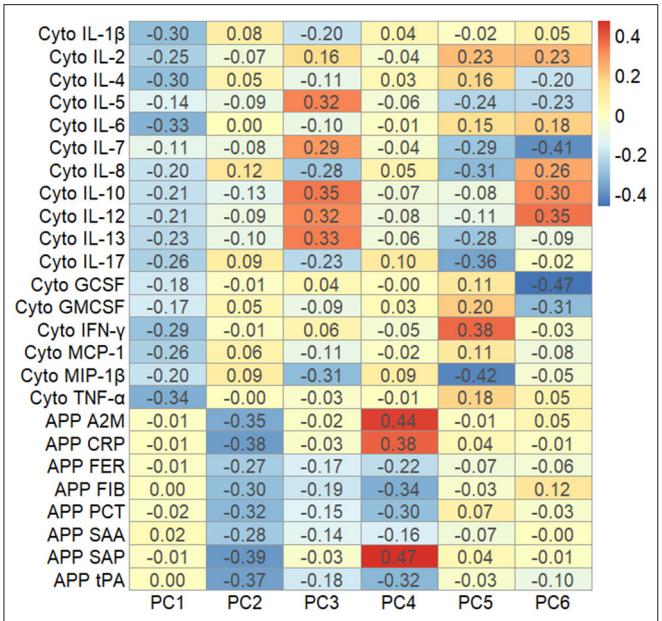


**FIGURE 2 |** Heat map showing the factor loadings of the six largest principal components (PC1-PC6) derived from the variation in z-scores of all cytokines and acute phase proteins, using samples from mothers to cases and controls drawn in the first trimester of pregnancy ($n = 747$). A2M, α-2 Macroglobulin; CRP, C-Reactive Protein; FER, Ferritin; FIB, Fibrinogen; IL-1β, Interleukin-1β; IL-2, Interleukin-2; IL-4, Interleukin-4; IL-5, Interleukin-5; IL-6, Interleukin-6; IL-7, Interleukin-7; IL-8, Interleukin-8; IL-10, Interleukin-10; IL-12, Interleukin-12; IL-13, Interleukin-13; IL-17, Interleukin-17; GCSF, Granulocyte Colony-Stimulating Factor; GMCSF, Granulocyte Monocyte Colony-Stimulating Factor; IFN-γ, Interferon-γ MCP-1, Monocyte Chemoattractant Protein 1; MIP-1β, Macrophage Inflammatory Protein 1β; PCT, Procalcitonin; SAA, Serum Amyloid A; Serum Amyloid P; tPA, Tissue Plasminogen Activator; TNF-α, Tumor Necrosis Factor-α.

interpretability. However, this resulted in the exclusion of 232 individuals for whom maternal sera were available. As a sensitivity analysis, we therefore repeated the categorical analysis (using the 90th percentile cutoff for the cytokines) including all available serum samples and additionally adjusted for gestational week at sampling (<10, 10–13, and >13 weeks). We also repeated the PCA using information from all available serum samples ($n =$ 979) and evaluated any associations with ASD.

# RESULTS

## Association of Covariates With ASD-Case Status

Compared to unaffected controls, ASD-cases were more likely to be male. Mothers to ASD-cases were more likely to be below 30 or above 40 years of age, have a history of psychiatric disease, be born outside the Nordic region, and have a lower socioeconomic status (**Table 1**).

## Quality Control Statistics for Cytokines

The proportion of imputed values (due to observations below or near the LLOQ) varied from 0% (MIP-1β) to 93.3% (IL-5) (**Supplementary Table 1**). Seven cytokines had a proportion of imputed values >70%. The average inter- and intra-assay coefficients of variation (CV) of manufacturer-provided controls were 21.2% (range 15.1–34.9%) and 6.1 (range 2.5–9.2%), respectively (**Supplementary Table 1**). Among unaffected controls, we observed moderate to high degrees of pairwise correlations between the different cytokines (**Supplementary Figure 2A**). Overall, TNF-α, IL-6 and IL-4 showed the highest pairwise correlations with the other cytokines. Similar patterns of correlation were seen when only values within the detectable range were included (**Supplementary Figure 2B**).

## Association of Cytokines With Covariates

We observed a significant ($p < 0.05$) linear relationship between IL-8, IL-17, GCSF, MCP-1 and MIP-1β, and gestational age, using all available samples ($n = 979$, **Supplementary Table 2**). Levels of IL-8, IL-17, MCP-1, and MIP-1β tended to decrease, whereas GCSF tended to increase, over the course of pregnancy. A linear relationship between IL-6 and gestational age was observed in samples restricted to the first trimester and an inverse linear association between gestational age and MIP-1β was observed in samples restricted to the 2nd and 3rd trimesters.

Among mothers to controls, sex of fetus, maternal region of birth, parental income, and maternal education, were associated with one, or more, of the cytokines at our pre-defined level to consider as a potential confounder ($p < 0.2$; **Figure 1**). The strongest associations were observed for maternal region of birth with IL-1β and MCP-1, parental income with IL-1β and IL-8, and maternal education with IL-2 ($p < 0.05$). Among mothers to ASD-cases, some of the associations overlapped with the control group (maternal region of birth with MCP-1, and income with IL-8), whereas others were unique for the ASD-case group (sex of fetus with MIP-1β, maternal smoking with IL-7, and sampling season with GCSF) (**Supplementary Figure 3**).

For the cytokines with a low proportion of samples falling within the limits of detection, all the considered covariates except maternal region of birth and sampling week were associated ($p < 0.2$) with at least one of the cytokines (testing the proportion of samples in the highest decile), among mothers to controls (**Supplementary Figure 4**). The specific patterns of association differed by case status (**Supplementary Figure 5**). For example, BMI was associated with TNF-α among mothers in the control group, but not among mothers to ASD-cases.

The covariates that met the a priori criteria for inclusion in the adjusted regression models were: sex of fetus; family income quintile; maternal education, BMI, psychiatric history, region of origin, and age. Maternal education was excluded from the models due to the high degree of correlation with parental income.

## Association of Cytokines With Odds of ASD

There were no significant differences in median concentrations of cytokines with low proportion of imputed <70% imputed values between ASD-cases and unaffected controls (**Table 2**; **Supplementary Figure 6**). For those cytokines with >70% of imputed values, the proportion of observations in the highest

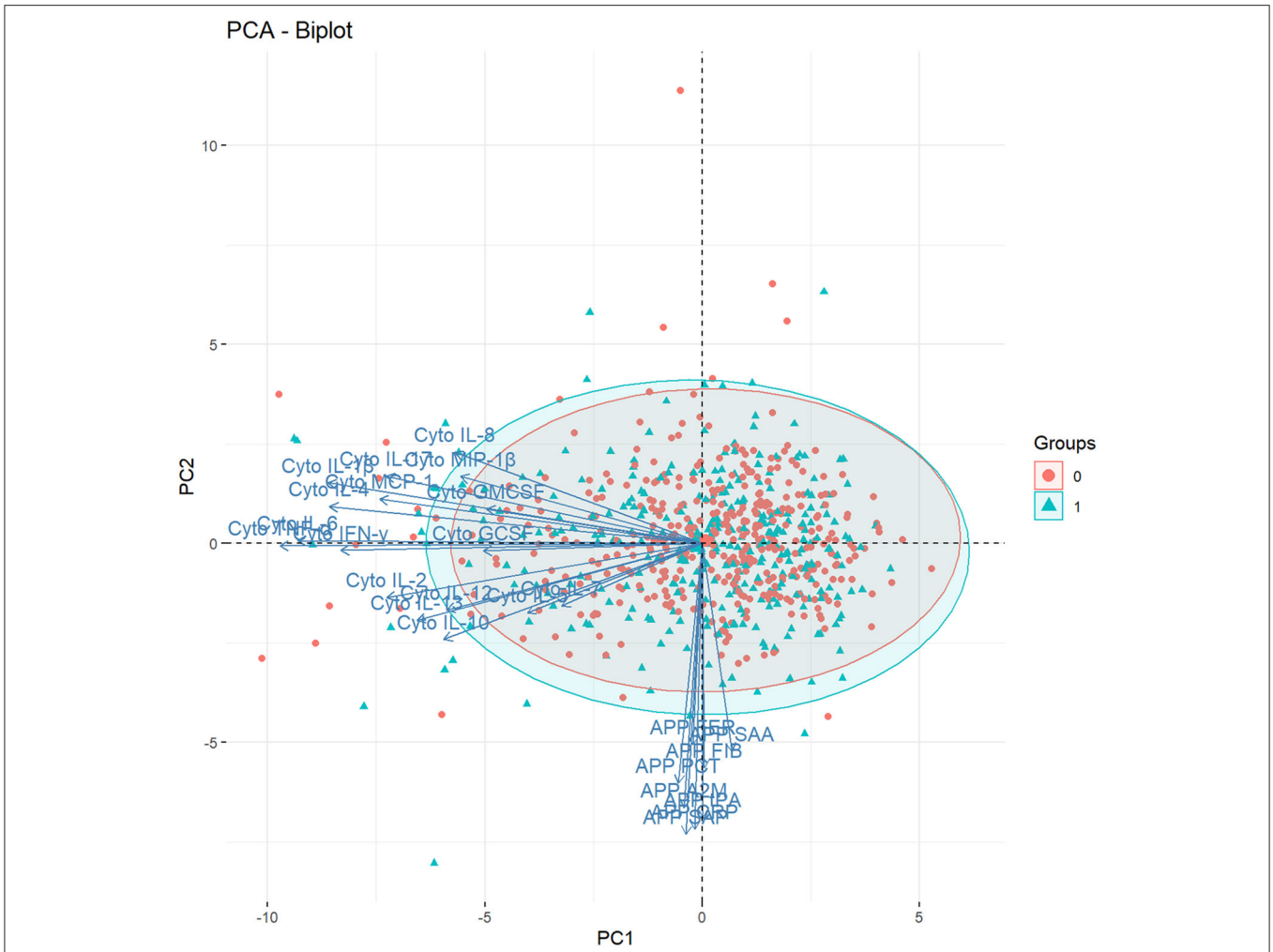


**FIGURE 3 |** Biplot showing the factor loadings by the individual cytokines and acute phase proteins on the first two principal components (PC1 and PC2), and the values of these components for each individual in the study [$n$ = 747, Groups: 0 = unaffected controls (red circles); 1 = ASD-cases (blue triangles)], in the two-dimensional component space generated by PC1 and PC2. A2M, α-2 Macroglobulin; APP, Acute Phase Protein; CRP, C-Reactive Protein; Cyto, Cytokine; FER, Ferritin; FIB, Fibrinogen; IL-1β, Interleukin-1β; IL-2, Interleukin-2; IL-4, Interleukin-4; IL-5, Interleukin-5; IL-6, Interleukin-6; IL-7, Interleukin-7; IL-8, Interleukin-8; IL-10, Interleukin-10; IL-12, Interleukin-12; IL-13, Interleukin-13; IL-17, Interleukin-17; GCSF, Granulocyte Colony-Stimulating Factor; GMCSF, Granulocyte Monocyte Colony-Stimulating Factor; IFN-γ, Interferon-γ MCP-1, Monocyte Chemoattractant Protein 1; MIP-1β, Macrophage Inflammatory Protein 1β; PCT, Procalcitonin; SAA, Serum Amyloid A; Serum Amyloid P; tPA, Tissue Plasminogen Activator; TNF-α, Tumor Necrosis Factor-α.

decile were significantly ($p < 0.05$) larger for IL-10 in cases compared to controls (**Table 2**). In the unadjusted categorical regression analysis, using the 90th percentile among controls as cut-off, significant associations ($p < 0.05$) were observed between IL-7, IL-10 and IL-13, and odds of ASD with ID, and between TNF-α and ASD with ADHD (**Supplementary Table 3**). In the adjusted regression models (**Table 3**), levels of the pro-inflammatory cytokines IL-1β (OR = 1.71, $p = 0.030$), IL-6 (OR = 1.68, $p = 0.037$), and TNF-α (OR = 1.79, $p = 0.020$), and the inhibitory cytokine IL-10 (OR = 1.74, $p = 0.024$) at or above the 90th percentile were associated with increased odds of any ASD diagnosis. Further, IL-1β (OR = 2.31, $p = 0.017$), IL-7 (OR = 2.28, $p = 0.012$), IL-10 (OR = 2.23, $p = 0.018$), IL-13 (OR = 2.42, $p = 0.006$), and MCP-1 (OR = 2.09, $p = 0.041$), were associated with increased odds of ASD with ID (**Table 3**). Finally, GMCSF (OR = 2.06, $p = 0.041$) and TNF-α (OR = 2.31, $p = 0.014$) were associated with ASD with ADHD (**Table 3**).

In adjusted cubic spline models, only analytes with < 70% imputed values were included (**Supplementary Figures 7–10**). A significant overall association ($p = 0.032$) was observed between IL-7 and ASD with ID (**Supplementary Figure 9**), with increased odds at the higher end of the distribution, consistent with the results in the categorical analysis. No other associations reached overall statistical significance, although some cytokines displayed similar patterns to those observed in the categorical analysis, with elevated odds of ASD at the high end of the distribution (MCP-1 and ASD with ID, **Supplementary Figure 9**; IL-6

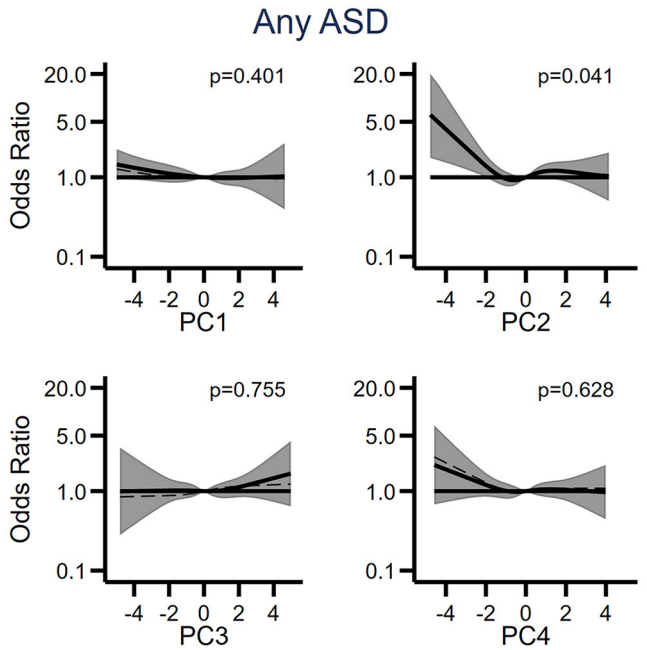


**FIGURE 4 |** The relationship between the first four principal components (PC1-PC4) derived from cytokines and acute phase proteins, and odds of ASD when comparing 318 individuals affected by ASD to 429 unaffected individuals selected from the cohort. Each panel displays the odds of ASD according to principal component scores, flexibly fit using restricted cubic spline models with four knots and a score = 0 as the referent. The dashed line represents the unadjusted estimate of the relationship between each principal component and odds of ASD. The solid line represents the fully adjusted model, adjusted for sex of fetus; family income quintile; maternal BMI, psychiatric history, region of origin, and age. The gray bands represent the 95% confidence interval for the fully adjusted model. *P*-values are shown for a Wald test with a null hypothesis that all spline terms were jointly equal to zero, as a test of whether each principal component was generally associated with the outcome. PC, principal component.

and MCP-1 and ASD with ADHD, **Supplementary Figure 10**, **Table 3**).

Although several of the associations between cytokines and the outcomes were significant at $p < 0.05$ (**Table 3**), none survived the Bonferroni-adjusted significance thresholds based on either 17 or 68 statistical comparisons. Nor did any of the associations survive correction for multiple comparisons using the Benjamini-Hochberg procedure, with FDR specified at 5, 10 or 25%. Our lowest observed *p*-value ($p = 0.006$ for the association between IL-7 and ASD with ID in the categorical analysis) would only reach statistical significance (i.e., fall below the critical value) at an FDR of 41.7%.

## Principal Component Analysis

The first four principal components, each explaining >5% of the variation, together explained over 50% of the variation in cytokines and APPs (**Supplementary Figure 11**). PC1 explained about 24% of the variation in the immune markers (**Supplementary Figure 12**), and was dominated by variation in cytokines, with the largest factor loadings from IL-1β, IL-4, IL-6 and TNF-α (**Figure 2**). PC2 explained 10% of the variation (**Supplementary Figure 12**) and was mostly influenced by the APP, with the largest factor loadings from α-2-Macroglobulin (A2M), C-Reactive Protein (CRP), Serum Amyloid A (SAA) and Tissue Plasminogen Activator (tPA) (**Figure 2**). All factor loadings of the APP on PC2 were negative, indicating that an increase in APP's would lead to lower PC2 values. The cytokines and APP were completely separable when the factor loadings of each individual immune marker were plotted in the 2-dimensional component space generated by PC1 and PC2 (**Figure 3**). However, there was no separation by case-status when PC1 and PC2 were plotted in the same component space (**Figure 3**). Similarly, we observed no separation by case-status for the remaining combinations of the four largest components (PC1-PC4) (**Supplementary Figures 13A–E**).

We retained the first four components (PC1-PC4), which were approximately normally distributed (**Supplementary Figure 14**) and examined their potential relation with ASD using restricted cubic spline models to account for potential non-linear relationships (**Figure 4**; **Supplementary Figures 15–17**). We observed a significant ($p = 0.041$) association between low levels of PC2 and any diagnosis of ASD (**Figure 4**), and similar trends of increased odds of the stratified outcomes (ASD only, ASD with ID and ASD with ADHD) at low levels of PC2 (**Supplementary Figures 15–17**). Because the loadings of PC2 increased with decreasing APP levels, the overall associations between PC2 and odds of ASD outcomes indicate an increase in odds of ASD with generally increasing APP levels. We did not observe any relationships between any of the other components and the outcomes.

## Random Forest Prediction Models

The random forest prediction models (number of trees = 100) including only the register-based covariates (e.g., sex, birth order, income) had an overall prediction accuracy of ASD of 60.7% (Pseudo-bootstrap 95% CI 55.8; 65.5; **Figure 5**). There was no notable improvement in the accuracy of the prediction of ASD when either the immune markers collectively [mean accuracy 58.6% (Pseudo-bootstrap 95% CI 53.4; 63.9); **Figure 5A**], or the six largest principal components [mean accuracy 62.3% (Pseudo-bootstrap 95% CI 57.0; 67.5); **Figure 5B**], were added to the register-based covariates in the random forest prediction models. The average sensitivity of detecting ASD for the respective models were 41.8% (register-based covariates only), 42.6% (PC1–PC6 and register-based covariates), and 33.6% (biomarkers and register-based covariates). The average specificity of each model was 74.9% (covariates only) 77.1% (PC1–PC6 and register-based covariates), and 77.4% (individual biomarkers and register-based covariates). The variable importance measurements showed that sex of the child was the most important predictor for ASD, followed by maternal psychiatric history (**Figures 6A,B**). The algorithm also deemed a high family income at birth, maternal age and BMI to be among the top predictors of ASD in this sample.

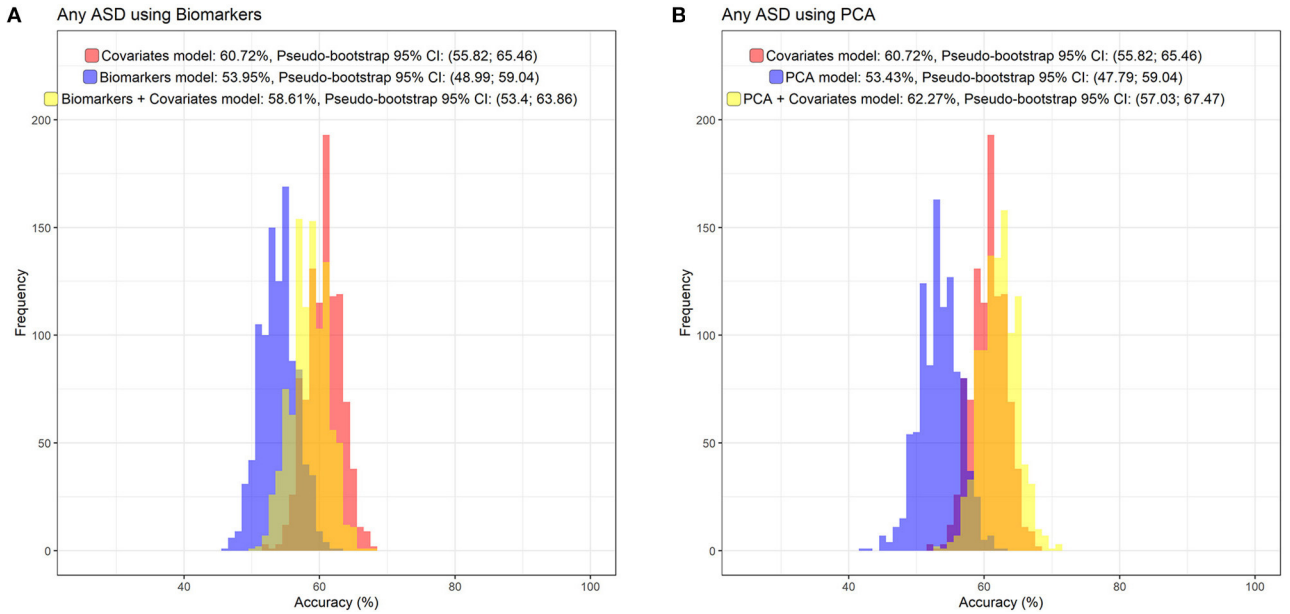


**FIGURE 5 |** The distribution of prediction accuracies of Random Forest prediction models, including the non-biomarker (i.e., register) covariates used in the fully adjusted regression models (sex of fetus; family income quintile; maternal education, BMI, psychiatric history, region of origin, and age), and adding the original cytokine and APP z-score variables **(A)**, or the four largest principal components **(B)**.

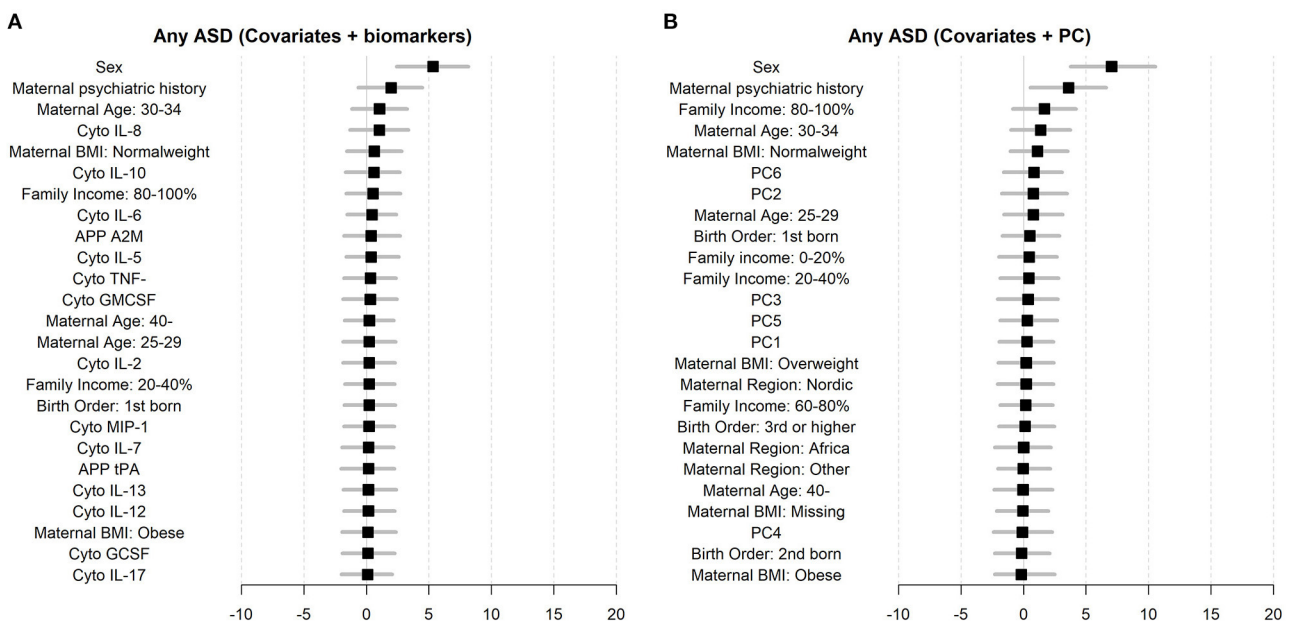


**FIGURE 6 |** Variable importance measures for biomarkers and registered covariates **(A)**, and the six largest principal components (PC1–PC6) and registered covariates **(B)**.

## Sensitivity Analysis

When samples from all trimesters were included ($n = 979$), the observed associations were generally consistent with the main categorical analyses (restricted to the first trimester) regarding direction and magnitude, although some differences were observed (**Supplementary Table 4**). The associations between IL-7 or MCP-1 and ASD with ID, and the association between GMCSF and ASD with ADHD were no longer significant (at $p = 0.05$), whereas the associations between IL-12 and any ASD diagnosis, and GMCSF and ASD with

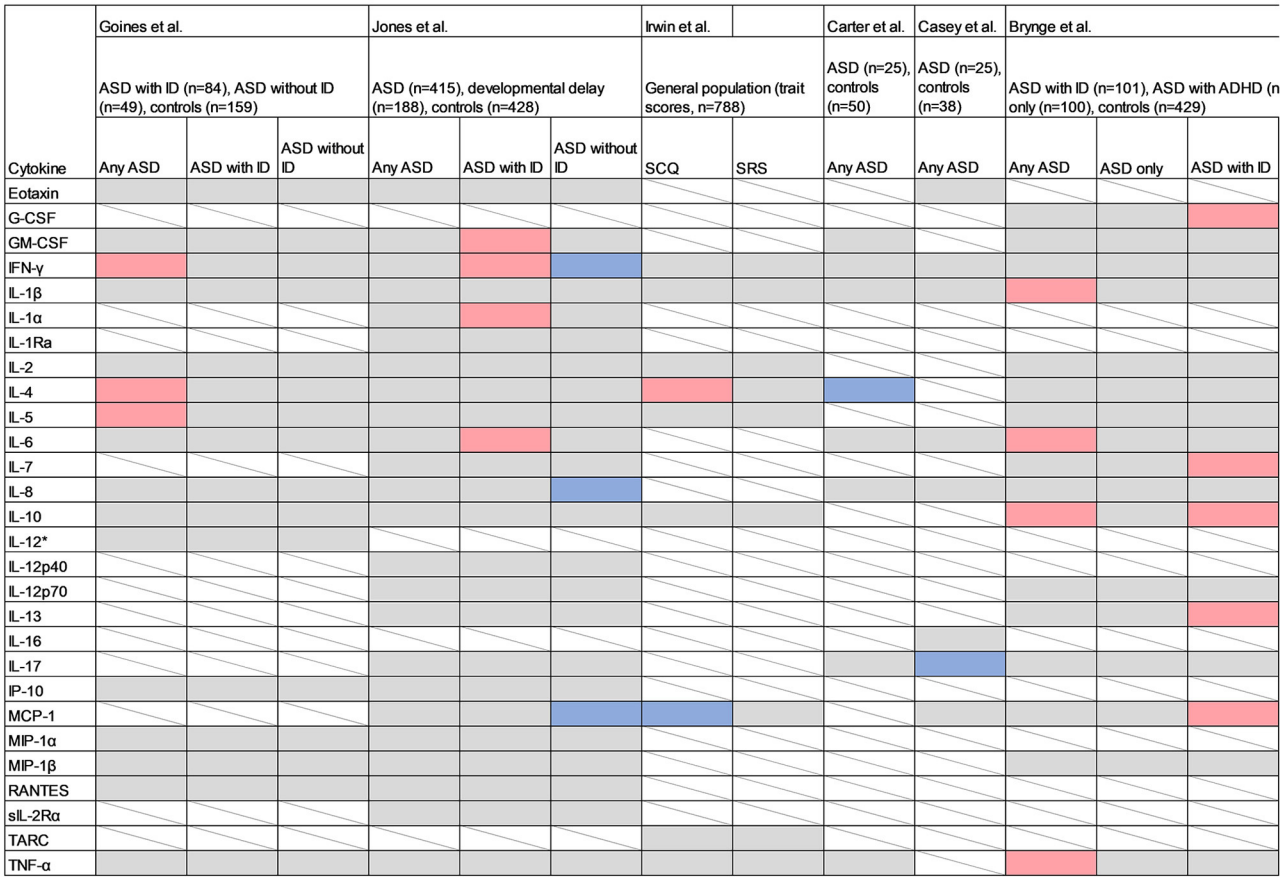


**FIGURE 7 |** Studies measuring cytokines in maternal serum samples. Red cell color indicates a positive association ($p < 0.05$) between the cytokine and the outcome, blue indicates an inverse association, grey indicates no association ($p > 0.05$), and white with strikethrough indicates that the cytokine was not measured. SCQ, Social Communication Questionnaire; SRS, Social Responsiveness Scale; IL-1α, Interleukin 1α; IL-1β, Interleukin 1β; IL-2, Interleukin 2; IL-4, Interleukin 4; IL-6, Interleukin 6; IL-7, Interleukin 7; IL-8, Interleukin 8; IL-10, Interleukin 10; IL-12p40, Interleukin 12p40; IL-12p70, Interleukin 12p70; IL-13, Interleukin 13; IL-16, Interleukin 16; IL-17, Interleukin-17; IL-17A, Interleukin-17A; GCSF, Granulocyte Colony-Stimulating Factor; GMCSF, Granulocyte Monocyte Colony-Stimulating Factor; IFN-γ, Interferon-γ; IP-10, Interferon gamma-induced protein 10; MCP-1, Monocyte Chemoattractant Protein 1; MIP-1α, Macrophage Inflammatory Protein 1α; MIP-1β, Macrophage Inflammatory Protein 1β; RANTES, Regulated on Activation, Normal T-cell Expressed and Secreted; sIL-2Rα, Soluble Interleukin 2 Receptor-Alpha; TARC, Thymus and Activation Regulated Chemokine; TNF-α, Tumor Necrosis Factor α.

ID, fell below the threshold for statistical significance ($\alpha = 0.05$, **Supplementary Table 4**). As in the main categorical analysis, none of the associations survived correction for multiple comparisons.

When using samples from all trimesters, the results of the PCA were similar to the main analysis, though the associations with ASD outcomes observed at low levels of PC2 were less prominent (**Supplementary Figure 18**).

## DISCUSSION

In this study, we measured 17 cytokines in first trimester maternal serum samples from 318 mothers to ASD-cases and 429 mothers to unaffected controls. After adjusting for a range of potential confounding factors, elevated levels of the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α and the inhibitory cytokine IL-10 were associated with diagnosis of ASD. The results varied across diagnostic sub-groups, based on the presence of comorbid ID or ADHD. While no significant associations between cytokines and ASD without ID or ADHD were observed, elevated levels of the pro-inflammatory cytokines IL-1β and MCP-1, the hematopoetic growth factor IL-7, the inhibitory IL-10, and the Th2-cytokine IL-13, were associated with ASD with co-occurring ID. Elevated levels of the monocyte growth factor GMCSF and the pro-inflammatory TNF-α were associated with ASD with co-occurring ADHD. Though several associations between individual cytokines and ASD were observed, there was no convincing general pattern of association with ASD after accounting for multiple comparisons. In PCA analysis, we observed separation of cytokines from additional immune markers measured in this cohort (APP),

though no convincing separation of cases and controls. Only one extracted principal component, whose loading was dominated by APP, was associated with ASD. Using the immune markers collectively did not improve the prediction of ASD in our sample, beyond that observed for models including only maternal and child characteristics obtained from register data.

## Comparison With Previous Studies

The previous studies investigating cytokines in maternal serum samples used samples collected later in pregnancy (2nd and 3rd trimesters) compared to the present study (**Figure 7**). One of the studies used a quantitative scale for measuring autistic traits in a general population sample, whereas the others used clinically evaluated neurodevelopmental diagnoses. Goines et al. analyzed 17 cytokines (using a different assay compared to the present study) in archived maternal serum samples, collected at gestational weeks 15–19, from mothers to cases with ASD ($n$ = 84), ID without ASD ($n$ = 49), and population controls ($n$ = 159) (23). IFN-γ, IL-4 and IL-5 were significantly associated with any diagnosis of ASD, independent of early/late onset of ASD or presence of ID. In 2016, the same research group performed a follow-up study, analyzing 22 cytokines from mothers to ASD-cases ($n$ = 415), developmental delay ($n$ = 188) and controls ($n$ = 428) (24). No significant associations were found for any of the cytokines and any ASD-diagnosis. When the sample was stratified based on co-morbidity, GMCSF, IL-1α, IL-6 and IFN-γ were significantly associated with increased odds of ASD with ID, and IFN-γ, IL-8 and MCP-1 were associated with decreased odds of ASD without ID. Some of the associated cytokines overlapped with those observed in the present study (**Figure 7**), but the results are inconsistent when considering the specific diagnostic sub-groups. For example, IL-6 was associated with an overall diagnosis of ASD in the present study, but only with a diagnosis of ASD with ID in the study by Jones et al.

In the "Seychelles Child Development Study Nutrition Cohort 2", Irwin et al. measured 13 inflammatory markers including 10 cytokines in a cohort of 788 mother-child pairs, at a mean of 28 weeks of gestation, and related these to the trait scales Social Communication Questionnaire (SCQ) and Social Responsiveness Scale (SRS), at age 7 (25). Both the exposures and outcomes were considered as continuous linear variables. A significant association was reported for IL-4 and autistic traits as measured by SCQ, which is in line with the findings by Goines et al. However, an inverse association between MCP-1 and SCQ contradicts the positive association between MCP-1 and ASD without ID reported by Jones et al. Because Irwin et al. evaluated autistic traits in the general population, any direct comparison with studies employing a case-control design are difficult.

In a recent case-control study, Carter et al. (26) investigated the association between eight maternal cytokines measured at 20 weeks of gestation in 25 mothers to ASD-cases and 50 mothers to controls, matched by infant sex, gestational age, birthweight and maternal BMI. The median cytokine concentrations were compared between groups using non-parametric tests. Decreased levels of mid-gestational IL-4 were associated with ASD, which contradicts the findings by Goines et al. and Irwin et al. (23, 25). Casey et al. combined maternal serum samples from two mother-child cohorts including 25 mothers to ASD-cases and 38 mothers to controls, and investigated associations between eight maternal cytokines at 15 and 20 weeks of gestation and odds of ASD in offspring (47). Decreased levels of IL-17A at 20 weeks of gestation were associated with increased odds of ASD.

This is the first study to date to investigate maternal serum cytokines during the first trimester of pregnancy and risk of ASD in the offspring. In the present study, we analyzed cytokines in a study sample nested within a large population-based cohort. None of the significant associations in the present study overlapped with those reported previously. There are methodological differences between the studies that may explain these discrepancies. As in previous case-control studies, our archived serum samples were collected as part of an antenatal screening program, and handling of samples before final storage at −80°C cannot be accounted for. This might have influenced the reliability of the measurements (48). Finally, multiple exposures and endpoints are assessed, and none of the previous studies correct for this. Hence, there is a possibility that the positive associations reported previously represent chance findings due to heterogeneity in inclusion criteria and sampling variability.

## Interpretation

In this cohort of pregnant women, we measured a wide range of cytokines, including mediators of both innate and adaptive immune pathways. We observe several associations between individual immune markers and ASD. However, the associations were weak to moderate in general, and none survived correction for multiple comparisons. This is in line with previous studies on maternal cytokines and ASD, although none of the previous studies corrected for multiple comparisons. Overall, we found no strong evidence for maternal immune activation among mothers to individuals with ASD in this cohort, although there was a tendency for odds ratios estimates to be above one, similar to other studies (23, 24).

Although we observe increased levels of several of the pro-inflammatory cytokines and ASD, the specific results differ considerably according to diagnostic sub-group. Indeed, recent data suggests that ASD without ID is more familial and may have a different genetic architecture than ASD with ID (4, 49, 50). The findings may indicate the presence of a first-trimester gestational immune stimulus among some mothers to ASD-cases. This observation might reflect the presence of an (unmeasured) external or inherent "driver" of cytokine levels in these mothers, such as infection or genetic variants.

If any of the cytokines are causally related to ASD, the mechanism by which they can potentially affect children's neurodevelopment remains to be established. In experimental animals, IL-6 can cross the placental barrier and influence fetal brain development potentially through transmission of an inflammatory signal to the fetal compartment (21, 51). Maternal cytokines can also affect the cytokine production by decidual cells of the placenta, with potential consequences for the fetal

levels. Several of the cytokines play important roles in key neurodevelopmental processes, e.g., neuronal migration and differentiation and synaptic pruning, and a shift in cytokine levels might interfere with such processes.

The overall picture indicates that instead of relying on individual markers, some general aspect of the maternal immune function and its interaction with the developing fetus may be relevant for ASD. To integrate the biological information from all our measured immune markers in one model, we employed an approach using PCA, also including measurements of maternal APP measured in the same cohort of women. A similar approach has been used in a few previous studies of cytokines in archived maternal or neonatal samples. Jones et al. used separate PCA for each diagnostic sub-group (ASD with ID, ASD without ID, developmental delay, controls) to investigate the presence of specific aggregated patterns of maternal cytokines. The authors concluded that there was no obvious specific cytokine-profile for any of the stratified groups. Krakowiak et al. conducted a PCA of 14 cytokines, though these were measured in neonatal samples from ASD-affected and unaffected children. Overall, there were no specific clustering patterns across diagnostic sub-groups (mild/moderate ASD, severe ASD, developmental delay, controls). Finally, Heuer et al. used partial least squares discriminant analysis (PLS-DA) to investigate if neonatal cytokine levels separated diagnostic groups (ASD cases, controls). The authors concluded that there was no outcome-specific profile based on a plot of cases and controls in component space, but a variable importance analysis suggested that the cytokines IL-6 and IL-8 were the most important cytokines, which corresponded to their logistic regression analyses. In our study, maternal levels both IL-6 and IL-8 were among the most important predictors in our random forest models, although the confidence intervals overlapped with zero.

We observed a non-linear relationship between one of the extracted principal components, PC2, and odds of ASD, with increased odds of ASD associated with low PC2 scores. Low PC2 scores correspond to high levels of several of the APP, given the direction of the loading of the APPs on the second principal component, indicating that children's odds of ASD generally increase with increasing levels of maternal APP. The remaining components, including the cytokine-dominated PC1, showed no significant associations with ASD-case status, and cases and controls were not otherwise separated based on the first four components. This indicates that maternal APP levels may contain more information relevant for the children's risk of ASD than maternal cytokine levels. This may relate to their longer half-lives, higher base-line concentrations, and effector functions within the maternal innate immune system (52).

Finally, adding the immune markers or their derived principal components to other registered covariates regarding maternal and child characteristics did not improve the prediction of ASD-case status in Random Forest prediction models. This indicates that knowledge regarding maternal immune biomarkers (including cytokines and APP) are unlikely to be informative in terms of early detection of ASD. However, the weak predictive capability does not rule out a role for maternal immune status in the etiology of ASD. The potential dysregulation of immune processes may be more subtle than what can be detected in our current sample, and the maternal serum samples from early pregnancy may not reflect the conditions in the developing fetal brain. There is a possibility that each of the markers has a very small effect size that we are unable to capture given our restricted sample size, and that each of the small effects add up to a cumulative effect that is only detectable in a substantially larger population, analogous to single nucleotide polymorphisms in genome wide association studies.

## Strengths and Limitations

We use a validated case-finding procedure within a healthcare system with universal coverage and regular developmental screening. This increases the likelihood of identifying ASD cases in the population. Using our large, well-characterized population-based cohort, we were able to assess confounding by a range of different environmental factors and investigate how they influence the serum levels of cytokines. By analyzing multiple immune markers, we increased the likelihood of detecting a signal of activation of the maternal immune system. On the other hand, by doing multiple statistical comparisons, we also increased the probability of chance findings. None of the associations survived correction for multiple comparisons, either using a traditional (Bonferroni) or a less conservative (FDR) approach. A strength of our study is the PCA and Random Forest analyses, which move beyond the conventional one-by-one analyses and allows for an integrated interpretation of the immune markers.

We could adjust for a wide range of potential confounders, such as maternal BMI and fetal sex, with documented associations with both the maternal immune status and autism (8, 30, 53). However, there is a possibility for residual confounding by unmeasured genetic or environmental factors. We do not have serum samples available from multiple pregnancies and can therefore not perform sibling-comparisons. Our previous work in NDBS suggests that this would likely be valuable in order to address issues of confounding by shared familial factors (32). Indeed, recent research suggests that adverse prenatal exposures are associated with maternal genetic liability for neurodevelopmental disorders (54), further stressing the importance of adjustment for genetic factors in studies of maternal immune markers and children's risk of ASD. Despite our large overall sample, the number of individuals in the stratified groups is limited, and we may be underpowered to detect more subtle relationships, particularly in the diagnostic sub-groups.

By restricting the analysis to the first trimester, we reduced heterogeneity and variation in cytokines related to the progression of pregnancy. On the other hand, the strategy also reduced the sample size and statistical power and prevented detection of differences during later stages of pregnancy. The majority of serum samples (76.3%) in this study were drawn in the first trimester. The observed associations between several of the maternal cytokines and gestational age, as well as other covariates, stress the importance of taking key covariates

into account in any assessment. Including samples from all trimesters in a sensitivity analysis yielded results similar to the main analysis in terms of the direction and magnitude of the estimated odds ratios, though some relationships were less apparent when considering the full cohort and a few became more apparent.

Cytokines are powerful regulators of the immune system and generally have short half-lives and low baseline levels at homeostatic conditions. Since cytokines are often involved in local (paracrine) cell-signaling pathways, systemic levels may not necessarily reflect concentrations at peripheral sites of inflammation, or in the embryo and the fetal/placental unit. Thus, measuring concentrations in blood/serum samples can be both technically challenging and difficult to interpret. Overall, there was a large proportion of non-detectable values for many of the cytokines. The inter-assay coefficient of variation was markedly higher for IFN-γ compared to the other cytokines, and the results for IFN-γ must therefore be interpreted with some caution.

Moreover, because these samples were collected as part of a clinical screening program, storage procedures may have varied. Samples could be stored at room temperature or in a refrigerator up to several days before freezing at −80°C, though this procedure was not within our control, and we do not have information on the handling of individual samples prior to their arrival in our laboratory. Since cytokines are vulnerable to degradation at ambient temperatures, there is a possibility that the levels measured in the samples do not reflect the actual levels at the time of sampling/venipuncture. The degradation of cytokines in the samples due to these issues would attenuate any real associations toward the null, and further reduce our ability to detect case/control differences.

## CONCLUSIONS

While we observed a number of individual associations between maternal cytokines measured in early pregnancy and children's risk of ASD, none survived corrections for multiple comparisons. Considering the individual associations, our results do not provide strong support for the maternal immune activation hypothesis in ASD, especially when compared to the often divergent results of previous studies. The relationships we observed varied with the presence or absence of co-occurring neurodevelopmental diagnoses, including both ADHD and ID. We also observed variation in the maternal cytokine levels with key covariates, such as maternal characteristics and the gestational week at serum sampling. This emphasizes the importance of considering both potential genetic and environmental influences on the maternal immune system when attempting to interpret associations between maternal immune biomarkers and children's risk of ASD in future studies.

When taking a more integrated view of biomarkers reflecting the maternal immune response in the first trimester, we observed that maternal cytokines as a class were not strongly associated with children's risk of ASD, though higher levels of another class of immune biomarkers, the acute phase proteins, were associated with children's risk for ASD. Using all of these markers together did not markedly improve prediction models for ASD, indicating a limited utility for maternal immune biomarkers in early detection strategies for ASD given the limitations of studies to date in terms of sample size and the relatively small effect sizes for the individual markers.

## DATA AVAILABILITY STATEMENT

The Swedish health and population register data used in this study are available from Statistics Sweden and the Swedish National Board of Health and Welfare. The authors are not allowed to distribute the data according to the ethical approval for this study and the agreements with Statistics Sweden and the Swedish National Board of Health and Welfare.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Stockholm Regional Review Board. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

## AUTHOR CONTRIBUTIONS

MB contributed to data acquisition, data analysis, and drafted the first manuscript. HK and RG contributed to study design, data acquisition, data analysis, and manuscript preparation. CD contributed to data acquisition, study design, and manuscript preparation. HS contributed to data acquisition, data analysis, and manuscript preparation. BL contributed to study design and manuscript preparation. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2022.917563/full#supplementary-material

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EDITED BY
Ulrik Lausten-Thomsen,
Copenhagen University Hospital Rigshospitalet, Denmark

REVIEWED BY
Victor Javier Lara-Diaz,
Tecnológico de Monterrey, Mexico
Kristin Skogstrand,
Statens Serum Institute, Denmark

*CORRESPONDENCE
Mingwen Jin
mary_kim1123@yahoo.co.jp

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# Development of a classifier to screen for severe sleep disorders in children

Mingwen Jin*, Masaharu Kato and Shoji Itakura

Center for Baby Science, Doshisha University, Kyoto, Japan

This study aimed to develop an automatic classifier for the identification of severe sleep disorders that require immediate intervention in children. Our study assessed 7,008 children (age: 0–83 months) in Japan, whose parents and nursery teachers recorded their 14-day sleep patterns. Sleep quality was assessed by pediatricians and scored as 1 (no severe sleep disorder) or 0 (severe sleep disorder). Discriminant analysis was performed for each age group using sleep quality (0 or 1) as the dependent variable and variables in the 14-day sleep log as independent variables. A stepwise method was used to select the independent variables to build the best model. The accuracy of the discriminant analysis for the age groups ranged from 71.3 to 97.3%. In summary, we developed an automatic classifier with sufficient application value to screen for severe sleep disorders in children. In the future, this classifier can be used to rapidly determine the presence or absence of severe sleep disorders in children based on their 14-day sleep logs, thus allowing immediate intervention.

KEYWORDS

**discriminant analysis, children, sleep problems, sleep disorder, classifier, sleep quality judgments**

## Introduction

Sleep disorders in children are common. They impair the social-emotional development of the child (1), increase his/her risk of obesity (2), and cause parental child-rearing stress (3) and maternal postpartum depression (4). As sleep problems and disorders are persistent and their frequency is high, it is important for pediatricians to screen for and identify these problems at different developmental stages (5). Notably, a large-scale study in Italy found that COVID-19 pandemic increased the incidence of sleep disorders in children aged 8–10 years; these disorders included inability to fall asleep or maintain sleep and occurrence of nightmares and/or sleep terrors (6). Owing to the persistence and high frequency of sleep disorders in children (5), it is necessary to assess children's sleep quality across various developmental stages, identify severe sleep disorders, and provide immediate intervention.

Sleep disorders in children are defined as follows: (1) sleep onset insomnia: it takes more than 60 min to fall asleep, and the time of sleep onset is after 10:30 pm; (2) sleep fragmentation: waking up more than three times during the night; (3) disturbances of continuous sleep: once awake, cannot sleep again for more than 60 min; (4) short sleep

duration: total sleep duration is <8 h; and 5) variation of wake up and bed times: the variation is large, with a difference of more than 60 min (7, 8). Assessment of sleep disorders in multiple children within a specific time period is difficult owing to the time-consuming nature of the assessment.

Subjective methods of monitoring sleep status include self-reported sleepiness evaluation, life habit evaluation (9), and the Brief Infant Sleep Questionnaire-Revised (10), whereas objective methods include the use of a sleep log (11), actigraphy (12), polysomnography (13), heart rate variability analysis (14), and non-contact sleep analysis (15). Among these methods, the use of a sleep log is simple and causes minimal stress to participants. In this study, we used the sleep log observation method to determine the sleep status and sleep quality of children in a large dataset (7,008 children). The purpose of this study was to conduct a discriminant analysis of the presence or absence of severe sleep disorders in children and to develop a classifier to screen for such disorders. The classifier does not aim to classify the type of sleep disorder, which can be done using the criteria laid out in the 3rd edition of the International Classification of Sleep Disorders (16).

# Materials and methods

## Participants and procedure

Company A operates 63 childcare facilities throughout Japan. As part of its efforts to improve sleep and life rhythm, it conducted voluntary sleep surveys of children (age: 0–83 months) in childcare facilities in November 2012, September 2013, and September 2014. The parents of all participants provided informed consent. Only those who agreed to participate in the surveys were asked to record their children's 14-day sleep patterns. Data obtained from the surveys were anonymized and incorporated into the database of the Doshisha University Center for Baby Science.

## Data collection

From the database, we collected data for 7,031 children. After excluding children with incomplete data, the number of eligible study participants was 7,008. There were two types of data: those derived from the 14-day sleep log [sex (partially missing), age, record date, day of the week, wake up time, bed time, number of night wakings, self-awakening frequency, and breakfast frequency] and those pertaining to sleep quality. Sleep quality judgments were made by a pediatrician based on information from the 14-day sleep log. A, B, C, and D designations indicate good sleep ($n$ = 2,391), sleep with signs of sleep disorder ($n$ = 2,853), mild sleep disorder ($n$ = 1,216), and severe sleep disorder that requires interventional treatment ($n$ = 548), respectively. Because the purpose of this study was to develop a classifier to screen for severe sleep disorders, we divided the judgments into two categories: judgments A, B, and C (no severe sleep disorder, designated as 1) and judgment D (severe sleep disorder, designated as 0).

## Age group

Newborns are characterized by short, repeated sleep periods, regardless of their day-night rhythm. At 1 month of age, they begin differentiating between day and night. Around 3 months of age, a diurnal pattern is established, with a longer period of sleep at night and shorter naps during the day. From 6 months of age, the number of nighttime feedings decreases, and infants sleep for about 6 h at night without waking.

Infants have defined sleep stages similar to adults. By 9 months of age, 70–80% of infants can sleep through the night. At ~1 year of age, they sleep twice a day, once in the morning, and once in the afternoon. By 1.5 years of age, if they are sleeping well at night, they sleep only once a day. Napping no longer occurs between 3 and 5 years of age. By age 4, they develop a circadian rhythm, and their percentage of REM sleep at night is equal to that of adults. Naps are thought to compensate for any lack of sleep at night.

Because sleep characteristics and sleep structure differ according to age (17, 18), the children in our study were divided into nine age groups: Group 0, 0–2 months; Group 1, 3–5 months; Group 2, 6–9 months; Group 3, 10–14 months; Group 4, 15–18 months; Group 5, 19–47 months; Group 6, 48–59 months; Group 7, 60–71 months; and Group 8, 72–83 months.

## Statistical methods

IBM SPSS version 27 (19) and R-studio version 4.1.1 (20) software were used to analyze the data. Descriptive statistics, Welch's analysis of variance (ANOVA) with the Tamhene *post-hoc* test, and discriminant analysis were used in this study.

# Results

## 14-Day sleep log data

Based on the clinical findings, the following 10 variables were extracted from the 14-day sleep log and analyzed using R-studio software.

### Wake up time

The wake up time is assumed to be between 3:00 and 10:00 in the morning, and the sleep-wake state within that time is

expressed as 0 (asleep) or 1 (awake) in 5-min increments. The average sleep-wake state (sleep-wake rate) at each time (5-min increments) was calculated for the entire observation period (14 days). The data were distributed between 0 and 1, with a typical pattern of 0 from 3:00 am to 5:00 am, gradually approaching 1 from 5:00 am to 8:00 am, and 1 from 8:00 am to 10:00 am. The data were then fitted to the sigmoid function using the maximum likelihood method as follows: the closer the wake up time (x) is to 3:00 am, the closer the sleep-wake state (y) is to a; the closer the wake up time is to 10:00 am, the closer y is to b (c is the time at which the function becomes point-symmetric, and d is the slope of the function). Because y is 0 when sleeping and 1 when awake, the change in y becomes a step function and d approaches 0 if the wake up time is constant throughout the 14-day observation period. In other words, the stability of the sleep-wake rhythm can be continuously expressed by the value of d. Further, the closer d is to 0, the smaller the variation in the wake up time; c is the time when the sleep and wake states become equal (50% each) when fitted with the sigmoid function (Figure 1). The formula for the calculation of y is as follows:

$$y = a + \frac{b - a}{1 + e^{(c-x)/d}} \quad (1)$$

It should be noted that when fitting a sigmoid function, the maximum likelihood method often fails to converge. One of the reasons is that the function to be fitted is non-linear and thus is greatly affected by initial values. Therefore, we added a small jitter to the average sleep-wake rate obtained during the observation period, calculated it 100 times, and used the average of the obtained values as the final estimated value.

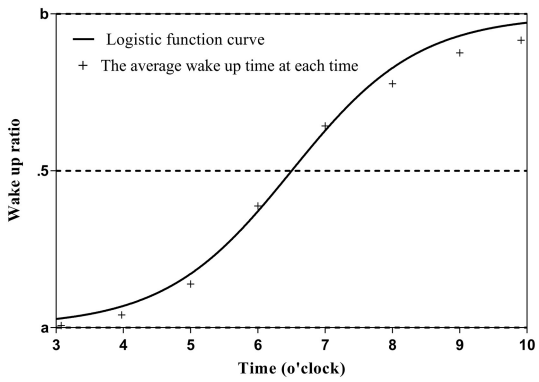


FIGURE 1
Estimation method of average waking time.

## Wake up time variation

d in the Wake up Time Sigmoid Function.

## Bed time

The bed time is assumed to be between 6:00 pm and 2:00 am, and the calculation method is the same as that for the wake up time. As an advantage, the maximum calculation method allows automatic determination of the wake up time and bed time even in conditions in which neither variable can be defined; such conditions include waking up once in the morning and going back to bed immediately afterward or going to bed in the evening and waking up shortly afterward. As a difference, the slope is positive for the wake up time estimate because y changes from 0 to 1 as x (the wake up time) increases, but is negative for the bed time estimate.

- Bed time variation: d in the bed time sigmoid function.
- Total sleep duration: the average of the total time spent in a sleep state in a 24-h period.
- Nocturnal sleep duration: average time spent in a sleep state between 7:00 pm and 9:00 am.
- Night wakings: mean number of awakenings during nocturnal sleep.

TABLE 1 Descriptive statistics of the various variables.

| Variables | Min. | Max. | M | SD | Ske. | Kur. |
|---|---|---|---|---|---|---|
| Wake up time | 4.23 | 10.26 | 6.84 | 0.58 | 0.17 | 0.74 |
| Wake up time variation | 0.00 | 1.00 | 0.26 | 0.16 | 1.12 | 3.37 |
| Bed time | 18.70 | 1.45 | 21.44 | 0.69 | 0.11 | 0.97 |
| Bed time variation | −1.00 | 0.01 | −0.27 | 0.17 | −1.20 | 3.20 |
| Total sleep duration | 7.00 | 18.21 | 11.27 | 0.84 | 0.04 | 1.32 |
| Nocturnal sleep duration | 5.25 | 11.88 | 9.18 | 0.58 | −0.04 | 1.09 |
| Night wakings | 0.00 | 5.31 | 0.14 | 0.38 | 5.34 | 37.86 |
| Evening nap | 0.00 | 1.62 | 0.11 | 0.15 | 2.69 | 11.43 |
| Breakfast | 0.00 | 1.00 | 0.94 | 0.18 | −4.14 | 17.41 |
| Self-awake | 0.00 | 1.00 | 0.69 | 0.31 | −0.66 | −0.76 |

TABLE 2 Results of Welch's ANOVA with Tamhene *post-hoc* testing for each variable.

| Groups | Months | *N* | Wake up time | Wake up time variation | Bed time | Bed time variation | Nocturnal sleep duration | Total sleep duration | Night wakings | Evening nap | Breakfast | Self-awake |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 | 0–2 m | 10 | | | | | | | | | | |
| 1 | 3–5 m | 37 | $F_{(8,170.28)} =$ | $F_{(8,170.01)} =$ | $F_{(8,170.18)} =$ | $F_{(8,170.44)} =$ | $F_{(8,170.13)} =$ | $F_{(8,170.13)} =$ | $F_{(8,174.11)} =$ | $F_{(8,170.30)} =$ | $F_{(8,169.87)} =$ | $F_{(8,170.33)} =$ |
| 2 | 6–9 m | 168 | 43.28 | 11.82 | 69.88 | 9.52 | 103.76 | 378.06 | 103.76 | 88.60 | 16.42 | 32.81 |
| 3 | 10–14 m | 687 | $p < 0.001$ | $p < 0.001$ | $p < 0.001$ | $p < 0.001$ | $p < 0.001$ | $p < 0.001$ | $p < 0.001$ | $p < 0.001$ | $p < 0.001$ | $p < 0.001$ |
| 4 | 15–18 m | 786 | 2, 3, 4 < 5, 6, 7, 8; | 1 > 4, 5, 6, 7, 8; | 2, 3, 4 < 5, 6, 7, 8 | 2, 3, 4 < 5, 6, 7, 8 | 3 > 8; 4 > 5, 6, 7, 8 | 2, 3, 4 > 5 > 6 > 7 > 8; 1 > 3, 4, 5, 6, 7, 8 | 1, 2 > 3, 4, 5, 6, 7, 8; 6 > 8 | 0 > 6, 7, 8; 1 > 2 > 3 > 4 > 5 > 6, 7, 8; 6 > 8 | 1 < 2 < 3, 4,5,6,7,8 | 2, 5 > 6,7,8; 3,4 > 5,6,7,8 |
| 5 | 19–47 m | 4138 | 7, 8; 3 < 4 | 2 > 5, 6, 7, 8; 3,4 > 6, 7, 8; 5 > 6, 7 | 5, 6, 7, 8 | | | | | | | |
| 6 | 48–59 m | 626 | | | | | | | | | | |
| 7 | 60–71 m | 407 | | | | | | | | | | |
| 8 | 72–83 m | 149 | | | | | | | | | | |

- Evening nap: mean number of sleep states at 3:00 and 7:00 pm.
- Breakfast: mean number of times breakfast was eaten.
- Self-awake: mean number of times children woke up by themselves.

Descriptive statistics were calculated using SPSS version 27 software. The descriptive statistics for the 10 variables are presented in Table 1.

## Sleep characteristics of different age groups

Using Welch's ANOVA with the Tamhene *post-hoc* test, we identified significant age-related differences in all of the variables in the 14-day sleep log (Table 2). Figures 2–11 show the box plots for each variable according to age using the alphabetical labeling method. If one or more of the alphabets in each group is the same, it means that there is no difference between the two groups. And if the alphabets are completely different, it means that there is a difference between the two groups.

Wake up time (Figure 2) and bed time (Figure 3) tended to increase with increasing age. Wake up times were earlier at ages 6–9 months than at ages 19–71 ($p < 0.001$) and 72–83 ($p = 0.016$) months, at ages 10–14 months than at ages 15–18 ($p = 0.003$) and 19–83 ($p < 0.001$) months, and at ages 15–18 months than at ages 19–71 ($p < 0.001$) and 72–83 ($p = 0.007$) months. Children aged 19–83 months had later bed times than those aged 6–9, 10–14, and 15–18 months ($p < 0.001$).

Wake up time variation tended to decrease with increasing age (Figure 4). It was greater at ages 3–5 months than at ages 15–18 ($p = 0.048$), 19–47 ($p = 0.029$), 48–59 ($p = 0.005$), and 60–83 ($p = 0.004$) months; at ages 6–9 months than at ages 19–47 ($p = 0.011$) and 48–83 ($p < 0.001$) months; at ages 10–14 months than at ages 48–83 months ($p < 0.001$); at ages 15–18 months than at ages 48–71 ($p < 0.001$) and 72–83 ($p = 0.032$) months; and at ages 19–47 months than at ages 48–71 months ($p < 0.001$).

Bed time variation tended to decrease with increasing age (Figure 5). It was greater at ages 3–5 months than at ages 10–14 ($p = 0.035$), 15–18 ($p = 0.024$), 19–47 ($p = 0.003$), 48–59 ($p = 0.004$), 60–71 ($p = 0.002$), and 72–83 ($p = 0.001$) months; at ages 6–9 months than at ages 19–47 ($p = 0.013$), 48–59 ($p = 0.032$), 60–71 ($p = 0.008$), and 72–83 ($p = 0.003$) months; at ages 10–14 months than at ages 19–47 ($p < 0.001$), 48–59 ($p = 0.013$), 60–71 ($p = 0.002$), and 72–83 ($p = 0.002$) months; and at ages 15–18 months than at ages 19–47 ($p < 0.001$), 48–59 ($p = 0.026$), 60–71 ($p = 0.004$), and 72–83 ($p = 0.006$) months.

Total sleep duration (Figure 6) and nocturnal sleep duration (Figure 7) tended to decrease with increasing age. Total sleep

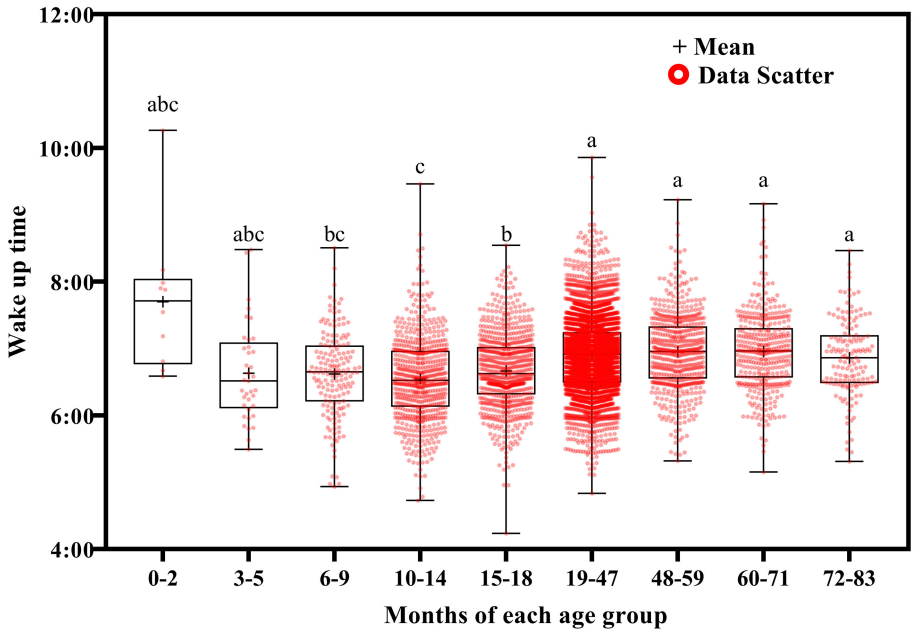


FIGURE 2
Results of the one-way ANOVA for wake up time stratified by age.

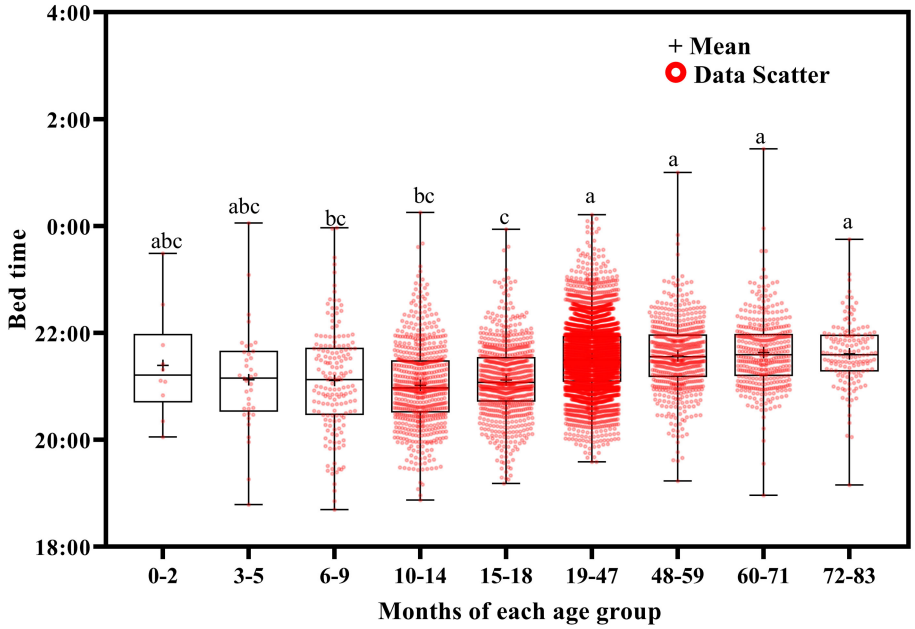


FIGURE 3
Results of the one-way ANOVA for bed time stratified by age.

duration was longer at ages 3–5 months than at ages 10–14 ($p = 0.021$), 15–18 ($p = 0.014$), and 19–83 ($p < 0.001$) months; at ages 6–18 months than at ages 19–83 months ($p < 0.001$); at ages 19–47 months than at ages 48–83 months ($p < 0.001$); at ages 48–59 months than at ages 60–83 months ($p < 0.001$); and at ages 60–71 months than at ages 72–83 months ($p < 0.001$). Nocturnal sleep duration was longer in children aged 10–14 months than in those aged 72–83

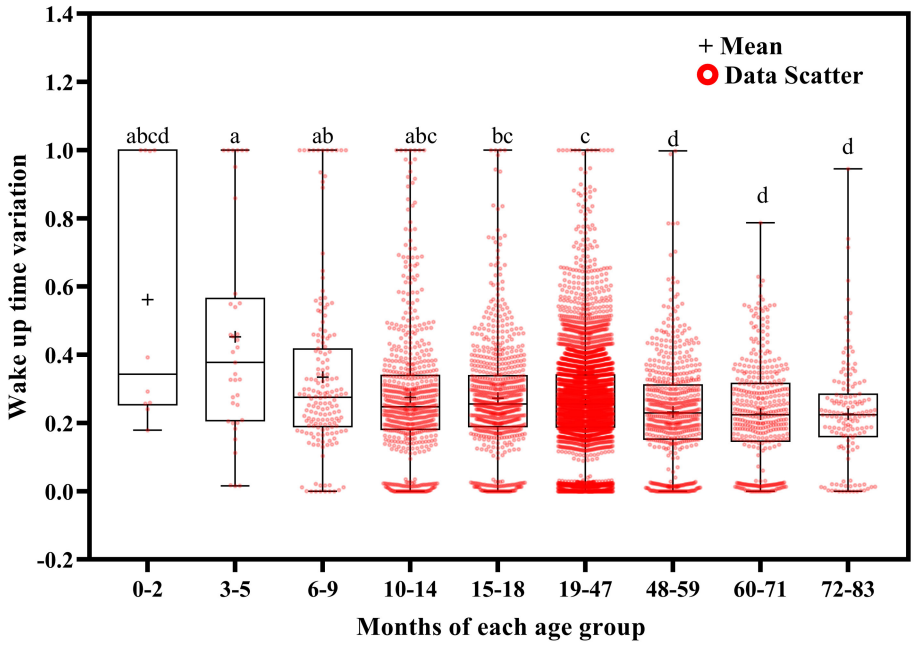


FIGURE 4
Results of the one-way ANOVA for wake up time variation stratified by age.

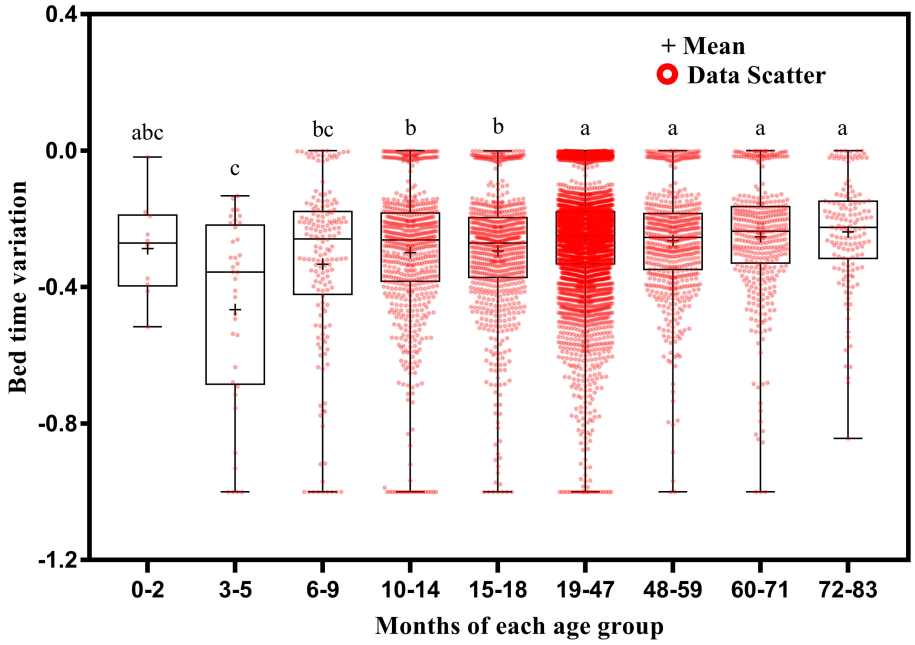


FIGURE 5
Results of the one-way ANOVA for bed time variation stratified by age.

months ($p = 0.029$) and in children aged 15–18 months than in those aged 19–47 ($p < 0.001$), 48–59 ($p = 0.005$), 60–71 ($p < 0.001$), and 72–83 ($p < 0.001$) months. The number of night wakings tended to decrease with increasing age (Figure 8). Night wakings were more frequent at ages 3–5 months than at ages 10–14 ($p = 0.002$) and 15–83 ($p < 0.001$)

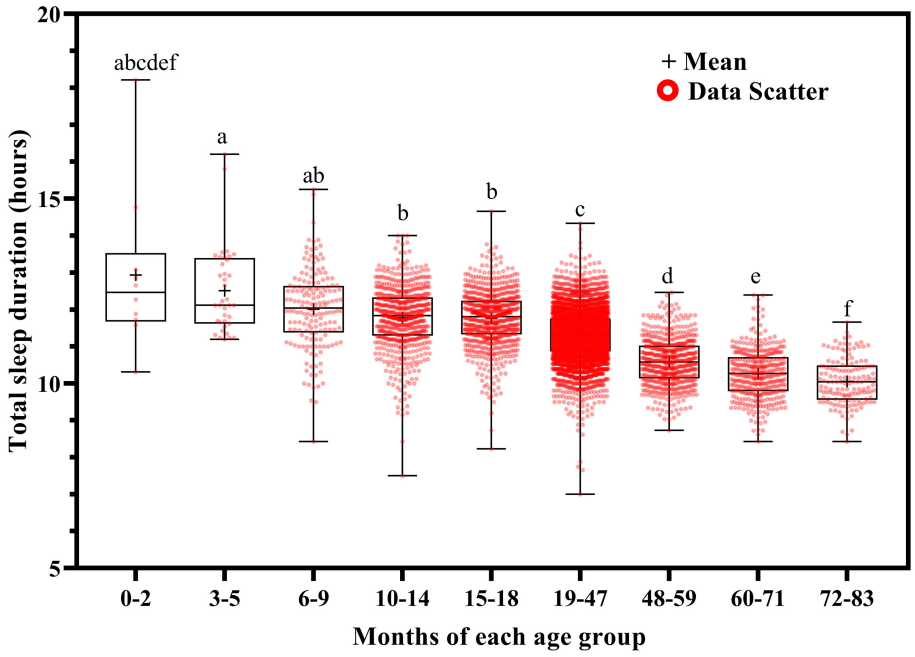


FIGURE 6
Results of the one-way ANOVA for total sleep duration stratified by age.

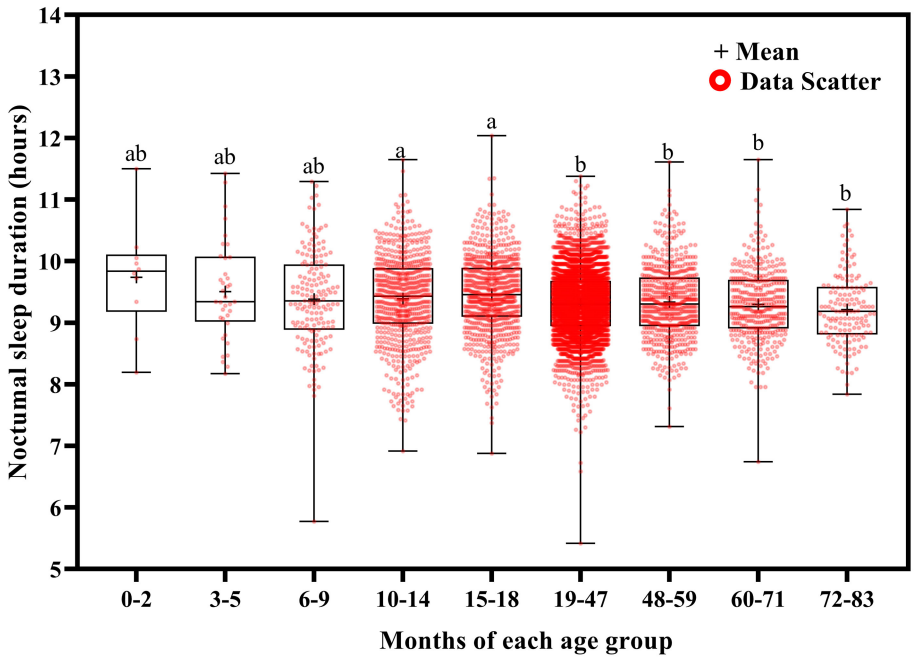


FIGURE 7
Results of the one-way ANOVA for noctumal sleep duration stratified by age.

months; at ages 6–9 months than at ages 10–83 months ($p < 0.001$); at ages 10–14 months than at ages 15–83 months ($p < 0.001$); at ages 15–18 months than at ages 19–83 months ($p < 0.001$); at ages 19–47 months than at ages 48–83 months ($p < 0.001$); and at ages 48–59 months than at ages 72–83 months ($p < 0.001$).

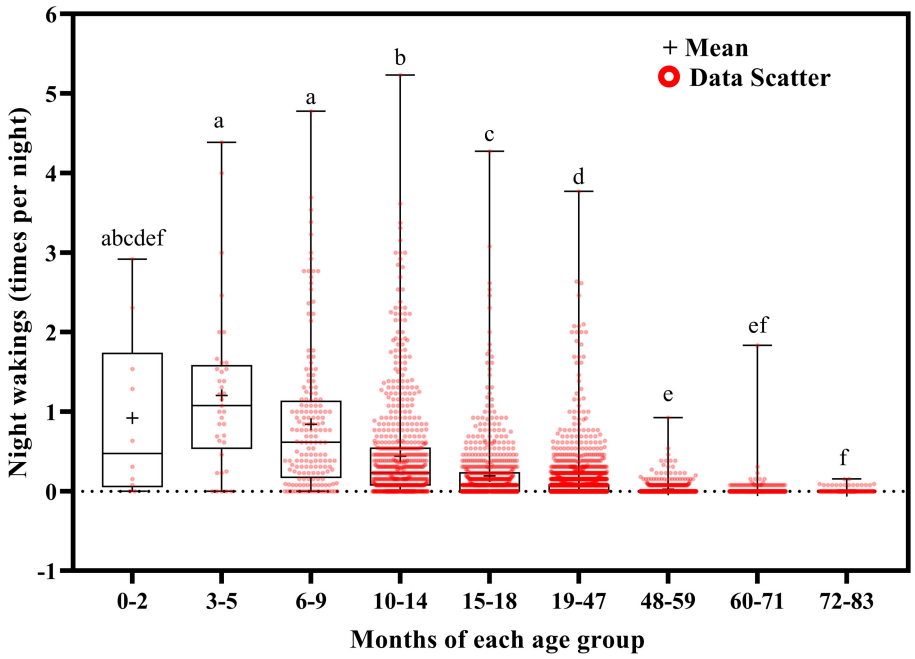


FIGURE 8
Results of the one-way ANOVA for night wakings stratified by age.

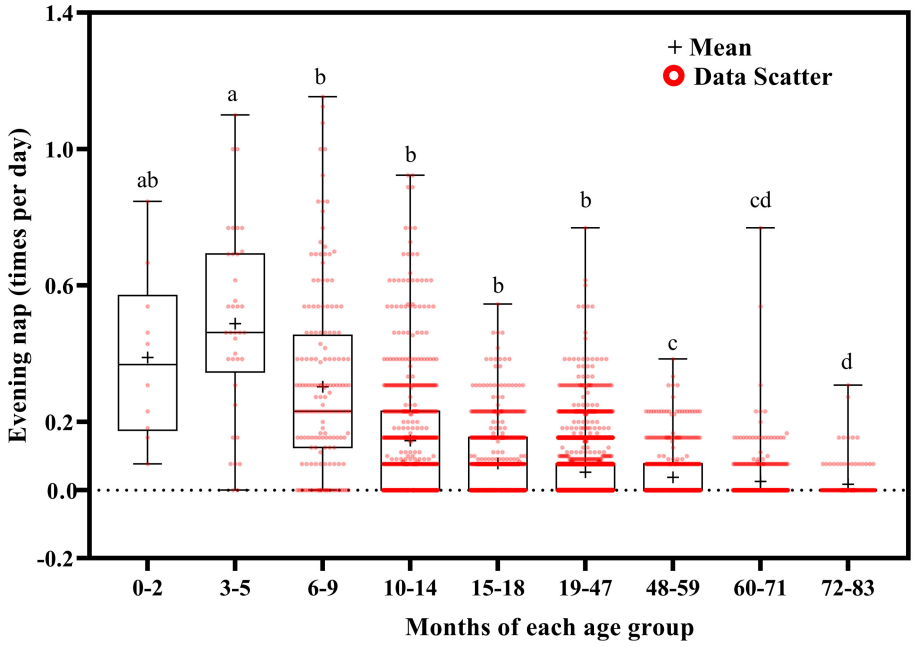


FIGURE 9
Results of the one-way ANOVA for evening nap stratified by age.

Evening nap frequency tended to decrease with increasing age (Figure 9). Evening naps were more frequent at ages 0–2 months than at ages 48–59 ($p = 0.050$), 60–71 ($p = 0.040$), and 72–83 ($p = 0.034$) months; at ages 3–5 months than at ages 6–9 ($p = 0.018$) and 10–83 ($p < 0.001$) months; at ages 6–9 months than at ages 10–83 months ($p < 0.001$); at ages 10–14 months

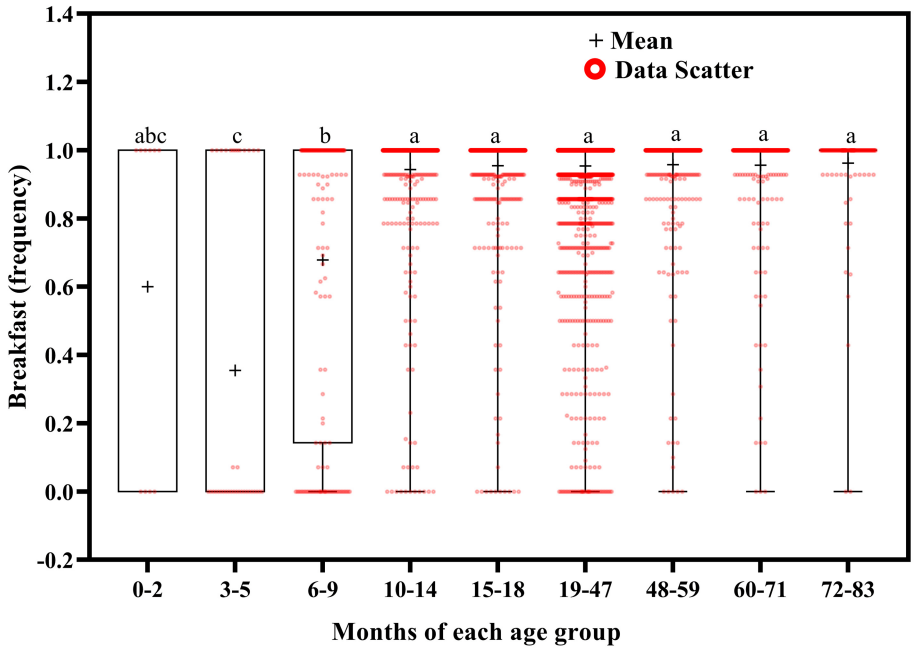


FIGURE 10
Results of the one-way ANOVA for breakfast stratified by age.

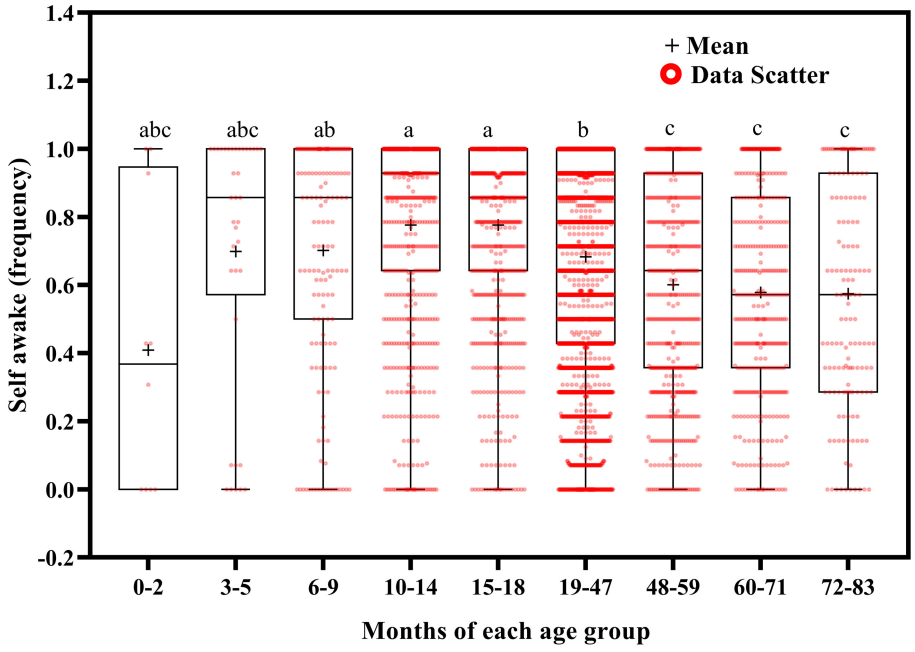


FIGURE 11
Results of the one-way ANOVA for self-awake stratified by age.

than at ages 15–83 months ($p < 0.001$); at ages 15–18 months than at ages 19–83 months ($p < 0.001$); at ages 19–47 months than at 48–83 months ($p < 0.001$); and at ages 48–59 months than at ages 72–83 months ($p = 0.001$).

Breakfast frequency tended to increase with increasing age (Figure 10). Children aged 3–5 months ate breakfast less often than those aged 6–9 ($p = 0.015$) and 10–83 ($p < 0.001$) months, and children aged 6–9 months

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TABLE 3 Results of the discriminant analysis stratified by age.

| Groups | Months | Eigenvalue | Canonical correlation coefficient | wilks' Lambda | $X^2$ | *df* | *p* | Discriminant variable(s) | Accuracy |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 3–5 m | 0.628 | 0.621 | 0.614 | 16.804 | 1 | 0.000 | Bed time | 97.30% |
| 2 | 6–9 m | 0.502 | 0.578 | 0.666 | 67.111 | 2 | 0.000 | Wake up time variation; Bed time | 88.70% |
| 3 | 10–14 m | 0.299 | 0.480 | 0.770 | 178.292 | 6 | 0.000 | Night wakings; Total sleep duration; Bed time; Bed time variation; Wake up time; Wake up time variation | 86.80% |
| 4 | 15–18 m | 0.298 | 0.479 | 0.770 | 204.112 | 4 | 0.000 | Wake up time; Wake up time variation; Bed time; Bed time variation | 89.30% |
| 5 | 19–47 m | 0.352 | 0.510 | 0.740 | 1246.358 | 6 | 0.000 | Breakfast; Night wakings; Wake up time; Wake up time variation; Bed time; Bed time variation | 88.10% |
| 6 | 48–59 m | 0.299 | 0.480 | 0.770 | 162.944 | 3 | 0.000 | Total sleep duration; Wake up time variation; Bed time | 88% |
| 7 | 60–71 m | 0.164 | 0.376 | 0.859 | 61.390 | 3 | 0.000 | Night wakings; Total sleep duration; Bed time | 71.30% |
| 8 | 72–83 m | 0.963 | 0.700 | 0.509 | 98.128 | 3 | 0.000 | Total sleep duration; Nocturnal sleep duration; Bed time | 80.50% |

ate breakfast less often than those aged 10–83 months ($p < 0.001$).

Self-awakening tended to decrease with increasing age (Figure 11). Children self-awoke more frequently at ages 10–18 months than at ages 19–83 months ($p < 0.01$); at ages 6–9 months than at ages 48–59 ($p = 0.027$), 60–71 ($p = 0.003$), and 72–83 ($p = 0.033$) months; and at ages 19–47 months than at ages 48–71 ($p < 0.001$) and 72–83 ($p = 0.005$) months.

## Generation of a classifier to screen for severe sleep disorders

Discriminant analysis was conducted for each age group except Group 0 (0–2 months), whose sample size ($n = 10$) was insufficient for analysis. Judgment of the children's sleep quality (1 and 0) served as the dependent variable. The significant influencing factors determined *via* the stepwise method served as the discriminant variables. In the stepwise method, a variable that minimizes Wilks' lambda for the entire dataset is submitted at each step, and the maximum number of steps is 20. The identity of the classifier that discriminates between the presence and absence of severe sleep disorders in each age group is shown in Table 3.

Group 1 (3–5 months) had a high canonical correlation coefficient (0.621), which indicates that the discriminant function can discriminate the children's sleep quality well. In addition, Wilks' lambda was significant ($p < 0.001$), indicating that the distance between severe and non-severe sleep disorders was sufficiently large. The significant discriminant variable

TABLE 4 Fisher function coefficients of 3–5 months age group.

| Discriminant variable | $y_0$ | $y_1$ |
|---|---|---|
| Bed time | 41.685 | 37.154 |
| (constant) | −492.019 | −391.015 |

TABLE 5 Fisher function coefficients of 6–9 months age group.

| Discriminant variables | $y_0$ | $y_1$ |
|---|---|---|
| Wake up time variation | 24.341 | 19.184 |
| Bed time | 34.666 | 31.853 |
| (constant) | −400.922 | −337.346 |

identified by the stepwise method for Group 1 was the bed time. In a cross-validation test, the accuracy was 97.3%. The Fisher function coefficients are shown in Table 4.

The values of $y_0$ and $y_1$ are calculated by substituting each discriminant variable(s). If $y_1 > y_0$, the judgment is 1, which means that the child does not have a severe sleep disorder. If $y_0 > y_1$, the judgment is 0, which means that the child has a severe sleep disorder and needs immediate intervention. The advantage of using the Fisher function instead of the standardized canonical discriminant function is that it allows direct calculation of the values of the variables obtained from the 14-day sleep log.

Group 2 (6–9 months), Group 3 (10–14 months), Group 4 (15–18 months), Group 5 (19–47 months), Group 6 (48–59 months), Group 7 (60–71 months), and Group 8 (72-83 months) also had a high canonical correlation coefficient (0.578, 0.480, 0.479, 0.510, 0.480, 0.376, and 0.700, respectively) and a significant Wilkes' lambda (all $p < 0.001$).

The significant discriminant variables for Group 2 were bed time and wake up time variation; the accuracy was 88.7%. The Fisher function coefficients are shown in Table 5.

The significant discriminant variables for Group 3 were night wakings, total sleep duration, bed time, bed time variation, wake up time, and wake up time variation; the accuracy was 86.8%. The Fisher function coefficients are shown in Table 6.

The significant discriminant variables for Group 4 were wake up time, wake up time variation, bed time, and bed time variation; the accuracy was 89.3%. The Fisher function coefficients are shown in Table 7.

The significant discriminant variables for Group 5 were breakfast, night wakings, bed time, bed time variation, wake up time, and wake up time variation; the accuracy was 88.1%. The Fisher function coefficients are shown in Table 8.

TABLE 6 Fisher function coefficients of 10–14 months age group.

| Discriminant variables | $y_0$ | $y_1$ |
|---|---|---|
| Night wakings | 21.095 | 20.517 |
| Total sleep duration | 48.222 | 48.774 |
| Bed time | −42.915 | −41.966 |
| Bed time variation | 20.686 | 17.265 |
| Wake up time | 80.051 | 77.281 |
| Wake up time variation | 4.665 | 7.182 |
| (constant) | −1016.801 | −967.353 |

TABLE 7 Fisher function coefficients of 15–18 months age group.

| Discriminant variables | $y_0$ | $y_1$ |
|---|---|---|
| Wake up time | −14.981 | −13.263 |
| Wake up time variation | 22.049 | 14.206 |
| Bed time | 63.949 | 60.297 |
| Bed time variation | −12.001 | −5.334 |
| (constant) | −667.184 | −594.396 |

TABLE 8 Fisher function coefficients of 19–47 months age group.

| Discriminant variables | $y_0$ | $y_1$ |
|---|---|---|
| Breakfast | 56.681 | 58.185 |
| Night wakings | 20.155 | 17.231 |
| Wake up time | −13.828 | −14.662 |
| Wake up time variation | 5.050 | 1.415 |
| Bed time | 80.974 | 77.699 |
| Bed time variation | −14.396 | −12.494 |
| (constant) | −892.961 | −814.159 |

TABLE 9 Fisher function coefficients of 48–59 months age group.

| Discriminant variables | $y_0$ | $y_1$ |
|---|---|---|
| Total sleep duration | 54.291 | 52.887 |
| Wake up time variation | 9.948 | 6.118 |
| Bed time | 89.612 | 85.382 |
| (constant) | −1308.482 | −1199.060 |

The significant discriminant variables for Group 6 were total sleep duration, bed time variation, and bed time; the accuracy was 88%. The Fisher function coefficients are shown in Table 9.

The significant discriminant variables for Group 7 were night wakings, total sleep duration, and bed time; the accuracy was 71.3%. The Fisher function coefficients are shown in Table 10.

The significant discriminant variables for Group 8 were total sleep duration, nocturnal sleep duration, and bed time; the

TABLE 10 Fisher function coefficients of 60–71 months age group.

| Discriminant variables | $y_0$ | $y_1$ |
|---|---|---|
| Night wakings | 39.119 | 35.671 |
| Total sleep duration | 48.610 | 47.691 |
| Bed time | 77.240 | 75.409 |
| (constant) | −1106.977 | −1057.470 |

TABLE 11 Fisher function coefficients of 72–83 months age group.

| Discriminant variables | $y_0$ | $y_1$ |
|---|---|---|
| Total sleep duration | 54.552 | 49.927 |
| Nocturnal sleep duration | 30.080 | 33.847 |
| Bed time | 111.102 | 108.746 |
| (constant) | −1633.058 | −1569.110 |

accuracy was 80.50%. The Fisher function coefficients are shown in Table 11.

# Discussion

## Sleep characteristics of different age groups

Using a large dataset of children's sleep status, we found that total sleep duration and nocturnal sleep duration decreased with increased age. In line with this finding, the American Academy of Sleep Medicine recommends the following sleep durations for children: 12–16 h at ages 4–11 months, 11–14 h at ages 1–2 years, 10–13 h at ages 3–5 years, and 9–12 h at ages 6–12 years (20).

In our study, increases in age were consistently associated with decreases in wake up time variation and bed time variation. By the age of 36 months, some children no longer need a nap. After the age of 48 months, children have almost no night wakings or evening naps; in fact, a nap is no longer necessary at this age if the child sleeps for 10–11 h at night. Pediatricians attribute this phenomenon to the gradual establishment of sleep patterns in children aged over 19 months (21).

We also found that as the children aged, they went to bed and woke up at later times and were less able to wake up by themselves. Possible causes include late bed time and poor sleep quality. A previous study demonstrated that children who engage in watching television and playing with smartphones or tablets go to bed at later times than those who do not (22). Stress and long work hours in a competitive society with a large amount of information may account in part for the deteriorating sleep quality (23), a phenomenon that seems to reflect the lifestyle of a modern society.

In this study, children older than 10 months consumed breakfast almost every day. This finding suggests that by 10 months of age, most children have developed a pattern of eating three times a day and that, despite the later wake up time, parents always serve their children breakfast before sending them to a nursery school.

The generally accepted developmental patterns are considered as follows.

In order to survive, the body has a mechanism that prioritizes "eating" over "sleeping." Therefore, sleep time was reduced when child was hungry. According to the Guidelines for Breastfeeding and Weaning Support (Revised 2019) published on the website of the Japanese Ministry of Health, Labor and Welfare (24), milk consumption by infants during one session increases with growth, with the frequency being ∼every 3 h around 1–2 months of age and every 4 h around 3–5 months of age. Around 3–4 months of age, the body's mechanisms gradually change to correspond to the repetitive light-dark rhythm of day and night, and the tendency of continuous sleep increases. By 5–6 months of age, the child's gastrointestinal tract has developed the ability to digest and absorb food, and weaning can begin while monitoring the child's condition. The amount of milk they drink at one time may increase, and by 6 months of age, they will feed less frequently at night and sleep for approximately 6 h at a time, without waking up in the middle of the night. Around 6–7 months of age, baby teeth begin to erupt and the child develops the ability to chew. Weaning is completed by 9–10 months of age, and the child begins to develop a rhythm of eating three times a day. Eating well provides energy for daytime activities and helps them sleep well at night.

Infants do not sleep throughout the night, so naps during daytime is important. However, napping in an uncontrolled manner can be detrimental. If the child sleeps well at night, the nap will be in the form of one morning and one afternoon nap at 1 year of age and one afternoon nap after 1 year 6 months of age. By the age of 4 years, the child no longer needs naps. However, for children without a good rhythm, they may nap in small increments, and although they may feel a little more energetic after sleeping, they may continue this repetitive state: waking up for a short while, playing, and then going back to sleep. In the absence of proper sleep, this unhealthy pattern is repeated. Therefore, it is important to establish a daily rhythm early.

Taken together, our findings, which were obtained by accessing a large dataset, are in line with the general characteristics of children's sleep.

Regarding the change in discriminant variables by age, bed time was found to be a discriminant variable for all age groups considering that earlier bed time ensures longer sleep. Next, wake up time variation was found to be a discriminant variable in all age groups except those aged 3–5 months and 60–83 months. Because a regular daily rhythm can foster good sleep behavior, wake up variation is an important indicator in determining sleep quality. In the 10–47 months age group, bed time, wake up time variation, bed time variation, and wake up time were found as discriminant variables. After 10 months of

age, children have completed weaning and are adjusting their life and circadian rhythm. Therefore, daily bed time, bed time variation, wake up time, and wake up time variation can be used to determine if daily rhythm is proper. However, since children in the 10–14 months age group frequently wake up during the night, night wakings is an important indicator, and total sleep duration should also be included as an indicator since increased night wakings can reduce sleep time. Therefore, for the 10–14 months age group, sleep quality should be assessed using a total of six variables: bed time, bed time variation, wake up time, wake up time variation, night wakings, and total sleep duration. In addition, children aged 19–47 months are more active during the day as their physical growth and motor skills develop. Breakfast guarantees energy for daytime activities, which leads to good sleep at night. Therefore, it is reasonable to assess sleep quality in the 19–47 months age group using a total of six variables: bed time, bed time variation, wake up time, wake up time variation, breakfast, and night wakings.

Since children after the age of 4 years no longer need naps if they are sleeping well at night, naps are also an important indicator to assess sleep quality. In this study, evening nap (15:00–19:00) was extracted from the 14-day sleep logs and is considered to interfere with nighttime sleep based on clinical findings. Therefore, it is necessary to assess the presence and hours of naps; total sleep duration, bed time, and wake up time variation were accepted as discriminant variables in the 48–59 months age group and were considered valid. In the 60–71 months age group, total sleep duration, bed time, and night wakings were found to be discriminant variables, and since wake up time variation was excluded from the discriminant variables in the 60–83 months age group, it can be inferred that children in this age group wake up at the same time almost every day. Therefore, bed time, total sleep duration, and night wakings are important indicators to assess sleep quality. However, the accuracy of the discriminant analysis for the 60–71 months age group was somewhat low, suggesting that the validity of the discriminant variables should be re-examined in future studies. In the 72–83 months age group, total sleep duration, bed time, and nocturnal sleep duration were found to be discriminant variables for children after the age of 6 years, because their life, circadian, and dietary rhythms have already been established.

According to the Sleep Guidelines for Health Promotion 2014 published by the Japanese Ministry of Health, Labor and Welfare (25), lack of sleep at night can lead to poor brain function, impaired concentration, attention, memory, and judgment, accompanied by daytime sleepiness, fatigue, inactivity, irritability, and anger. Accidents, injuries, and failures increase and cause low self-esteem. Furthermore, chronic sleep deprivation has been shown to increase the risk of diabetes, obesity, hypertension, and dementia. Therefore, good quality sleep, especially at night, is of utmost importance and is a prerequisite for healthy life.

## Situations in which the classifier can be used to screen for severe sleep disorders

Using a statistical model generated *via* discriminant analysis, we herein developed a classifier for screening severe sleep disorders in children. Our classifier allows us to quickly screen for, and thus immediately treat, severe sleep disorders on the basis of data contained in a 14-day sleep log. Its accuracy was almost 80% or higher for all age groups; thus, it is a valid screening tool. As an advantage, it can be used to simultaneously screen many cases. We assume that our classifier can be used in the situations described below.

### Use by local governments in regular health checkups conducted for children

Despite the high prevalence of sleep disorders in the general population, healthcare utilization among patients with sleep disorders is relatively low. This finding, which is based on information in the Cerner Health Facts database, suggests that awareness of sleep disorders and access to medical care for sleep disorders are poor (26). In addition, previous studies suggest that primary care providers and pediatricians may underdiagnose sleep disorders in children (27, 28). Therefore, it is necessary to educate parents about the importance of sleep for children during the health checkups mandated by local governments. In Japan, local governments conduct health checkups for children aged 3–4, 9–10, 18 months, and 3 years. At that time, parents could be asked to record their children's sleep patterns for the 14 days preceding the checkup and to submit the log at the checkup; the classifier would then be applied. Parents would then be informed of their children's sleep problems and given instructions on how to manage their children's sleep and sleep hygiene; they would also be advised to visit a pediatrician if necessary. This procedure would facilitate early detection and treatment of sleep disorders in children.

### Use by parents via an application

Parents, especially first-time parents, sometimes feel anxious and lonely because they cannot talk to anyone about their child-raising concerns. To aid parents, we are developing an application that uses artificial intelligence to enable consultations between parents and pediatricians, senior mothers from NPOs, etc. on child-rearing issues. Parents will be able to monitor the sleep quality of their children anytime and anywhere by installing the application on their smartphones. The application, which will include the classifier, will alert parents to their children's sleep problems and will encourage those in need to consult a pediatrician.

As a ripple effect, alleviating sleep disorders would be expected to alleviate postpartum depression in mothers and prevent child abuse. Studies showed that the quality of children's

sleep significantly predicted the quality of maternal sleep, and they highlight the importance of screening for and treating pediatric sleep disruptions (29). Another study showed that significant differences in postpartum depression were found between mothers of children with and without significant sleep disorders (30). Mothers of children with sleep disorders have poorer sleep quality and are easily fatigued during the day and night, affecting their care of the child, feeding, and response to the child's sleep behavior. Persistent sleep disorders can lead to a series of long-term effects such as growth retardation and behavioral problems, which increase parenting stress and lead to emotional distress, creating a vicious cycle. Therefore, screening for severe sleep disorder in children and promptly providing intervention treatment and sleep hygiene guidance can help alleviate postpartum depression in mothers (31). In addition, parental depression is one of the risk factors for child abuse (32, 33). Therefore, alleviating maternal postpartum depression can help prevent child abuse.

In conclusion, we expect that the classifier developed in this study will facilitate the screening of children's sleep disorders in multiple situations. A previous study showed that early detection and treatment of sleep disorders aided the treatment of mental disorders (34); therefore, use of the classifier may help improve mental status. In the future, we will further improve the accuracy of the automatic classifier by incorporating machine-learning techniques.

## Data availability statement

The datasets analyzed in this study can be found at https://akachan.doshisha.ac.jp/forresearcher/bu/bu-about (Doshisha University Center for Baby Science Joint Usage/Research Center).

## Ethics statement

The studies involving human participants were reviewed and approved by Doshisha University. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

## Author contributions

Conceptualization, supervision, project administration, and funding acquisition: MK and SI. Methodology, software, data curation, visualization, and writing-review and editing: MK and MJ. Validation: MJ, MK, and SI. Formal analysis and writing-orginal draft preparation: MJ. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY
Nis Borbye-Lorenzen,
Statens Serum Institute, Denmark

REVIEWED BY
Cihad Dundar,
Ondokuz Mayis University, Turkey
Hüsamettin Vatansev,
University of Selcuk, Turkey

*CORRESPONDENCE
Zhongliang Wang
4829855@163.com

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# Predictive value of the systemic immune-inflammation index for cancer-specific survival of osteosarcoma in children

Haiping Ouyang and Zhongliang Wang*

Department of Orthopedics, Chongqing Key Laboratory of Pediatrics, Ministry of Education Key Laboratory of Child Development and Disorders, National Clinical Research Center for Child Health and Disorders, China International Science and Technology Cooperation base of Child development and Critical Disorders, Children's Hospital of Chongqing Medical University, Chongqing, China

**Background:** Osteosarcoma (OS) is the primary malignant bone tumor that most commonly affects children and adolescents. Recent years effective chemotherapy have improved the 5-year survival in osteosarcoma patients to up to 60%-70%. Still, there is a lack of novel therapeutic strategies to enhance further survival. Our study aimed to evaluate the clinical significance of pretreatment inflammatory-based parameters, including PLT, NLR, and SII, as prognostic indicators of survival in pediatric osteosarcoma patients.

**Methods:** A total of 86 pediatric osteosarcoma patients between 2012 and 2021 in the Department of Orthopedics or tumor Surgery of Children's Hospital affiliated to Chongqing Medical University were retrospectively analyzed. The clinicopathological variables and systematic inflammatory biomarkers, including NLR, PLR and SII, was performed by the A Receiver operating characteristic (ROC) curve and Cox proportional risk regression model. According to the results of multivariate analysis, a prognostic nomogram was generated, and the concordance index (C-index) was calculated to predict the performance of the established nomogram. The survival curve was plotted by the Kaplan-Meier method.

**Results:** Univariate analysis showed that TNM stage, tumor size, NLR value, PLR value, SII value, neutrophil count and platelet count were related to CSS ($p < 0.05$). According to multivariate analysis, only TNM stage ($p = 0.006$) and SII values ($p = 0.015$) were associated with poor prognosis.To further predict survival in pediatric osteosarcoma patients, multivariate Cox regression analysis was used to predict cancer-specific survival at 1, 3 and 5 years. And constructed a nomogram model to predict children's CSS. The C-index of the nomogram is 0.776 (95%CI, 0.776–0.910), indicating that the model has good accuracy.

**Conclusion:** Preoperative SII and TNM staging are independent prognostic markers for pediatric osteosarcoma patients. SII may be used in conjunction with TNM staging for individualized treatment of pediatric osteosarcoma patients in future clinical work.

KEYWORDS

**pediatric osteosarcoma, systemic immune-inflammation index, cancer-specific survival, prognosis, event-free survival**

## Introduction

Osteosarcoma is the primary malignant bone tumor that most commonly affects children and adolescents (1). The incidence rates of Osteosarcoma for all races and both sexes are 4.0 for the range 0–14 years and 5.0 for the content 0–19 years per year per million persons (2). Osteosarcoma exhibits a propensity to occur in the metaphysis of long bones and most commonly occurs in the distal femur (43%), proximal tibia (23%), or humerus (10%) (3). The lung is the most common site of metastasis, with over 85% of metastatic disease occurring there, while the bone is the second most common site of distant metastasis (3). Osteosarcomas may progress rapidly with poor prognosis and high mortality. Recurrence and metastasis are the significant causes of death and poor prognosis in children with Osteosarcoma. Recent years effective chemotherapy have improved the 5-year survival in osteosarcoma patients to up to 60%-70%. Still, there is a lack of novel therapeutic strategies to enhance further survival (4). The traditional approaches such as tumor size, metastasis, histological subtype, and tumor stage have been considered inaccuracy and inadequacy as prognostic parameters in routine clinical practice (5). Therefore, it is crucial to find reliable prognostic factors for pediatric osteosarcoma patients. Tumor-promoting inflammation has been recognized as an enabling characteristic of cancer (6). The interplay between local immune response and systemic inflammation plays vital roles in cancer progression and patient survival (7). Therefore, inflammatory parameters are strong candidates for predicting tumor prognosis. Measuring neutrophils, lymphocytes, and platelets on a total blood count may help understand systemic inflammatory responses. However, individual inflammatory parameters are susceptible to other factors, so a combination of inflammatory indicators such as neutrophil to lymphocyte ratio (NLR) platelet to lymphocyte ratio (PLR) may theoretically be more reliable. Recently, neutrophils, lymphocytes, and platelets have been used in a joined tool, a systemic immune-inflammation index (SII), to obtain the prognostic information in patients with various malignant tumors, such as hepatocellular carcinoma, esophageal squamous cell carcinoma, gastric cancer, non-small-cell lung cancer, colorectal cancer, and epithelial ovarian cancer (8–13). However, the relationship between these inflammatory markers and childhood osteosarcoma is poorly understood. Therefore, our study aimed to evaluate the clinical significance of pretreatment inflammatory-based parameters, including PLT,NLR and SII, as prognostic indicators of survival in pediatric osteosarcoma patients.

TABLE 1 Clinicopathological characteristics of children with OS.

| | ALL $N = 86$ | Dead $N = 24$ | Alive $N = 62$ | p |
|---|---|---|---|---|
| Age | 10.7 (2.93) | 9.58 (3.59) | 11.1 (2.55) | 0.068 |
| Sex | | | | 0.831 |
| Male | 54 (62.8%) | 16 (66.7%) | 38 (61.3%) | |
| Female | 32 (37.2%) | 8 (33.3%) | 24 (38.7%) | |
| Region | | | | 1.000 |
| Urban | 44 (51.2%) | 12 (50.0%) | 32 (51.6%) | |
| Rural | 42 (48.8%) | 12 (50.0%) | 30 (48.4%) | |
| Medical.insurance | | | | 0.392 |
| No | 42 (48.8%) | 14 (58.3%) | 28 (45.2%) | |
| Yes | 44 (51.2%) | 10 (41.7%) | 34 (54.8%) | |
| Primary.site | | | | 0.179 |
| Limb | 80 (93.0%) | 24 (100%) | 56 (90.3%) | |
| Axial | 6 (6.98%) | 0 (0.00%) | 6 (9.68%) | |
| lateral | | | | 0.057 |
| Left | 46 (53.5%) | 10 (41.7%) | 36 (58.1%) | |
| Right | 34 (39.5%) | 14 (58.3%) | 20 (32.3%) | |
| Not pairs | 6 (6.98%) | 0 (0.00%) | 6 (9.68%) | |
| Stage | | | | <0.001 |
| I | 28 (32.6%) | 2 (8.33%) | 26 (41.9%) | |
| II | 24 (27.9%) | 2 (8.33%) | 22 (35.5%) | |
| III | 21 (24.4%) | 9 (37.5%) | 12 (19.4%) | |
| IV | 13 (15.1%) | 11 (45.8%) | 2 (3.23%) | |
| Surgery | | | | 0.310 |
| Limb salvage | 82 (95.3%) | 22 (91.7%) | 60 (96.8%) | |
| Amputation | 4 (4.65%) | 2 (8.33%) | 2 (3.23%) | |
| Chemotherapy | | | | 0.496 |
| No | 12 (14.0%) | 2 (8.33%) | 10 (16.1%) | |
| Yes | 74 (86.0%) | 22 (91.7%) | 52 (83.9%) | |
| Radiotherapy | | | | 0.573 |
| No | 82 (95.3%) | 24 (100%) | 58 (93.5%) | |
| Yes | 4 (4.65%) | 0 (0.00%) | 4 (6.45%) | |
| Size | 71.0 (37.4) | 89.9 (28.6) | 63.7 (38.0) | 0.001 |
| PLR | 128 (73.6) | 166 (86.8) | 113 (62.6) | 0.011 |
| NLR | 1.61 (0.72) | 1.90 (0.73) | 1.49 (0.69) | 0.021 |
| SII | 677 (464) | 913 (462) | 586 (436) | 0.005 |
| Platelet | 407 (157) | 474 (146) | 381 (154) | 0.013 |
| Neutrophile | 5.14 (1.48) | 5.66 (1.24) | 4.94 (1.53) | 0.026 |
| Lymphocyte | 3.56 (1.17) | 3.35 (1.43) | 3.65 (1.06) | 0.367 |
| Metastasis | | | | 1.000 |
| No | 78 (90.7%) | 22 (91.7%) | 56 (90.3%) | |
| Yes | 8 (9.30%) | 2 (8.33%) | 6 (9.68%) | |
| Survival. months | 33.6 (23.4) | 21.2 (21.9) | 38.4 (22.3) | 0.002 |

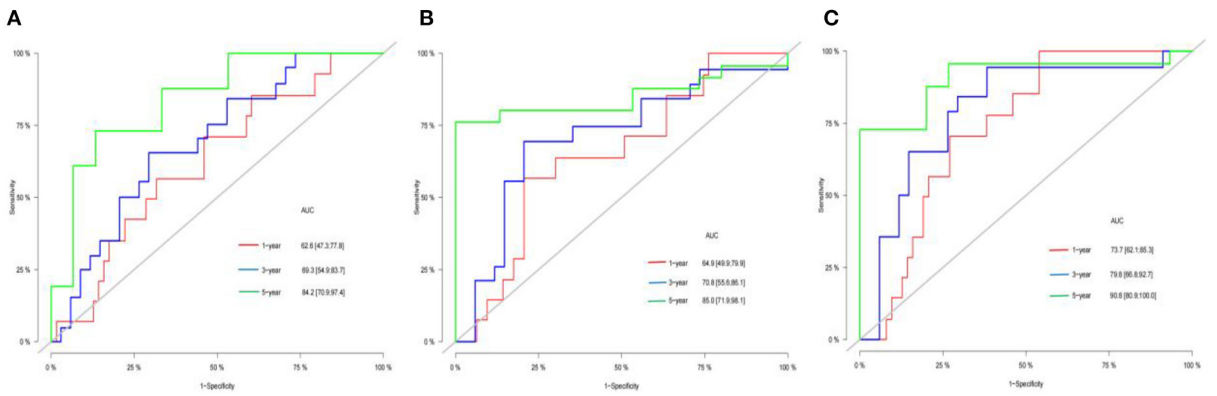


FIGURE 1
AUC of the NLR(**A**), PLR(**B**), and SII(**C**) for 1-, 3-, 5-year CSS of children with OS.

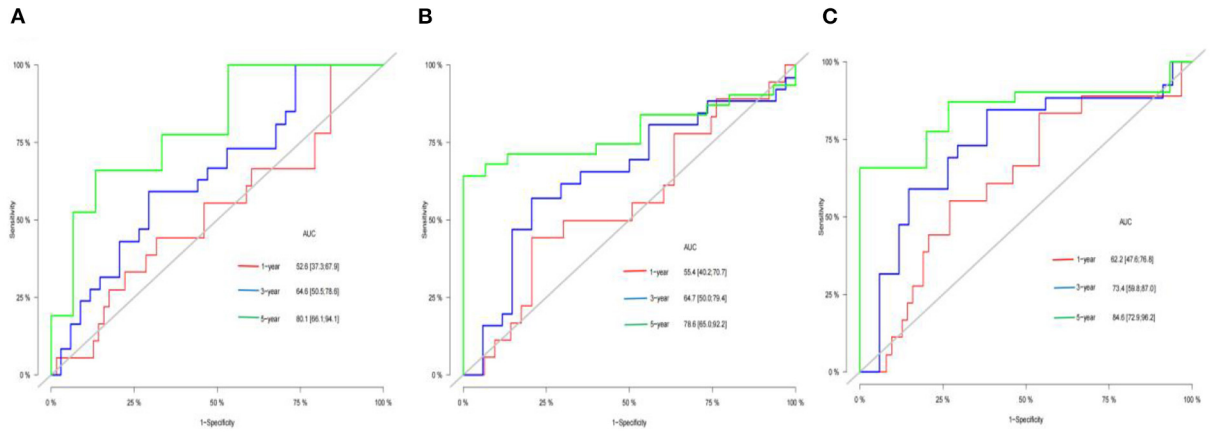


FIGURE 2
AUC of the NLR(**A**), PLR(**B**), and SII(**C**) for 1-, 3-, 5-year EFS of children with OS.

# Patients and methods

## Data source and data extraction

We conducted a retrospective analysis on pediatric osteosarcoma patients who underwent radical surgery or limb salvage surgery in the Department of Orthopedics or tumor Surgery of Children's Hospital affiliated to Chongqing Medical University from May 2012 to September 2021. The Ethics Committee approved this study of Children's Hospital Affiliated to Chongqing Medical University. Written informed consent was obtained for the study from the parents of the patients.

Inclusion criteria were: 1) pathologically diagnosed as Osteosarcoma. 2) No previous anticancer treatment. 3) Have detailed medical data and laboratory results, And 4) available follow-up. Exclusion criteria were: 1) pre-existing blood disorders. 2) There are inflammatory diseases such as infection before treatment. 3) Incomplete medical records and laboratory results; Or 4) use non-steroidal anti-inflammatory drugs, as this may interfere with blood tests. Finally, we collected the medical data of 86 pediatric osteosarcoma patients in our hospital.

## Data collection

We collected relevant clinicopathological data, including gender, age, region, medical insurance, primary tumor site, left and right sides, TNM stage, operation, chemotherapy, radiotherapy, metastasis, and survival time. Routine laboratory data included preoperative blood samples in determining neutrophil, lymphocyte, and platelet levels and calculating NLR, PLR, and SII indices. NLR and PLR were defined as the total number of neutrophils or platelets divided by the total number of lymphocytes. SII was calculated by the formula SII = (P×N)/L, where P,N and L represented peripheral blood plate, neutrophil, and lymphocyte counts, respectively.

TABLE 2 AUC of the NLR, PLR, and SII for 1-, 3-, 5-year CSS and EFS of children with OS.

| | AUC | 95%CI |
|---|---|---|
| NLR 1 year CSS | 0.626 | 0.473–0.778 |
| NLR 3-year CSS | 0.693 | 0.549–0.837 |
| NLR 5-year CSS | 0.842 | 0.709–0.974 |
| PLR 1 year CSS | 0.649 | 0.449–0.799 |
| PLR 3-year CSS | 0. 708 | 0.556–0.861 |
| PLR 5-year CSS | 0.850 | 0.719–0.981 |
| SII 1 year CSS | 0.731 | 0.621–0.853 |
| SII 3-year CSS | 0.798 | 0.668–0.927 |
| SII 5-year CSS | 0. 908 | 0.809–1.000 |
| NLR 1 year EFS | 0.526 | 0.373–0.679 |
| NLR 3-year EFS | 0.646 | 0.505–0.786 |
| NLR 5-year EFS | 0.801 | 0.661–0.941 |
| PLR 1 year EFS | 0.554 | 0.402–0.707 |
| PLR 3-year EFS | 0.647 | 0.500–0.794 |
| PLR 5-year EFS | 0.786 | 0.650–0.922 |
| SII 1 year EFS | 0.622 | 0.476–0.768 |
| SII 3-year EFS | 0.734 | 0.598–0.870 |
| SII 5-year EFS | 0.846 | 0.729–0.962 |

## Follow-up

All pediatric osteosarcoma patients require regular follow-up after surgery. According to the institution's practice, we follow up once every 3 months in the first three years, once every 6 months in the fourth to 15 years, and once a year after that. Contact the patient by outpatient examination or telephone. Physical examination, blood test, surgical site X-ray, chest CT are routine clinical examination items in our hospital. Follow-up was completed until death or November 2021. Overall survival was considered the interval from surgery to the date of tumor-related death or loss of follow-up or last contact.

The event-free survival (EFS) period was defined as the time from the start of study treatment to metastasis, recurrence, or death. Cancer-specific survival (CSS) is the interval between the initial diagnosis of Pediatric Osteosarcoma and the occurrence of Pediatric osteosarcoma-specific death.

## Statistical analysis

All analyses were performed using SPSS 26.0 and R Software 4.1.0. Optimal prognostic cut-off values for NLR, PLR, and SII were calculated using the A Receiver operating characteristic (ROC) curve corrected by the Jorden index. These values were used as thresholds to group all patients above or below the points. The survival curve was plotted by the Kaplan-Meier method. Cox proportional risk regression model was used for univariate and multivariate analysis. Only significant prognostic parameters from the univariate Cox balanced risk model were included in the multivariate analysis to determine independent prognostic factors in pediatric osteosarcoma patients. Based on independent risk factors, nomograms that predicted CSS of pediatric osteosarcoma patients were built.The concordance index (C-index) was calculated to predict the performance of the established nomogram. $P < 0.05$ was considered statistically significant.

# Result

## Baseline patient characteristics

The basic characteristics of 86 pediatric osteosarcoma patients in this study are summarized in Table 1. Twenty-four of the pediatric osteosarcoma patients had died, and 62 of the pediatric osteosarcoma patients were alive. Fifty-four (62.8%) were boys, and 32 (37.2%) were girls. Forty-four (51.2%) pediatric osteosarcoma patients were from urban areas, and 42 (48.8%) pediatric osteosarcoma patients were from rural areas. 42 (48.8%) had health insurance, and another 44 (51.2%) did not. Eighty patients (93.0%) developed osteosarcomas in the extremities and six (6.98%) in the trunk. Forty-six patients (53.5%) had primary lesions in the left limb, and 34 patients (39.5%) had primary lesions in the right limb. According to TNM staging of Osteosarcoma, 28 (32.6%) had stage 1, 24 (27.9%) had stage II, 21 (24.4%) had stage III, and 13 (15.1%) had stage IV. Eighty-two (95.3%) had limb salvage surgery, while another four (4.65%) had amputation surgery. 74 (86.0%) had received chemotherapy, and 12 (14.0%) had not received chemotherapy. Only 4 (4.65%) received radiotherapy, and the remaining 82 (95.3%) did not. The mean tumor size was 71.0 mm. Platelet count, neutrophil count, and lymphocyte count were 407, 5.14, and 3.56, respectively. The mean values of PLR, NLR, and SII were 128, 1.61, and 677, respectively. No metastasis occurred in 78 patients (90.7%), and metastasis occurred in 8 patients (9.30%). Up to the last follow-up time, the mean survival time of 86 pediatric osteosarcoma patients was 33.6 months, including 21.2 months for the deceased pediatric osteosarcoma patients and 38.4 months for the living patients.

## ROC curve analysis of inflammatory indices of pediatric osteosarcoma patients

NLR, PLR, and SII were used to predict 1, 3 and 5-year event-free survival and cancer-specific survival in pediatric osteosarcoma patients. The accuracy of NLR, PLR and SII in predicting cancer-specific survival and event-free survival at 1, 3 and 5 years were shown in Figures 1, 2, respectively, by

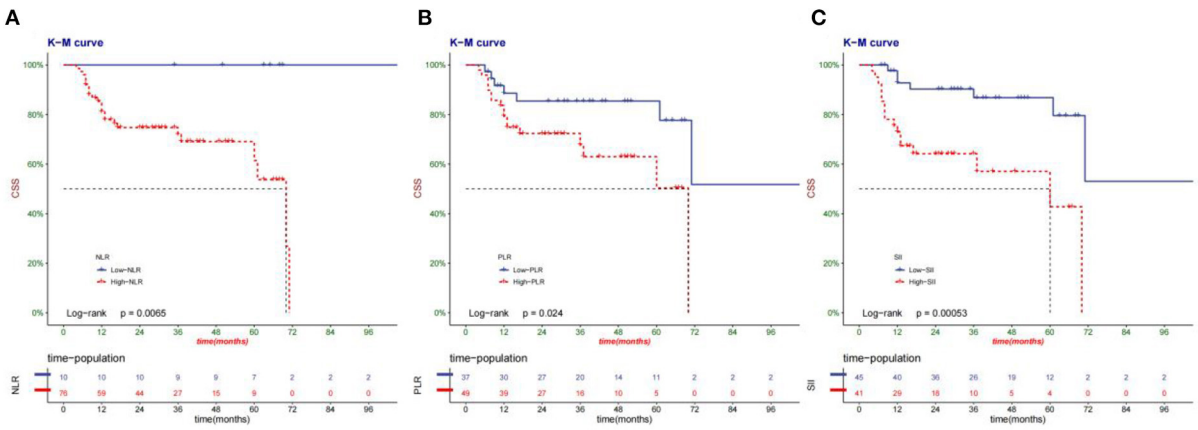


FIGURE 3
Kaplan-Meier curve of the CSS of patients according to NLR**(A)**, PLR**(B)**, and SII**(C)** group.

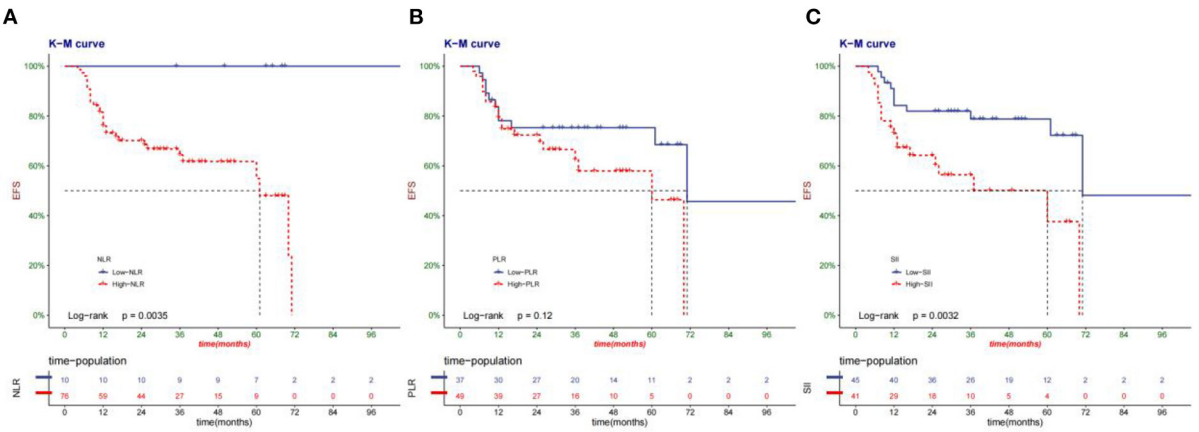


FIGURE 4
Kaplan-Meier curve of the EFS of patients according to NLR**(A)**, PLR**(B)**, and SII**(C)** group.

ROC curve analysis. With the extension of time, AUC gradually increased in Table 2. The optimal cut-off point was 0.80(NLR), 97.9(PLR)and 565(SII) according to ROC analysis. Among 86 included patients, NLR≥0.80, PLR≥97.9, SII≥565 were considered as high groups based on the above cut-off results.

## Cancer-specific survival and event-free survival

The median survival time of patients in this group was 33.6 months. Compared with high-NLR, PLR, and SII, low-NLR, PLR, and SII had a higher cancer-specific survival rate (Figure 3). Low-NLR, PLR, and SII have higher event-free survival than high-NLR, PLR, and SII (Figure 4).

## Univariate and multivariate cox regression analysis

Univariate analysis showed that TNM stage, tumor size, NLR value, PLR value, SII value, neutrophil count and platelet count were related to CSS ($p < 0.05$). In contrast, age, sex, region, health care, primary site, and laterality were not associated with CSS. According to multivariate analysis, only TNM stage ($p = 0.006$) and SII values ($p = 0.015$) were associated with poor prognosis, while NLR and PLR were not (Table 3). To further predict survival in pediatric osteosarcoma patients, multivariate Cox regression analysis was used to predict cancer-specific survival at 1, 3 and 5 years. And constructed a nomogram model to predict children's CSS (Figure 5). The C-index of the nomogram is 0.776 (95%CI, 0.776–0.910), indicating that the model has good accuracy.

TABLE 3 Univariate and multivariate analyses of CSS.

| | Univariate | | | Multivariate | | |
|---|---|---|---|---|---|---|
| | HR | 95%CI | P | HR | 95%CI | P |
| Age | 0.89 | 0.78–1.02 | 0.097 | | | |
| Sex | | | | | | |
| Male | Reference | | | | | |
| Female | 1.1 | 0.46–2.64 | 0.825 | | | |
| Region | | | | | | |
| Urban | Reference | | | | | |
| Rural | 1.4 | 0.6–3.28 | 0.437 | | | |
| Medical.insurance | | | | | | |
| No | Reference | | | | | |
| Yes | 0.59 | 0.26–1.34 | 0.212 | | | |
| Primary.site | | | | | | |
| Limb | Reference | | | | | |
| Axial | 0 | 0–Inf | 0.998 | | | |
| lateral | | | | | | |
| Left | Reference | | | | | |
| Right | 1.71 | 0.74–3.98 | 0.211 | | | |
| Not pairs | 0 | 0–Inf | 0.998 | | | |
| Stage | | | | | | |
| I | Reference | | | Reference | | |
| II | 3.48 | 0.31–38.79 | 0.31 | 3.322 | 0.298–37.08 | 0.329 |
| III | 17.68 | 2.23–140.52 | 0.007 | 18.106 | 2.273–144.207 | 0.006 |
| IV | 38.48 | 4.92–300.73 | 0.001 | 28.818 | 3.647–227.687 | 0.001 |
| Surgery | | | | | | |
| Limb salvage | Reference | | | | | |
| Amputation | 1.96 | 0.45–8.44 | 0.369 | | | |
| Chemotherapy | | | | | | |
| No | Reference | | | | | |
| Yes | 2.82 | 0.62–12.82 | 0.179 | | | |
| Radiotherapy | | | | | | |
| No | Reference | | | | | |
| Yes | 0 | 0–Inf | 0.997 | | | |
| Size | 1.02 | 1.01–1.03 | 0.003 | | | |
| PLR | 1.01 | 1–1.01 | 0.001 | | | |
| NLR | 2.07 | 1.27–3.39 | 0.004 | | | |
| SII | 1 | 1–1.001 | 0.001 | 1.001 | 1–1.002 | 0.015 |
| Platelet | 1 | 1–1.01 | 0.015 | | | |
| Neutrophile | 1.47 | 1.11–1.95 | 0.008 | | | |
| Lymphocyte | 0.73 | 0.51–1.06 | 0.102 | | | |

## Discussion

Osteosarcomas may progress rapidly with poor prognosis and high mortality. Recurrence and metastasis are the major causes of death and poor prognosis in children with Osteosarcoma. A new inflammatory indicator, the systemic immune inflammation index (SII), which combines inflammatory markers such as lymphocytes, neutrophils, and platelet count, has recently emerged and has been shown to predict poor prognosis in patients with hepatocellular carcinoma (8). This study evaluated preoperative systemic inflammatory markers in pediatric osteosarcoma patients, including SII PLR NLR, to understand the relationship between these markers and prognosis and survival in pediatric osteosarcoma patients. Univariate analysis showed that TNM stage, tumor size, NLR value, PLR value, SII value, neutrophil count and platelet count were related to CSS. Multifactorial analysis showed that only the TNM stage and SII values were associated with poor prognosis rather than NLR and PLR. Event-free survival and cancer-specific survival at 1, 3 and 5 years were higher in the low SII group than those in the high SII group.Based on the comprehensive indicators of peripheral blood neutrophil, platelet and lymphocyte count,the survival and prognosis value of SII for cancer patients may be derived from the function of these three cells, and there is increasing evidence that neutrophil and platelet's increase are related to carcinogenesis (14–17). Neutrophils not only promote the invasion of cancer cells Value-added and transfer, but also can help the cancer cells evade immune surveillance (18). Platelet protects cancer cells from immune clearance and promote their stranded in endothelial cells, to support the establishment of secondary lesions (19). In contrast, lymphocytes play an important role in the tumor defense by inducing cell death and inhibiting cell proliferation and migration (20). These mechanisms will help us better understand the role of neutrophil platelets and lymphocytes in cancer and their relationship with immunity and inflammation. Liu et al. concluded that elevated NLR PLR was associated with poor prognosis of Osteosarcoma, but they did not analyze the relationship between SII and prognosis of Osteosarcoma (21). Compared with PLR and SII, Yang et al. showed that NLR was a more reliable predictor of survival of Osteosarcoma, and no independent correlation was found between SII and survival of patients with Osteosarcoma (22). It should not be ignored that the difference in the efficacy of predictors in the literature may be due to cancer staging. Huang et al. suggested that high SII was an independent prognostic marker of postoperative survival of Osteosarcoma, which was consistent with our results (23). Another major difference between this study and the above studies (21, 23) is that the subjects are pediatric osteosarcoma patients, while the above studies are mainly adult osteosarcoma patients.This makes this study more significant in predicting the prognosis of pediatric osteosarcoma patients.The current treatment methods for Osteosarcoma are mainly chemotherapy, surgery and radiotherapy. Standard systemic therapy includes methotrexate based chemotherapy, including doxorubicin cisplatin and ifosfamide. Meta-analyses show that triple therapy is superior to double therapy and the importance of using high doses of methotrexate (24). Surgical resection after induction of chemotherapy is the standard for local control of

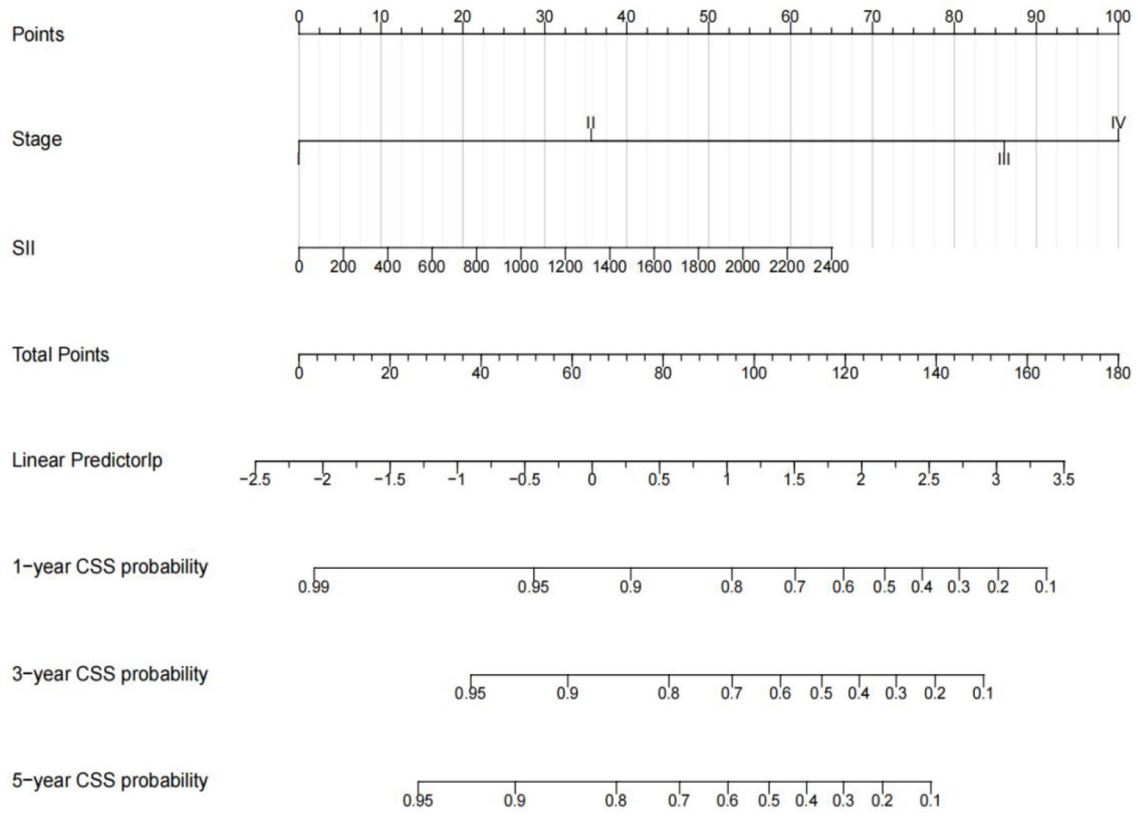


FIGURE 5
Nomograms for 1-, 3-, 5-year CSS of children with OS. Input individual patient variables. Each variable corresponds to a point, and the point of all variables can be added to find the corresponding total point. Below the total point is the survival rate for each patient.

osteosarcoma.Biopsies are performed at the time of diagnosis to confirm the pathological diagnosis and retrospective data suggest that local control is better when biopsies are performed by the same surgeon at a center experienced in surgical excision (25). With current treatment, about three-quarters of the patients diagnosed with Osteosarcoma are cured, and 90% to 95% of patients diagnosed with Osteosarcoma can be successfully treated by limb salvage surgery instead of amputation (26). Osteosarcoma is not a radiation sensitivity diseases. Therefore, radiotherapy is not considered a clear line of resectable tumors treatment. Instead, it is primarily used as a supplementary stage after marginal or incomplete resection, or for the final treatment of unresectable disease.In intratumoral or non-operative cases, patient who received adjuvant radiotherapy at the primary site had better overall survival than those who didi not receive radiotherapy (27).

However, in recent years, the 5- and even 10-year survival rates for pediatric osteosarcomas have not made breakthrough progress, so we need to find simple, easy, low cost and reliable non-invasive biochemical markers to predict the long-term prognosis of patients with Osteosarcoma in children. SII may give us a new direction to predict the survival rate of children patients with Osteosarcoma in different time. This may provide a new train of thought for clinicians to treat patients and further improve the long-term survival of patients. More studies are needed to determine the exact value of SII in pediatric osteosarcoma patients. However, the study has some limitations. First, we conducted a retrospective single-center study, and the sample size is relatively small. More studies are needed to confirm our results further. Second, although the predictive value of SII is confirmed, we did not compare the discriminative power of SII with other prognostic markers, such as PNI and CRP. Third, the patients are mainly from southwest China, which may lead to selection bias. More pediatric osteosarcoma patients from all over China are needed to study the relationship between SII and prognosis.

## Conclusion

This study is a retrospective study involving 86 pediatric osteosarcoma patients. Our results confirm that preoperative

SII and TNM staging are independent prognostic markers for pediatric osteosarcoma patients. SII may be used in conjunction with TNM staging for individualized treatment of pediatric osteosarcoma patients in future clinical work. However, multicenter prospective studies and more patients are needed to validate our results.

## Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

## Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Review Board of the Children's Hospital of Chongqing Medical University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

## Author contributions

HO and ZW designed the study, collected and analyzed the data, drafted the initial manuscript, revised the article critically, reviewed and edited the article. All authors approved the final manuscript.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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# Lactoferrin and Human Neutrophil Protein (HNP) 1–3 Levels During the Neonatal Period in Preterm Infants

*Kirstin B. Faust[1]*, Katja Moser[2], Maren Bartels[1], Ingmar Fortmann[1], Kathrin Hanke[1], Christian Wieg[2], Guido Stichtenoth[1], Wolfgang Göpel[1], Egbert Herting[1] and Christoph Härtel[3]*

*[1] Department of Paediatrics, University of Lübeck, Lübeck, Germany, [2] Department of Neonatology and Pediatric Intensive Care, Hospital Aschaffenburg-Alzenau, Aschaffenburg, Germany, [3] Department of Pediatrics, University of Würzburg, Würzburg, Germany*

Antimicrobial polypeptides (APPs) are part of the innate immune system, but their specific role in the context of preterm birth is not yet understood. The aim of this investigation was to determine the systemic expression of APPs, i.e., lactoferrin (LF) and human neutrophil protein (HNP) 1–3 in preterm infants in the period of highest vulnerability for infection and to correlate these biomarkers with short-term outcome. We therefore conducted a prospective two-center study including plasma samples of 278 preterm infants and 78 corresponding mothers. APP levels were analyzed on day 1, 3, 7, and 21 of life via enzyme-linked immunosorbent assay (ELISA). The levels of LF and HNP1–3 remained stable during the first 21 days of life and were not influenced by maternal levels. Elevated APP levels were found at day 1 in infants born to mothers with amniotic infection syndrome (AIS vs. no AIS, mean ± SD in ng/ml: LF 199.8 ± 300 vs. 124.1 ± 216.8, HNP 1–3 16,819 ± 36,124 vs. 8,701 ± 11,840; $p = 0.021$, $n = 179$). We found no elevated levels of APPs before the onset of sepsis episodes or in association with other short-term outcomes that are in part mediated by inflammation such as necrotizing enterocolitis (NEC) or retinopathy of prematurity (ROP). Interestingly, infants developing bronchopulmonary dysplasia (BPD) showed higher levels of HNP1–3 on day 21 than infants without BPD (13,473 ± 16,135 vs. 8,388 ± 15,938, $n = 111$, $p = 0.008$). In infants born without amniotic infection, levels of the measured APPs correlated with gestational age and birth weight. In our longitudinal study, systemic levels of LF and HNP 1–3 were not associated with postnatal infection and adverse short-term outcomes in preterm infants.

**Keywords: antimicrobial polypeptides, lactoferrin, blood levels, preterm infants, infection, inflammation, HNP 1–3**

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****Correspondence:***
*Kirstin B. Faust*
*kirstin.faust@uksh.de*

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## INTRODUCTION

Preterm infants are vulnerable for short- and long-term morbidity originating from perinatal infections and the associated inflammatory response. Their infection risk is mainly attributed to immaturity and imbalance of the neonatal immune system but also related to the need of invasive therapeutic measures. In this setting, the innate immune system as first-line defense mechanism is crucial (1).

Antimicrobial polypeptides (APPs), a large group of proteins with pleiotropic functions, are part of the innate immune system and have direct antimicrobial capacity (2, 3). APPs may also modulate immune defense pathways (4) and inflammatory processes (5). APPs are produced by epithelial cells of airway tissues and the gastrointestinal tract (6, 7). Human defensins are found at the fetomaternal interface, i.e., amnionic fluid, placenta, (8) vernix caseosa (9) and human milk (e.g., lactoferrin (LF) and LL-37) (10). Other APPs are primarily expressed by neutrophils and reach high systemic levels such as lactoferrin (LF), human neutrophil peptides (HNP) (11) or bacterial permeability increasing protein (BPI) (12).

In preterm infants, infection and inflammation may influence systemic levels of APPs (6, 7, 13–15). In line with that, LF has been proposed to have a beneficial effect by counter-regulating systemic inflammation in neonatal sepsis (16), which was not confirmed by other investigators (17). While some data exist on the influence of gestational age on the constitutive expression of LF, human beta defensin-2 and BPI (12, 18–20), expression patterns of HNP in preterm infants are unknown. As APPs are potential candidates for supplementation in vulnerable infants, we studied the systemic levels of LF and HNP in preterm infants during their highest period of vulnerability for infection, i.e., day 1–21 of life. The main objectives were to evaluate whether infants with lower plasma concentrations for LF and HNP have an increased risk for infection and whether APP levels are associated with inflammation-mediated short-term outcomes.

# PATIENTS AND METHODS

## Study Population

This prospective two-center convenience sample study included a cohort of 278 preterm infants (gestational age 22 5/7–34 6/7 weeks) in the perinatal centers of the University of Luebeck Hospital ($n$ = 158 infants, $n$ = 78 corresponding mothers) and the hospital at Aschaffenburg-Alzenau ($n$ = 120 infants).

## Ethical Approval

Ethical approval was given for all study parts by the University of Luebeck ethical committee and the Bavarian Medical Board ethical committee. Informed written consent was given by parents as legal representatives on behalf of their infants. While withdrawal of peripheral full blood counts was part of clinical routine, a maximum of additional 0.5 ml blood/kg body weight was obtained for research purposes in accordance with current guidelines of the European Medical Agency. Maternal blood samples were obtained within routine laboratory testing at delivery.

## Clinical Data and Definitions

Data were collected from clinical record files of mother and infant pairs. Gestational age was defined according to postmenstrual age (obstetrical dating). Cause of preterm delivery was defined by the attending obstetrician. Specifically, clinical chorioamnionitis (amniotic infection syndrome, AIS) was diagnosed when more than two of the following clinical signs were noted: maternal fever > 38.0°C, or fetal tachycardia > 180/min, maternal increase in white blood cell count > 10/nL or C-reactive protein levels (> 10 mg/L) without other focus of infection, painful uterus, foul-smelling amnionic fluid, preterm labor, preterm rupture of membranes or early onset sepsis in the newborn.

Small for gestational age (SGA) was defined as a birth weight less than 10th percentile according to gender-specific standards for birth weight by postmenstrual age in Germany (21).

Clinical sepsis was defined as condition with at least two signs of systemic inflammatory response (temperature > 38°C or < 36.5°C, tachycardia > 200/min, new onset or increased frequency of bradycardias or apneas, hyperglycemia > 140 mg/dL, base excess < -10 mval/L, changed skin color, increased oxygen requirements; laboratory sign: C-reactive protein > 10 mg/L, immature/neutrophil-ratio > 0.2, white blood cell count < 5/nL, platelet count < 100/nL) plus the decision of the attending neonatologist to treat with antibiotics for at least 5 days, but without growth of bacteria in blood culture (22). Blood culture proven sepsis was defined as clinical sepsis with growth of bacteria in blood culture. If coagulase-negative staphylococci (CoNS) were isolated as single pathogen in one peripheral blood culture, two clinical signs and one laboratory sign were required to fulfill the definition of blood culture confirmed sepsis. EOS was defined as sepsis occurring in the first 72 h of life. LOS was defined as sepsis occurring later than 72 h of life.

Necrotizing enterocolitis (NEC) surgery was defined according to modified Bell criteria (≥ stage 2) requiring surgery. Bronchopulmonary dysplasia (BPD) was defined as need of oxygen or respiratory support (continuous positive airway pressure (CPAP) or mechanical ventilation) at 36 weeks' postmenstrual gestational age and retinopathy of prematurity (ROP) as ROP requiring treatment.

## Sample Collection and Analysis of Biomarkers

Plasma samples of preterm infants ($n$ = 278) were collected in tubes containing 16 I.E. heparin/ml from arterial cord blood and peripheral blood on days 1, 3, 7, and 21 of life. For 78 cases, plasma samples were also collected from corresponding mothers during the first 48 h after birth within routine analysis. After centrifugation aliquots were stored at –20°C until analysis. Maximum storage time before centrifugation was 24 h. Levels of APPs were determined in plasma probes using commercial ELISA kits (Hycult Biotechnology, Netherlands), HK317 for human HNP 1–3 and HK329 for human LF according to manufacturer's instructions. Arterial pH, glucose, total white blood cell count (WBC) and differential blood count were determined by routine hospital laboratory analysis.

## Statistical Analysis

Mann–Whitney-$U$-test and Kruskal–Wallis test were applied for statistical analysis of differences between non-paired groups. Wilcoxon test was applied for statistical analysis of differences between paired datasets. Correlations were tested by the Spearman's rho test. The level of significance was defined as

**TABLE 1 |** Clinical characteristics of study cohort (WBC, white blood cell; PPROM, Preterm prelabor rupture of membranes; CTG, cardiotocography; HELLP, hemolysis, elevated liver enzymes, and low platelets syndrome).

| **Number of infants** | **278** |
|---|---|
| **Gestational age** (median/IQR/min-max, weeks) | 29.4/12.1/22.7–34.8 |
| **Birth weight** (median/IQR/min-max, grams) | 1,325/3,070/365–3435 |
| **Gender** (male, n/%) | 156/56 |
| **Multiples** (n/%) | 109/39 |
| **Small for gestational age** (n/%) | 34/12 |
| **WBC count/nl** (mean/median/SD) | |
| **d1** | 13.0/10.9/8.5 |
| **d3** | 10.8/8.5/7.0 |
| **d7** | 12.9/11.1/7.2 |
| **d21** | 14.2/12.7/7.4 |
| **Neutrophil count/nl** (mean/median/SD) | |
| **d1** | 4.4/2.9/4.8 |
| **d3** | 6.4/5.0/5.4 |
| **d7** | 6.3/4.5/6.1 |
| **d21** | 5.8/4.3/5.5 |
| **Causes of preterm delivery** (n/%) | |
| Preterm labor/PPROM | 164/59 |
| Pathological Doppler/CTG | 40/14 |
| Pre-eclampsia/HELLP | 55/20 |
| Others | 19/7 |
| **Spontaneous delivery** (n/%) | 46/16.2 |
| **AIS** (n/%) | |
| Suspected | 115/41 |
| Severe | 57/21 |
| **Early onset sepsis** (n/%) | |
| Clinical | 13/13 |
| Blood culture proven | 3/1 |
| **Late onset sepsis** (n/%) | |
| Clinical | 19/8 |
| Blood culture proven | 30/11 |
| **Bronchopulmonary dysplasia (BPD)** (n/%) | 34/12 |
| **Necrotizing enterocolitis (NEC)** (n/%) | 10/4 |
| **Intraventricular hemorrhage (IVH, any grade)** (n/%) | 28/10 |
| **Retinopathy of prematurity (ROP)** (n/%) | 9/3 |

*One center did not perform routine blood counts on day 3.*

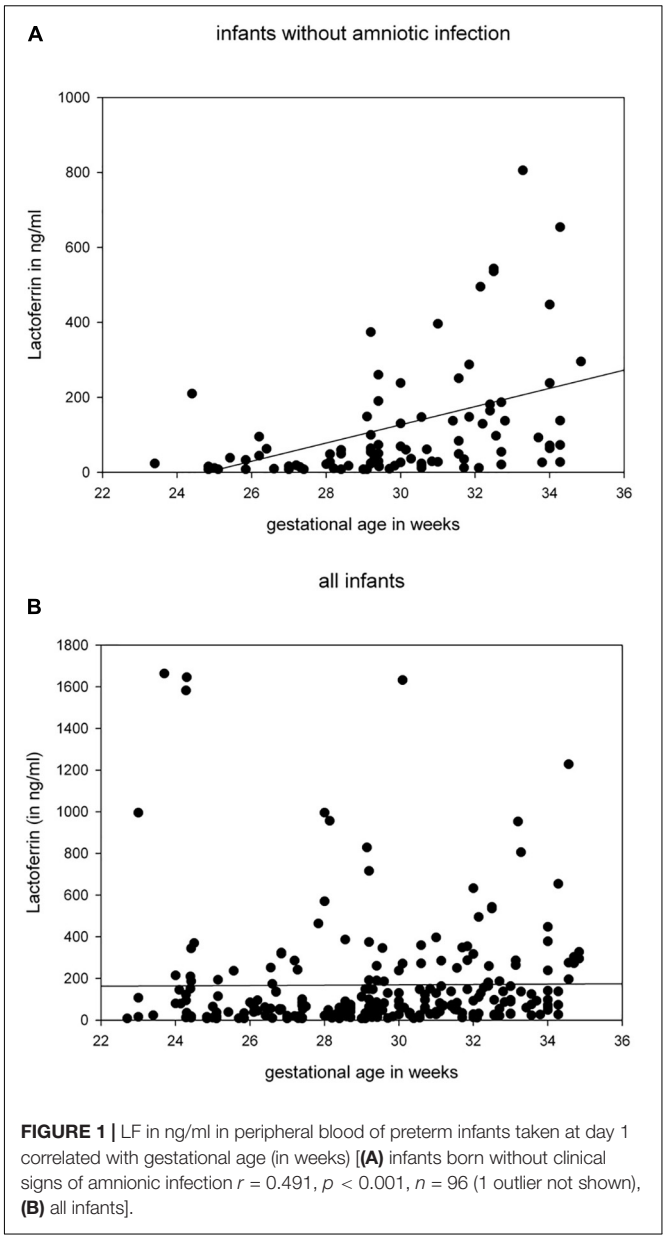


**FIGURE 1 |** LF in ng/ml in peripheral blood of preterm infants taken at day 1 correlated with gestational age (in weeks) [**(A)** infants born without clinical signs of amnionic infection $r = 0.491$, $p < 0.001$, $n = 96$ (1 outlier not shown), **(B)** all infants].

$p < 0.05$ in single comparisons and $p < 0.01$ for correlations. Statistical analysis was performed using SPSS_28.0 statistical software (SPSS Inc., Chicago, United States).

# RESULTS

## Clinical Characteristics and Antimicrobial Polypeptide Levels

Clinical characteristics of our study cohort are described in **Table 1**. We found weak correlations between gestational age or birth-weight and expression of LF levels ($r = 0.23$, $p < 0.001$, $n = 241$) in plasma of preterm infants on day 1. When we restricted our analysis to infants born without evidence of AIS ($n = 106$), we noted correlations between gestational age and levels of LF (**Figure 1**) as well as HNP1-3 ($r = 0.31$, $p = 0.01$, $n = 69$ (**Supplementary Table 1**). In line with that, LF ($r = 0.50$, $p < 0.001$, $n = 96$) and HNP1-3 levels ($r = 0.30$, $p = 0.012$, $n = 69$) were associated with birth weight in this subgroup. We found no influence of gender, multiple births or SGA on APP levels (data not shown).

## Higher Antimicrobial Polypeptide Levels in Mothers as Compared to Their Infants at Birth

We detected no correlation between APP levels in infants and corresponding mothers for LF and HNP 1-3. In general, maternal APP levels were higher than cord blood or peripheral blood levels of preterm infants on day 1 [Mother/cord blood/neonatal

peripheral blood d1, mean (SD) in ng/ml: LF 889 (472) / 220 (503) / 170 (272), $p < 0.001$; HNP1-3 30,750 (20,487) / 7,729 (7,611) / 13,690 (29,468), $p < 0.001$)].

## Elevated Antimicrobial Polypeptide Levels in Infants Born in the Context of Amniotic Infection Syndrome

We found significantly higher levels of LF in infants born because of AIS on day 1 of life (**Figure 2**) but also for HNP1–3 (AIS vs. no AIS, mean ± SD in ng/ml: HNP 1–3 16,819 ± 36,124 vs. 8,701 ± 11,840; $p = 0.021$, $n = 179$). Mode of delivery and antenatal exposure to steroids had no impact on the levels of APPs, and no correlation was found for surrogate parameters for neonatal stress such as umbilical arterial pH or APGAR score at 5 and 10 min of life and APP levels in cord blood, day 1 or day 3 (data not shown).

## Expression of Antimicrobial Polypeptide Levels in the Period of Highest Vulnerability for Sepsis

In preterm infants, cord blood and peripheral blood levels (day 1) of APPs differed remarkably, a moderate correlation was found for LF levels only ($r = 0.45$, $p = 0.03$, $n = 23$). There was a significant correlation between levels of each APP of day 1 and day 3 (LF $r = 0.44$, $p < 0.001$, $n = 179$, HNP1–3 $r = 0.37$, $p < 0.001$, $n = 119$). The mean level of APPs remained relatively stable during the first 21 days of life (**Figure 3**). APP levels correlated with total white blood cell and neutrophil counts at all measured time points (**Supplementary Table 2**).

## Antimicrobial Polypeptide Levels Preceding Sepsis Are Not Different From Matched Samples of Unaffected Infants

No significant differences were found for APP levels preceding EOS or LOS. A trend to decreased LF levels was found for preterm infants with EOS at day 3 [mean (SD) in ng/ml, 51.9 (64.4) vs. 116.1 (208.7), $n = 204$, $p = 0.071$ and **Supplementary Table 3**].

Infants developing BPD showed higher levels of HNP1–3 on day 21 than infants without BPD [mean (SD) in ng/ml, 13,473 ± 16,135 vs. 8,388 ± 15,938, $n = 111$, $p = 0.008$]. We found no significant difference between APP levels in plasma of preterm infants with or without NEC or ROP (**Supplementary Table 3**).

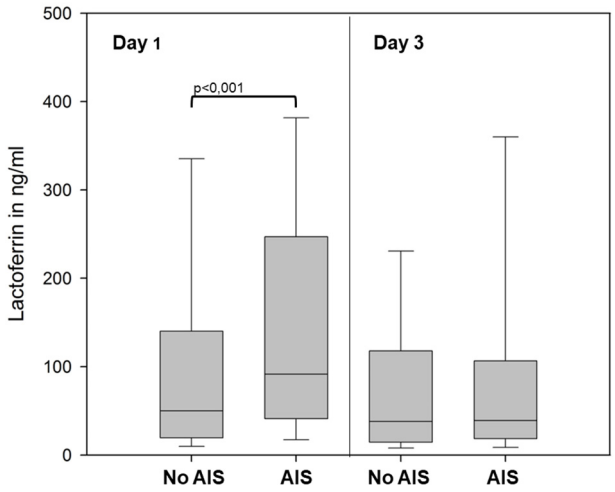


**FIGURE 2 |** LF levels in peripheral blood of preterm infants born with (AIS, $n = 145$) or without (No AIS, $n = 96$) signs of amnionic infection at days 1 and 3. Level of significance is marked above the brackets. Data is represented as box plot (median, 25th/75th, and 10th/90th percentiles).

# DISCUSSION

In this prospective study we noted a correlation between amniotic infection and APP levels in preterm infants while we found no distinct pattern of APPs in association with postnatal infection. APP levels were dependent on gestational age and birth weight but also correlated with white blood cell numbers.

Amnionic infection syndrome (AIS) was associated with elevated levels of all measured APPs (LF and HNP 1–3) in peripheral blood of preterm infants at day 1. This confirms previous results showing elevated APPs in cord blood of preterm infants born due to AIS (13). Our findings may be related to transplacental passage of elevated maternal APPs. Previous studies found higher levels of APPs in plasma of mothers with amnionic infection (23, 24) as well as APP transfer at the fetomaternal interface (25). In our study, we found no correlation between APP levels of mothers and infants, while maternal levels were higher than infant levels. Additionally, APP levels in cord blood did not correlate well with levels in peripheral blood of the infants, possibly due to technical contamination with maternal blood in the cord blood samples. We therefore encourage using peripheral blood samples for future investigations.

Previous studies (12, 13, 18, 20) had shown that APP levels in cord blood may be influenced by endogenous factors such as gestational age or birth weight. This was also demonstrated in peripheral blood during the first days of life, but perinatal inflammation had a more pronounced effect in our study context. We were not able to confirm a significant decline of APP levels over time (17) in peripheral APP levels in the subgroup of infants without AIS. A subgroup of extremely preterm infants born after 23 or 24 weeks of gestation expressed very low levels of APPs if not exposed to AIS, but were able to produce APP levels similar to near-term neonates in the presence of AIS.

Within the limits of our study, we were not able to confirm our hypothesis that neonatal infection is associated with low systemic LF levels. No significant differences were found between the LF levels between infants with or without sepsis, but we noted a trend to decreased LF levels in preterm infants with EOS on day 3. Lower APP levels in neonates after birth may depend on low nutritional input, and there is evidence that oral supplementation with bovine LF may be beneficial in the prevention of NEC or LOS in very preterm infants (26). However, this is still a matter of debate and has not entered clinical routine yet. Our data do not support the use of LF as biomarker preceding sepsis.

Likewise, we found no association of HNP-levels with postnatal inflammation such as LOS, NEC or ROP. However,

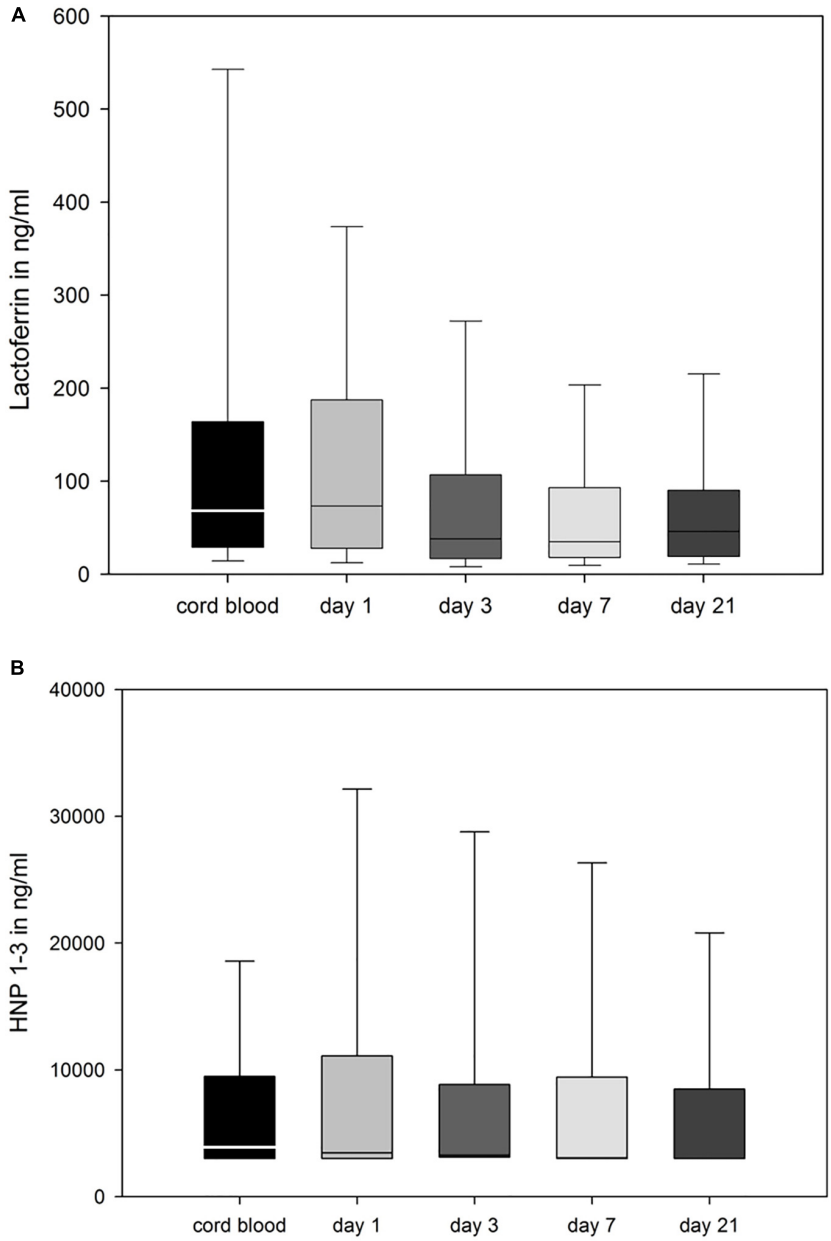


**FIGURE 3 |** LF **(A)**, and HNP 1–3 **(B)** in ng/ml in cord blood and peripheral blood of the infant taken at days 1, 3, 7, and 21. Data is represented as box plot (median, 25th/75th, and 10th/90th percentiles).

HNP 1–3 was elevated in infants diagnosed with another inflammation-mediated disease, BPD, comparable to serum concentrations of several cytokines (27). Interestingly, previous studies found an elevation of serum HNP in children with poor pulmonary outcome (15), assuming its function as an alarmin may be of use as a prognostic marker of the inflammation mediating the lung disease. This aspect needs to be confirmed in prospective trials in order to evaluate whether HNP adds information within the multifactorial risk profile for BPD. The role of HNPs in the immune defense is currently under discussion (2, 28), so our data may add new information to this topic.

Our study has several limitations. First, alterations of APP levels caused by pre-analytic sampling (plasma—as used in our study—vs. serum) and storage cannot be excluded. Secondly, we studied sepsis as primary endpoint, while our data cannot provide conclusive evidence on less frequent entities such as ROP, NEC, IVH or BPD. Therefore, multicenter approaches are needed to generate large scale data on the diagnostic or even prognostic value of HNP1–3. Finally, the APP monitoring was performed within routine blood sampling on day 1, 3, 7, 21 in order to minimize the burden of invasive measures for vulnerable preterm infants. Hence no samples from the exact time point

of clinical suspicion of sepsis were obtained in the majority of affected infants.

## CONCLUSION

In conclusion, gestational age and the context of AIS correlate with APP-levels in preterm infants. Whether HNP 1–3 is a potential biomarker for diagnostic tests or therapeutic monitoring in BPD needs to be defined in further clinical studies.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the University of Lübeck Ethical Committee. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

## AUTHOR CONTRIBUTIONS

CH and KF conceptualized the study, carried out the initial data analyses, drafted the initial manuscript, and approved the final manuscript as submitted. KM and MB performed the experiments. KH, IF, CW, GS, WG, and EH supervised and coordinated the data and sample collection, supported the study design and the development of data collection instruments. All authors contributed to the manuscript, approved the final version as submitted and agreed to be accountable for all aspects of the work.

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## SUPPLEMENTARY MATERIAL

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EDITED BY
Ulrik Lausten-Thomsen,
Copenhagen University Hospital Rigshospitalet, Denmark

REVIEWED BY
Karl Bechter,
University of Ulm, Germany
Hu Deng,
Peking University, China

*CORRESPONDENCE
Rebecca Alison Fabricius
rebecca.alison.fabricius@regionh.dk

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# Cytokine profile of pediatric patients with obsessive-compulsive and/or movement disorder symptoms: A review

Rebecca Alison Fabricius[1]*, Camilla Birgitte Sørensen[1], Liselotte Skov[1] and Nanette Mol Debes[1,2]

[1]Department of Pediatrics and Adolescent Medicine, Copenhagen University Hospital, Herlev, Denmark, [2]Department of Clinical Medicine, University of Copenhagen, Copenhagen, Denmark

Cytokines are an important modulator of the immune system and have been found to be altered significantly in many neurological and psychiatric disorders, like obsessive compulsive disorder (OCD) and movement disorders. Also, in pediatric autoimmune neuropsychiatric disorders associated with group A streptococcal infections (PANDAS), which are characterized by abrupt debut of symptoms of OCD and /or movement disorder symptoms, alterations in the immune system have been suggested. The aim of this paper was to review the current literature on the cytokine profile of pediatric patients with symptoms of OCD and/or movement disorder symptoms. A search of PubMed and Medline was performed with specific keywords to review studies measuring cytokines in pediatric patients with symptoms of OCD and/or movement disorders. Nineteen studies were found, twelve of which included a healthy control group, while four studies had control groups of children with other disorders, primarily neurological or psychiatric. One study compared cytokines measurements to reference intervals, and two studies had a longitudinal design. Many cytokines were found to have significant changes in patients with symptoms of OCD and/or movement disorders compared to both healthy controls and other control groups. Furthermore, differences were found when comparing cytokines in periods of exacerbation with periods of remission of symptoms in study participants. The cytokines that most studies with healthy control groups found to be significantly altered were TNF-α, IL-1β and IL-17. Although the exact role of these cytokines in OCD and movement disorder symptoms remains unclear, the available literature suggests a proinflammatory cytokine profile. This offers interesting perspectives on the pathogenesis of OCD and/or movement disorder symptoms in children, and further research into the implications of cytokines in neuropsychiatric disorders is warranted.

KEYWORDS

**cytokines, autoimmune, pro-inflammatory, obsessive-compulsive, movement disorders**

## Introduction

Autoimmunity is the failure of the immune system to recognize the organism as itself. The classic component of autoimmune disorder is the inflammation (1), which is a normal physiological defense against infection and tissue damage. However, in many autoimmune disorders an abnormal inflammatory response is associated with tissue and organ damage (2). Autoimmunity can be induced through many different mechanisms. One common etiology is post-infectious, as is seen in Guillain Barré Syndrome, rheumatic fever, and glomerulonephritis. Although many pathogens can cause autoimmunity, group A streptococci (GAS) is especially potent (3). Many autoimmune diseases, for example systemic lupus erythematosus, have comorbid psychiatric symptoms, suggesting a connection between disorders of the immune system and psychiatric disorders (1). Major depressive disorder has been studied extensively. In patients suffering from depression cardinal features of inflammation, such as elevated cytokines in peripheral blood and cerebrospinal fluid (CSF) as well as other acute inflammatory mediators, have been seen (4). In other psychiatric disorders such as obsessive-compulsive disorder (OCD), chronic tic disorder (CTD) and Tourette's Syndrome (TS) it has also been suggested that a subgroup of patients might have immune-related and/or post-infectious autoimmune etiology (5).

Historically, several studies have described patients with OCD and/or movement disorder after infections. In 1978, Kondo and Kabasawa reported a sudden and abrupt debut of a tic disorder after fever in a 11 year- old boy who had elevated antistreptolysin antibodies and responded well to treatment with corticosteroids (6). In the 1980s and 1990s, patients with OCD symptoms developing simultaneously with Sydenham's Chorea (SC) related to GAS infections were described (5). In 1990, children with movement disorders were found to have elevated antistreptococcal titers, and a link between an antecedent GAS infection and movement disorders was suggested (7). In 1995, Allen et al. (8) reported four cases of abrupt, severe onset or a worsening of OCD and/or movement disorder in form of tics. All patients had had recent infections, GAS or viral, and the essential symptoms were determined to be pediatric, infection-triggered, autoimmune neuropsychiatric disorders (PITANDS) (8). Pediatric autoimmune neuropsychiatric disorders associated with streptococcal (group A) infections (PANDAS) were first described in 1998 by Swedo et al. (9) and were described as presence of OCD and/or a tic disorder temporally associated with a GAS infection.

The role of cytokines in neuroinflammation and as pathophysiological mechanism in psychiatric disorders is of interest. Cytokines are small glycoproteins which can be produced by many different cells in all organs. They play an important role in brain development and promotion of normal brain function (10) and can, amongst many other things, create or hinder inflammation and recruit cellular components of the immune system (11). However, they can turn detrimental for the brain if strongly activated by infection or injury, as high levels of pro-inflammatory cytokines can negatively impact memory, neural plasticity and neurogenesis (10).

However, not much is known about the cytokine profile in children with neuropsychiatric symptoms. An improved understanding of the cytokine profile of these patients could offer insight into the pathogenesis of these disorders. In this article we review the available literature, to determine the cytokine profile of children with neuropsychiatric symptoms as seen in OCD, TS, SC, CTD, PANS and PANDAS.

## Method

In the present review, a literature search in the Pubmed, PMC and MEDLINE databases was performed. The initial search with the following terms; {[PANDAS (Body-Key Terms)] OR [PANS (Body-Key Terms)] OR [OCD (Body-Key Terms)] OR [Sydenham's chorea (Body-Key Terms)] OR [Tourette's disorder (Body-Key Terms)]} AND {[cytokine (Body-Key Terms)] OR [immune(Body-Key Terms)]} was conducted through the PubMed Central (PMC) database and yielded 342 results. A supplementary search with the terms "(cytokine) AND [(OCD) OR (PANDAS) OR (PANS) OR (Tourette's disorder) OR (tics) OR (Sydenham's chorea)] AND [(pediatric) or (children)]" was carried out on Pubmed.gov database, which includes PMC and MEDLINE, to see if any supplementary materials not found in the original search could be added. This yielded 90 results.

A total of 432 article titles and abstracts were assessed for relevance for the review. Exclusion criteria were studies written in other languages than English, letters to the editor, conference presentations, editorials, comments, or opinions. Seventy articles were included for close reading of the full text. Furthermore, 52 articles of potential interest were added through the references of the aforementioned articles.

In total, 122 article abstracts were systematically read to clarify if the articles documented any kind of cytokine measurement in subjects with either PANDAS, PANS, OCD, TS or SC, and 33 articles were identified. From these 33 articles, 19 had pediatric populations and 14 had only adult populations and were therefore excluded.

## Results

Of the 19 articles describing cytokines in pediatric patients with obsessive/compulsive symptoms and/or movement disorder symptoms, 12 had included a healthy control group, 5 had control groups with other disorders or no control group and 2 had a longitudinal study design.

The studies examining cytokines in pediatric patients compared to healthy controls are summarized in Table 1. The

TABLE 1 Studies examining cytokines in pediatric patients with obsessive-compulsive and/or tic symptoms compared to healthy controls.

| Article | Diagnosis | No of patients (no of healthy controls) | Medication | Tissue tested | Study design | Cytokines tested | Significant results |
|---|---|---|---|---|---|---|---|
| Bos-Veneman et al. (12) | TS or CTD | 66 (71) | 56% of patients used psychotropic medication, antipsychotic primarily | Serum | Cytokines measured with multiplex cytokine array, read on luminex platform | IL-2, IL-4, IL-5, IL-12, sIL-2R, TNF-α, IFN-γ, sVCAM-1 and sICAM-1 | No differences found, but lower frequency of detectable IFN-g levels in patients, medication had no association with levels of cytokines |
| Cheng et al. (13) | TS | 40 (40) | Unknown how many patients received any medication | Plasma | Cytokines measured using solid phase sandwich ELISA | IL-6, sIL-6R, IL-1β, sgp130 and IL-17 | IL-1β, IL-6, IL-17 and sgp130 were increased, and sIL-6R was decreased. Also found higher proportion IL-6 positive lymphocytes and IL-17 positive lymphocytes |
| Çolak Sivri et al. (14) | OCD, no coexisting tics | 44 (40) | Patients were psychiatric medication naive, apart from 5 who had a history of medication but were unmedicated at time of study | Serum | Cytokines and chemokines measured using ELISA | IL-12, IL-17, TGF-β, TNF-α, sTNFR1, sTNFR2, IL-1β, CCL3, CCL,24, CXCL8, BDNF | TNF-α higher for OCD, while IL-12 lower |
| Gabbay et al. (15) | TS (+/- OCD) | 32 (16) | 22% patients were medication naive, 78% were taking psychotropic medication at assessment | Plasma | Cytokines measured using ELISA, except for TNF-α, IL-1β, IL-12, IL-6 and IL-2 here specific high sensitivity human quantikine assays were used | TNF-α, IL-12, IL-β, IL-6 and IL-2 | TS+OCD subgroup elevated IL-12 compared to control and IL-2 increased in TS+OCD compared to TS-OCD. No changes when adjusted for psychotropic medication |
| Gariup et al. (16) | OCD and tics | 8 in OCD and tics group (34) | All patients received some form of medication | Serum | Cytokines measured with Luminex ultra-sensitive kit, except IP-10 and MCP-1 were measured with ELISA | IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL 10, GM-CSF, IFN-γ, TNF-α, IFN-γ-IP-10, MCP-1 | IL-1β and IL-8, IP-10 higher for OCD and tics |
| Leckman et al. (17) | OCD (+/- PANDAS) | 46 (31) | Majority of patients were receiving medication to control tics and/or OCD symptoms | Serum | Cytokines measured at study entry and exacerbations with multiplex ELISA | IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, INF-α, INF-γ, TNF-α | TNF-α and IL-12 levels higher in patients, non-PANDAS more likely than PANDAS to have elevated TNF-α. Patients not receiving medication had highest baseline level of IL-12 and TNF-α. IL-5 was higher for non-medicated than medicated and controls |
| Li et al. (18) | TS | 58 (30) | Treatment experience including dopaminergic receptor antagonists was exclusion criteria | Serum | ytokines measured with ELISA | IL-6, IL-8, and TNF-α | Decrease in levels of both IL-6 and IL-8 and increase in the level of TNF-α |

*(Continued)*





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TABLE 1 Continued

| Article | Diagnosis | No of patients (no of healthy controls) | Medication | Tissue tested | Study design | Cytokines tested | Significant results |
|---|---|---|---|---|---|---|---|
| Matz et al. (19) | TS | 46 (43) | 65 % of patients received psychotropic medication | Serum | Cytokines measured with Bio-Plex cytokine assay, except for IL1-ra and CD14 a specific quantikine Immunoassay was used for each | TNF-α, IL-6, CD14 and IL1-ra | TNF-α, IL1-ra and CD14 lower for TS children (unclear how many had comorbid OCD). No differences between medicated and non-medicated patients. |
| Pranzatelli et al. (20) | TS with streptococcus markers | 5 (26) | Patients were one week off medication when examined, all were medicated | Serum and CSF | Cytokines measured with ELISA | Intracellular IFN-γ and IL-4, CXCL13, CXCL10, CCL19, CCL21 and CCL22 | No differences found. |
| Rodriguez et al. (21) | OCD | 102 (47) | 80% of patients were medicated | Monocytes | LPS stimulated cytokines measured with multiplex luminex assay | IL-1β, IL-6, GM-CSF, TNF-α and IL-8 | Higher production of IL-1β, IL-6, GM-CSF, TNF-α and IL-8. Levels were higher for unmedicated patients than medicated, which were higher than controls |
| Simşek et al. (22) | OCD | 34 (34) | Psychotropics were exclusion criteria, unclear if all were medication naive | Serum | Cytokines were measured with BD cytometric Bead Array analysis | IL-2, IL-4, IL-6, IL-10, IL-17A, IFN-γ and TNF-α | Patients had increase in IL-2, TNF-α and IL-17A |
| Yeon et al. (23) | TS | 26 | 62% of patients were unmedicated | Serum | Cytokines were measured using ELISA | MCP-1, IL-1β, IL-17A, IL-6, IL-12p70, and TNF-α | IL-17A, IL-12p70, IL-6 and TNF-α were increased in patients. TNFα was found to increase in unmedicated patients compared to patients taking medication |

BDNF, brain derived neurotrophic factor; CCL, CC motif chemokine ligand; CD, cluster of differentiation; CSF, cerebrospinal fluid; CTD, chronic tic disorder; CXCL, C-X-C motif chemokine ligand; ELISA, enzyme linked immunosorbent assay; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; IL1-ra, IL-1 receptor antagonist; IP, interferon gamma-induced protein; LPS, lipopolysaccharide; MCP, membrane cofactor protein; OCD, obsessive-compulsive disorder; PANDAS, pediatric autoimmune neuropsychiatric disorder associated with streptococcal infections; sgp, soluble glycoprotein; sI/VCAM, soluble intercellular/vascular cell adhesion molecule; sTNFR, soluble TNF receptor; TNF, tumor necrosis factor; TS, Tourette syndrome.



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TABLE 2 Overview of cytokine changes in studies in pediatric patients with tics and/or obsessive-compulsive symptoms compared to healthy controls.

| Cytokine | Increase | Decrease | No differences |
|---|---|---|---|
| TNF-α | Çolak Sivri et al. (14), Leckman et al. (17), Rodriguez et al. (21), Simşek et al. (22), Li et al. (18), and Yeon et al. (23) | Matz et al. (19) | Bos-Veneman et al. (12) and Gariup et al. (16) |
| IL-6 | Rodriguez et al. (21), Cheng et al. (13), and Yeon et al. (23) | Li et al. (18) | Leckman et al. (17) and Simşek et al. (22) |
| IL-1β | Gariup et al. (16), Rodriguez et al. (21), and Cheng et al. (13) | | Çolak Sivri et al. (14) and Yeon et al. (23) |
| IL-2 | Gabbay et al. (15) and Simşek et al. (22) | | Bos-Veneman et al. (12), Gariup et al. (16), and Leckman et al. (17) |
| IL-17(A) | Cheng et al. (13), Simşek et al. (22), and Yeon et al. (23) | | Çolak Sivri et al. (14) |
| IL-12 | Gabbay et al. (15) and Leckman et al. (17) | Çolak Sivri et al. (14) | Bos-Veneman et al. (12) |
| IL-8 | Gariup et al. (16) and Rodriguez et al. (21) | Li et al. (18) | |
| GM-CSF | Rodriguez et al. (21) | | Gariup et al. (16) |
| IP-10 | Gariup et al. (16) | | |
| IL-12p70 | Yeon et al. (23) | | |
| IL1-ra | | Matz et al. (19) | |
| CD-14 | | Matz et al. (19) | |
| sgp130 | Cheng et al. (13) | | |
| sIL-6R | | Cheng et al. (13) | |

CD, cluster of differentiation; GM-CSF, granulocyte-macrophage colony-stimulating factor; IP, interferon gamma-induced protein; IL, interleukin; IL1-ra, IL-1 receptor antagonist; sgp, soluble glycoprotein; sIL-6R, soluble IL-6 receptor, TNF, tumor necrosis factor.

studies primarily used ELISA but also other immune assays to examine the different cytokine levels in primarily serum or plasma. The studies subjects differed in included number, ages, diagnosis and in extent of use of psychotropic medication. The cytokines the studies have chosen to examine also differed between studies, however many studies chose to examine TNF-a, IL-6, IL-1b, IL-2, IL-17A and IL-12, and many of the studies found significant alterations of these cytokines when compared to healthy controls. The cytokines with significant results from Table 1 are summarized in Table 2.

Five studies have included controls with other disorders, other neurological diseases, patients undergoing tonsillectomy (24–28). In general, these studies have included less participants ($N$ = 12–24) and they tested a broad range of cytokines through primarily ELISA. The medication status was not always described. The significant results included a higher level of IL-2 in patients with OCD compared to controls with ADHD or schizophrenia (24). Elevated IL-6 and IL-17A in D2R specific T-cells from subset of patients with SC, TS or PANS were seen compared to controls with neurological disease (25). Compared with children with non-inflammatory neurological diseases like epilepsy, IL-4 was found to be increased in patients with acute and persistent SC and Il-10 and IL-12 were only elevated in patients with acute SC (26). Another study analyzed cytokines in tonsils from PANDAS children compared to children undergoing tonsillectomy for either obstructive sleep apnea or chronic tonsilitis (29). A significant increase of TNF-a and eoxtaxin-3 was found in patients with PANDAS, while IL-8, IP-10, IL-17A IL-10 and IL-12 were significantly decreased (27). Another study compared serum cytokines from PANS children to standard reference values and found them to be within the normal reference (28).

Two studies had a longitudinal design (30, 31). The first study investigated children with TS and CTD with or without OCD and compared periods of exacerbation of symptoms to periods of remission. TNF-α was higher in exacerbation compared to remission. They found no differences in serum cytokine levels between tic-OCD patients and tic+OCD patients (30). The second longitudinal study compared PANDAS debut to periods of exacerbations and no significant differences in cytokines were found (31).

## Discussion

The aim of this study was to review the cytokine profile of pediatric patients with neuropsychiatric symptoms as seen in OCD, TS, SC, PANDAS, PANS or CTD. We found that cytokines for these patient groups appear to be affected in a proinflammatory direction. Of special interest were TNF-α, IL-17 and IL-1β, as most studies measuring these cytokines found a significant increase compared to healthy controls. The studies that had included healthy controls found significant increases in especially the cytokines TNF-α, IL-17 and IL-1β, but many other cytokines were also reported as being significantly increased or decreased in patients compared to healthy controls, as shown in Table 2. The studies with other control groups had more heterogenous results, most likely due to smaller sample sizes and

more heterogeneity in the control groups. Only two cytokines were reported significantly altered in more than one study with a non-healthy control group (Il-17 and IL-10), however these were reported as significantly increased in one study and significantly decreased in the other. The studies with longitudinal design were also challenged by their sample size. One study found significantly increased TNF-α in periods of exacerbations, while the other did not.

TNF-α is a proinflammatory cytokine, as it can initiate a strong inflammatory response in nucleated cells, but it can also act as an immunosuppressive mediator by limiting the inflammatory responses. Furthermore, it has a role in inhibiting the development of autoimmune diseases (32). TNF-α has been found to be of importance in many neurological and psychiatric disorders. A recent study found that maternal OCD was related to a significantly higher level of cord blood TNF-α which also was positively correlated with maternal anxiety level (33). Some polymorphisms of the TNF-α gene have found to be associated to OCD susceptibility (29, 34) and others to TS (35). In this review, increased levels of TNF-α were seen across different diagnoses. Significantly increased levels were seen in two studies with patients with TS (18, 23) and in three studies with patients with OCD (14, 21, 22). Furthermore, one longitudinal study found that TNF-α levels were increased in periods of exacerbations in children with TS/CTD (30). Although these findings not have been replicated by other studies (12, 19, 31), they do suggest that TNF-α might be involved in children with obsessive-compulsive and/or tic-related symptoms. Based on the findings in this review, we suggest that a dysregulation or increase of TNF-α, perhaps on a genetic basis, could be associated with obsessive-compulsive and tic symptoms.

IL-1β is also a proinflammatory cytokine, and has been of interest in various central nervous system (CNS) diseases, like multiple sclerosis (36). In children with febrile seizures, elevated levels of IL-1β (as well as TNF-α) in CSF were seen (37). In a meta-analysis, an association was found between the risk of febrile seizures and epilepsy and polymorphism in the IL-1β (511) gene (38). IL-1β has been found to be associated with Post-Traumatic Stress Disorder and bipolar disorder (39, 40). However, the literature on IL-1β and its association to obsessive-compulsive and/or tic symptoms is scarce. One systematic review and meta-analysis found significant reduction in IL-1β compared to healthy controls; importantly this was almost exclusively based on data from adults (41). In a Chinese population with OCD no association was found in IL-1β−511 polymorphism compared to healthy controls (42).

IL-17 (often also called IL-17A) is a proinflammatory cytokine and is mainly expressed by CD4+ TH17 cells (32). In CNS, IL-17 is a mediator between immune cells and tissue, and it was found that an artificial overexpression of IL-17 activates glial cells and enhances neuroinflammation (43). IL-17 has been reported to synergize with other proinflammatory cytokines, such as TNF-α, and potentiate their effects (44, 45). IL-17 has also been reported to be associated to different neurological and psychiatric disorders. In Parkinson's Disease patients, increased levels of IL-17 were correlated with higher levels of anxiety and depression (46). Depression has also been associated with IL-17. One study found IL-17 to be significantly increased in peripheral blood in depressive patients compared to healthy controls (47), although a correlation between severity of depression and IL-17 levels was not found (48). In children with autism, IL-17 levels were elevated compared to healthy controls, and were significantly correlated with the severity of autism (49). Although dysregulation of IL-17 has been found to be triggering several autoimmune diseases in murine models (50), literature on its role in obsessive-compulsive or tic symptoms remains sparse (41). We suggest therefore that studies investigating cytokines in children with obsessive-compulsive and/or tic symptoms in the future should include this cytokine in order to elucidate its role.

An important consideration regarding the methods used in the included articles is that most of the studies measured peripheral cytokines, in serum, or plasma, or tonsil tissue. It can be argued that peripheral cytokines are unreliable surrogate markers of the cytokines in the CNS, as peripheral cytokines can be influenced by many other variables such as age, body mass index, medication, smoking, stress and circadian fluctuation (51). On the other hand, it is important to recognize that the blood-brain barrier (BBB), initially giving reason for the immune privilege hypothesis, can be impaired in various ways, for example by inflammatory cytokines which appear to play a crucial role in allowing antibodies to cross the BBB by impacting (52) stability of the BBB (53). TNF-α induces formation of gaps in BBB by internalizing tight junction protein *via* upregulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF κB) and its transcription myosin light chain kinase (54, 55). IL-17 also disrupts the BBB tight junctions and promotes the transmigration of CD4+ lymphocytes through TH17 cells' ability (activated by IL-17) to permeabilize the BBB (56). Peripheral inflammation has also been reported to affect the BBB permeability in other psychiatric disorders (schizophrenia, bipolar disorder and major depressive disorder) (57).

The included studies have used various immune assays for measuring cytokines, performed on various biological materials (serum, plasma, CSF and tonsil tissue) from differing patient groups (TS, OCD, SC, CTD, PANS and PANDAS with different comorbid combinations). Only some of the studies reported on medication status, and not all of them included medication status as confounder. These considerations, and the relatively small number of patients and controls make meta-analysis and subgroup analysis challenging.

In summary, there appears to be an increase in proinflammatory cytokines most clearly for TNF-α, but probably also for IL-17 and IL-1β, in children with obsessive-compulsive and movement disorder symptoms compared to healthy controls. These cytokines can through their effect on the BBB give rise to neuroinflammation. This can potentially offer important insights into the pathogenesis of

obsessive-compulsive and tic symptoms, as it implies that at least a subgroup of the affected patients could have an autoimmune pathogenesis. This could offer new treatment options for the afflicted children. However, more knowledge on the role of the immune system, including that of pro-inflammatory cytokines, is needed in the future. The knowledge from the existing studies is still limited and challenged by the heterogeneity of used methods and their relatively small sample size, and thus larger studies are needed to thoroughly examine the cytokine profile of children with obsessive-compulsive and movement disorder symptoms.

## Author contributions

RF conducted the review and authored the manuscript with supervision and revisions from CS, ND, and LS. All authors contributed to the article and approved the submitted version.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY
Marie Bækvad-Hansen,
Statens Serum Institut (SSI), Denmark

REVIEWED BY
Hidde Huidekoper,
Erasmus Medical Center, Netherlands
Carolina Fischinger Moura De Souza,
Clinical Hospital of Porto Alegre, Brazil

*CORRESPONDENCE
Alessandro Rossi
alessandro.rossi@unina.it

†These authors have contributed equally to this work and share first authorship
‡These authors share last authorship

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# Long-term monitoring for short/branched-chain acyl-CoA dehydrogenase deficiency: A single-center 4-year experience and open issues

Alessandro Rossi[1*†], Mariagrazia Turturo[2†], Lucia Albano[3], Simona Fecarotta[1], Ferdinando Barretta[2,3], Daniela Crisci[3], Giovanna Gallo[3], Rosa Perfetto[3], Fabiana Uomo[2], Fabiana Vallone[3], Guglielmo Villani[2,3], Pietro Strisciuglio[1], Giancarlo Parenti[1], Giulia Frisso[2,3‡] and Margherita Ruoppolo[2,3‡]

[1]Department of Translational Medicine, Section of Pediatrics, University of Naples "Federico II", Naples, Italy, [2]Department of Molecular Medicine and Medical Biotechnology, University of Naples "Federico II", Naples, Italy, [3]CEINGE Biotecnologie Avanzate s.c.ar.l, Naples, Italy

**Introduction:** Short/branched-chain acyl-CoA dehydrogenase deficiency (SBCADD) is an inherited disorder of L-isoleucine metabolism due to mutations in the *ACADSB* gene. The role of current diagnostic biomarkers [i.e., blood 2-methylbutyrylcarnitine (C5) and urine 2-methylbutyrylglycine (2MBG)] in patient monitoring and the effects of proposed treatments remain uncertain as follow-data are lacking. This study presents first systematic longitudinal biochemical assessment in SBCADD patients.

**Methods:** A retrospective, observational single-center study was conducted on newborns born between 2017 and 2020 and suspected with SBCADD. Biochemical, molecular, clinical and dietary data collected upon NBS recall and during the subsequent follow-up were recorded.

**Results:** All enrolled subjects ($n = 10$) received adequate protein intake and L-carnitine supplementation. Nine subjects were diagnosed with SBCADD. During the follow-up [median: 20.5 (4–40) months] no patient developed symptoms related to SBCADD. No patient normalized serum C5 and urine 2MBG values. In 7/9 SBCADD patients mean serum C5 values decreased or stabilized compared to their first serum C5 value. A major increase in serum C5 values was observed in two patients after L-carnitine discontinuation and during intercurrent illness, respectively. Urine 2MBG values showed moderate intra-patient variability.

**Discussion:** The relatively stable serum C5 values observed during L-carnitine supplementation together with C5 increase occurring upon L-carnitine discontinuation/intercurrent illness may support the value of serum C5 as a monitoring biomarker and the benefit of this treatment in SBCADD patients. The role of urine 2MBG in patient monitoring remains uncertain. As all

patients were asymptomatic, no association between biochemical parameters and clinical phenotype could be investigated in this study.

KEYWORDS

amino acid, 2-methylbutyrylglycinuria, biomarkers, monitoring, treatment, carnitine, diet

# Introduction

Short/branched-chain acyl-CoA dehydrogenase (SBCAD) deficiency (SBCADD) (OMIM# 600301, also known as 2-methylbutyrylglycinuria, OMIM#610006) is an autosomal recessive metabolic disorder due to mutations in the *ACADSB* gene (1, 2). This gene encodes for the mitochondrial SBCAD, which catalyzes (1) the third reaction in the degradation pathway of L-isoleucine (that is the conversion of 2-methylbutyryl-CoA to tiglyl-CoA) and (2) the first oxidative step of short straight-chain acyl-CoAs (such as butyryl-CoA and hexanoyl-CoA). SBCAD could also use valproyl-CoA as a substrate, potentially playing a role in valproate metabolism (3, 4).

Most individuals with SBCADD, including those ascertained *via* NBS, show no health problems (5). However, a small percentage of individuals can develop signs and symptoms soon after birth or later in childhood, including poor feeding, lethargy, vomiting, irritability (6, 7). These symptoms can eventually progress to serious conditions such as dyspnea, seizures, and coma (5, 7). Additional features include poor growth, muscle weakness, delay in motor skills (6, 8–10), intellectual disability (11).

SBCADD is associated with elevated 2-methylbutyryl carnitine (C5) and 2-methylbutyrylglycine (2MBG) concentrations in blood and urine, respectively. Since C5 is detectable by tandem mass spectrometry (MS/MS) on dried blood spots (DBS) (and serum), SBCADD may be identified through expanded newborn screening (NBS) (5, 12, 13). Assessment of urine 2MBG represents (one of) the major confirmatory tests (3).

Despite the progress in patient ascertainment, multiple uncertainties on the management of SBCADD still exist (13, 14). Particularly, the role of currently employed diagnostic biomarkers (i.e., blood C5 and urine 2MBG) in patient monitoring is unknown as follow-up data are largely missing. In addition, there are no conclusive data on the efficacy of proposed treatments, namely L-carnitine supplementation and dietary protein restriction (15). With the gradual development of NBS programs worldwide, the number of individuals diagnosed with SBCADD as well as the phenotype variability is expected to increase over next years (16). Thus, it is important to define appropriate patient monitoring and management options.

The aim of the current study was to define the applicability of currently employed diagnostic biomarkers as monitoring tools by presenting the first systematic longitudinal biochemical assessment in SBCADD patients.

# Methods

## Subjects

This was a retrospective, observational single center study conducted at the Regional NBS reference center Campania, Italy, where the NBS samples of all newborns born in Campania Region are processed. Collectively, 160,015 subjects were analyzed in the study period. Subjects were enrolled if they met all the following inclusion criteria: (I) date of birth from 01 January 2017 to 31 December 2020; (II) increased C5 value (reference value 0.02–0.26 μmol/L) found on the NBS DBS sample; (III) increased urine 2MBG value (reference value < 2 mmol/mol Creatinine) found upon NBS recall. Exclusion criteria were: (I) normal urine 2MBG value found upon NBS recall; and/or (II) detectable urine isovaleryglycine found upon NBS recall.

The Regional Operative Procedure for newborns suspected with an inherited metabolic disease at NBS is presented in Supplementary Figure 1. Briefly, newborns suspected with SBCADD are included in a follow-up program and undergo: (1) routine clinical and biochemical assessment, (2) molecular testing of the *ACADSB* gene, and (3) oral L-carnitine supplementation (100 mg/kg/day) initiated upon NBS recall. Additionally, all caregivers are instructed on avoiding prolonged fasting and checking blood glucose concentrations upon metabolic stress conditions (e.g., intercurrent illness, decreased oral intake).

## Methods

All enrolled subjects were clinically and biochemically evaluated upon NBS recall and subsequently every 3–6 months. Collected data were retrieved from patients' records compiled during routine visits and included: DBS or serum C5 concentration, urine 2MBG concentration, serum glucose, ammonia and CK concentrations, liver function tests, blood gases, heart ultrasound, electrocardiogram, weight, height, and psychomotor development assessment as well as

information on medical history, daily protein intake, L-carnitine supplementation, and *ACADSB* variants.

C5 and 2MBG concentrations were assessed on morning samples (≥2-h fasting). C5 concentration was evaluated through acylcarnitine analysis. Acylcarnitine analysis was performed on DBS samples upon NBS recall and on serum samples during subsequent evaluations by tandem-mass spectrometry (LC/MS-MS) as previously described (17, 18). Urine 2MBG concentration was evaluated through urine organic acids (UOA) profile assessed by gas chromatography-mass spectrometry (GC-MS), as previously described (19). Additional biochemical parameters were evaluated by using routine assays with commercially available kits.

The intra-day precision was evaluated by analyzing three replicate analyses of three different serum and was estimated to be 0.7% coefficient of variation (CV). The inter-day precision was evaluated by analyzing three replicate analyses of three different serum over a 5-day period and was estimated to be 5%CV.

For each subject the serum C5 and urine 2MBG trend was defined as follows: (i) increased, if the difference between the first serum C5 value and the mean serum C5 value measured during the follow-up was > +10%, (ii) stable, if the difference between the first serum C5 value and the mean serum C5 value measured during the follow-up was between −10 and +10% and (iii) decreased, if the difference between the first serum C5 value and the mean serum C5 value measured during the follow-up was < −10%. The 10% threshold was chosen being around 10 times the intraday CV and twice the interday CV.

Molecular testing was performed on DNA extracted from EDTA peripheral venous blood samples. All exons and part of the flanking intron regions of *ACADSB* gene were amplified by polymerase chain reactions and sequenced for mutation analysis, according to standard procedure (20). Variations were reported following the Human Genome Variation Society (HGVS) nomenclature (http://www.HGVS.org/varnomen) and annotated according to NCBI SNPs Database (http://www.ncbi.nlm.nih.gov, accessed March 2022), ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar, accessed March 2022), Human Gene Mutation Database (HGMD) Professional (http://www.hgmd.cf.ac.uk/, accessed March 2022), and American College of Medical Genetics and Genomics (ACMG) guidelines for variant classification (21).

## Results

Ten subjects were enrolled in the present study. Median follow up was 20.5 (range: 4–40) months. No abnormal findings in serum glucose, ammonia, CK, blood gases concentrations, liver function tests, heart ultrasound, electrocardiogram were detected in any of the subjects during the follow-up. All subjects showed regular growth and psychomotor development. No subjects received a protein restricted diet (median protein intake: 3.5 g/kg/day) (Table 1). Weaning occurred at 4–6 months in all subjects (median protein intake: 1.7 g/kg/day, range: 1.5–2.0 g/kg/day). All subjects were started with oral L-carnitine supplementation (100 mg/kg/day) upon NBS recall and continued the treatment for the whole duration of the follow-up, unless stated otherwise.

Nine subjects (P1–P9) showed homozygosity or compound heterozygosity for *ACADSB* variants and were diagnosed with SBCADD (Table 1). One subject (P10) only carried the heterozygous c.1159G>A (p.Glu387Lys) (reference SNP ID rs188094280) *ACADSB* variant. Although such variant is classified as pathogenetic according to the ACMG guidelines, this subject could not be conclusively diagnosed with SBCADD. Among the 9 detected variants, 4 were not previously described as associated to SBCADD, being missing in ClinVar and HGMD databases. According to the novel ACMG criteria, all variants were classified as (likely) pathogenic, [(L)P], except c.1102T>C, which was classified as VUS/likely pathogenic. However, this variant was previously reported in the HGMD database as associated with SBCADD patient (22).

The median DBS C5 and urine 2MBG values upon NBS recall in SBCADD patients were 0.63 μmol/l (reference value 0.02–0.26 μmol/l) and 15.0 mmol/creatinine moles (reference values <2 mmol/creatinine moles), respectively. Their median serum C5 and urine 2MBG values during follow-up were 1.0 μmol/l (reference values 0.05–0.24 μmol/l) and 21.0 mmol/creatinine moles (reference values <2 mmol/creatinine moles), respectively. Figure 1 presents the individual serum C5 values evaluated during the follow-up of SBCADD patients. Serum C5 values stayed above the reference values in all subjects. In 4/9 SBCADD patients (P3, P4, P7, P9) the individual serum C5 value decreased (range −20/−55%). In 3/9 SBCADD patients (P1, P5, P8) the individual serum C5 value stabilized (range −2/+6%). In 2/9 SBCADD patients (P2, P6) the individual serum C5 value increased (range +25/+39%). Serum C5 values showed a similar trend in subjects P3 and P4 (twin subjects). P5 showed the highest baseline serum C5 value among SBCADD patients. In this subject a major increase in serum C5 value was noted at 8 months, when an intercurrent illness (vomiting and diarrhea) without metabolic decompensation occurred. In P6 a major increase in serum C5 value occurred at 8 months. Tracing back the patient's history, it was ascertained that from this point the family had decided to discontinue L-carnitine supplementation because of its unpleasant taste. In P2 a transient increase in serum C5 value was noted at 3 and 14 months. At 3 months an intercurrent illness (vomiting, reduced food intake) occurred. No known factors possibly associated with the subsequent increase occurring at 14 months could be found. In P9 oral L-carnitine supplementation was discontinued by the family from 8 months because of its unpleasant taste; subsequently a (slight) increase in serum C5 value was noted. Individual serum free

TABLE 1 Biochemical, clinical and molecular features of SBCADD patients.

| Subject | Initial C5 value[b] (μmol/L) | Initial urine 2MBG[c] (mmol/creatinine moles) | Follow up duration (months) | Clinical aspects | Daily Protein intake (g/Kg/day)[d] | Genotype *ACADSB* cDNA (protein variation) | Family segregation | Variant classification | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Reference SNP ID | ClinVar | HGMD* | ACMG** |
| P1 | 0.84 | 8 | 30 | Asymptomatic | 3.9 | **c.641C>A (p.Ala214Glu)** | **F: c.641C>A** | rs887880417 | NR | NR | **LP** |
| | | | | | | c.1102T>C (p.Ser368Pro) | M: c.1102T>C | rs774205809 | VUS | CM052826 | VUS/LP |
| P2 | 0.57 | 20 | 24 | Asymptomatic | 3.5 | c.1128+3A>T (possible splicing | F: c.1128+3 A>T | rs760423996 | CI | NR | LP |
| | | | | | | alteration) | M: c.443C>T | rs58639322 | CI | CM052824 | P |
| | | | | | | c.443C>T (p.Thr148Ile) | | | | | |
| P3[a] | 0.68 | 8 | 40 | Asymptomatic | 3.4 | c.1128+3A>T (possible splicing | F: c.1128+3A>T | rs760423996 | CI | NR | LP |
| | | | | | | alteration) | M: c.1159G>A | rs188094280 | CI | CM080029 | LP |
| | | | | | | c.1159G>A (p.Glu387Lys) | | | | | |
| P4[a] | 0.54 | 11 | 40 | Asymptomatic | 3.4 | c.1128+3A>T (possible splicing | F: c.1128+3A>T | rs760423996 | CI | NR | LP |
| | | | | | | alteration) | M: c.1159G>A | rs188094280 | CI | CM080029 | P |
| | | | | | | c.1159G>A (p.Glu387Lys) | | | | | |
| P5 | 0.68 | 60 | 17 | Asymptomatic | 3.8 | c.443C>T (p.Thr148Ile) | NP | rs58639322 | CI | CM052824 | P |
| | | | | | | c.443C>T (p.Thr148Ile) | | | | | |
| P6 | 0.63 | 15 | 12 | Asymptomatic | 3.5 | c.443C>T (p.Thr148Ile) | F: c.443C>T | rs58639322 | CI | CM052824 | P |
| | | | | | | c.1159G>A (p.Glu387Lys) | M: c.1159G>A | rs188094280 | CI | CM080029 | P |
| P7 | 0.69 | 20 | 4 | Asymptomatic | 3.5 | c.908G>C (p.Gly303Ala) | F: c.908G>C | rs1316417761 | NR | CM052825 | LP |
| | | | | | | c.443C>T (p.Thr148Ile) | M: c.443C>T | rs58639322 | CI | CM052824 | P |
| P8 | 0.48 | 11 | 4 | Asymptomatic | 3.8 | c.443C>T (p.Thr148Ile) | F: c.443C>T | rs58639322 | CI | CM052824 | P |
| | | | | | | **c.439A>T (p.Asn147Tyr)** | M: c.439A>T | rs747291865 | NR | NR | **LP** |
| P9 | 0.43 | 22 | 30 | Asymptomatic | 3.2 | **c.247A>G (p.Met83Val)** | F: c.247A>G | rs751301851 | NR | NR | **LP** |
| | | | | | | **c.293T>G (p.Phe98Cys)** | M: c.293T>G | NR | NR | NR | **LP** |

C5 values detected on DBS samples and urine 2MBG values found upon NBS recall are shown. For each genetic variant the following information is shown: variant nomenclature at cDNA and protein level, according to the HGVS (Human Genome Variation Society) guidelines; reference single nucleotide polymorphism (SNP) ID number (rs); clinical significance by ClinVar database, HGMD database (*Human Genetic Mutation Database) and ACMG (**American College of Medical Genetics) classification, respectively. Novel variants are presented in bold. CI, conflicting interpretation; LP, likely pathogenic; NP, not performed; NR, not reported; P, pathogenic; VUS, variant of uncertain significance.

[a]Twin subjects.

[b]Reference value: 0.02–0.26 μmol/l.

[c]Reference value: <2 mmol/creatinine moles.

[d]Before weaning.



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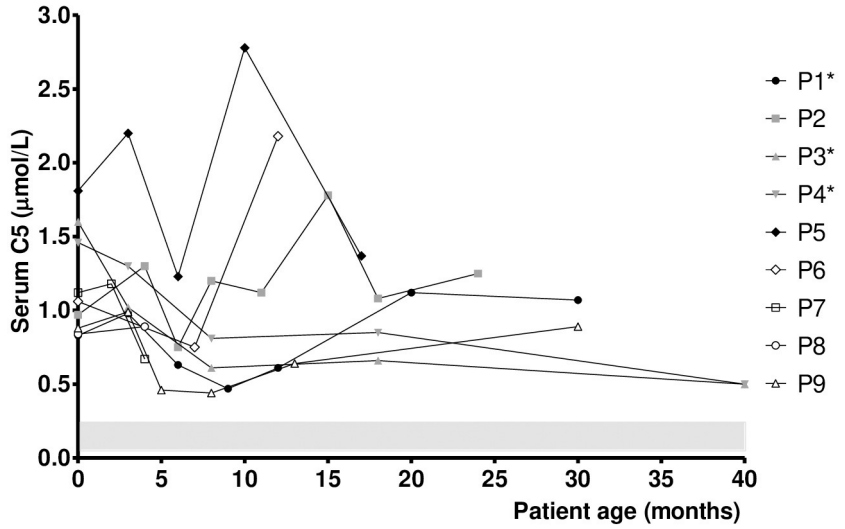


FIGURE 1
Serum C5 values in SBCADD patients. For each subject, the first time point shows the serum C5 value detected upon NBS recall before starting the L-carnitine treatment (100 mg/kg/day). Subsequent time points show serum C5 value after starting with L-carnitine treatment. C5 reference range is highlighted (shaded area). *Data for P1 at 24 months, P3 and P4 at 12 and 24 months were not collected due to COVID19 pandemic.

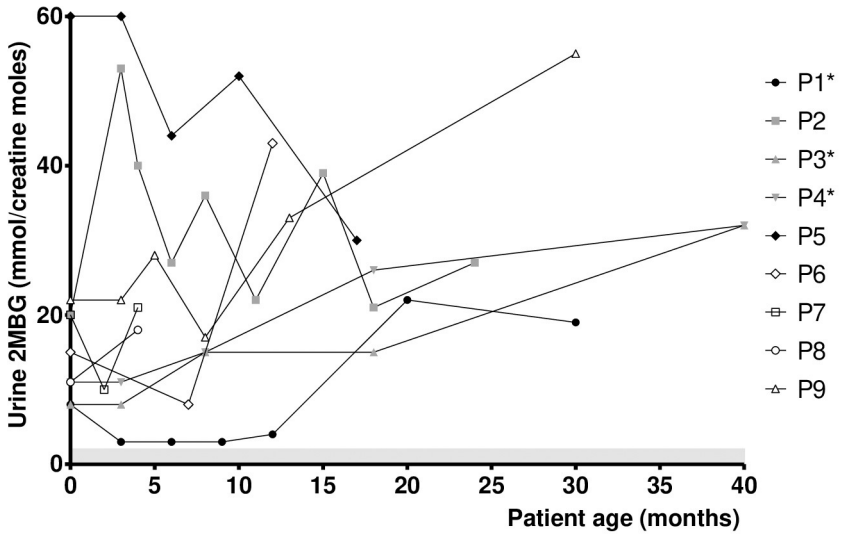


FIGURE 2
Urine 2MBG values in SBCADD patients. For each subject the first time point shows the urine 2MBG value detected upon NBS recall before starting the L-carnitine treatment (100 mg/kg/day). Subsequent time points show urine 2MBG value after starting with L-carnitine treatment. 2MBG reference range is highlighted (shaded area). *Data for P1 at 24 months, P3 and P4 at 12 and 24 months were not collected due to COVID19 pandemics.

carnitine (C0) and C5 values in SBCADD patients are presented in Supplementary Figure 2.

Figure 2 presents the individual urine 2MBG values in SBCADD patients during the follow up. Urine 2MBG values stayed above the reference values in all subjects. In 7/9 SBCADD patients (P1-P4, P6, P8, P9) the individual urine 2MBG value increased (range +13/+119%). In 2/9 SBCADD patients (P5, P7) the individual urine 2MBG value decreased (−22%). A moderately variable trend in urine 2MBG values was noticed in all subjects. Urine 2MBG values in P3 and P4 (twin subjects) widely overlapped. No relationship between urine 2MBG values and L-carnitine intake, dietary information, or medical history could be noticed in 7/9 SBCADD patients (P1–P5, P7, P8). In P5 no major increase in urine 2MBG value occurred at 8 months, at

the time when an increase in serum C5 value was noticed (see Figure 1). Conversely, an increase in urine 2MBG values was found in P6 at 12 months, after L-carnitine discontinuation. A (slight) increases in urine 2MBG values was also noticed in P9 after oral L-carnitine supplementation was discontinued.

In addition to 9 SBCADD patients, one subject (P10) was also enrolled who displayed increased C5 value on NBS DBS (0.45 μmol/L, reference 0.02–0.26) and increased urine 2MBG (15 mmol/creatinine moles, reference <2 mmol/) but could not be conclusively diagnosed with SBCADD. This subject was monitored for 7 months displaying both serum C5 and urine 2MBG values above the reference values. Such values were lower than median values found in SBCADD patients (mean serum C5: 0.85 μmoles/l; mean urine 2MBG: 12.0 mmol/creatinine moles).

## Discussion

SBCADD is an inherited disorder of amino acid metabolism in which L-isoleucine degradation is impaired (1). Due to the limited number of reported patients, its clinical relevance remains uncertain (13). Based on literature data 90% of SBCADD patients are asymptomatic (23). As most of these patients were ascertained by NBS, the inclusion of SBCADD in NBS programs is controversial (22). Nonetheless, there exist a group of SBCADD patients who develop (severe) health problems (6–8, 10, 11, 14). The reason why these patients become symptomatic remains unclear. Researchers speculate that some features, such as lethargy and muscle weakness, occur because L-isoleucine cannot be properly used for energy production in SBCADD (14). In addition, the enzyme defect may result in the accumulation of toxic compounds in the brain (24). Currently, neither recognized long-term monitoring biomarkers nor conclusive data on the efficacy of proposed treatments (i.e., L-carnitine supplementation, dietary protein restriction) are available for SBCADD.

In this study longitudinal biochemical data were systematically collected in SBCADD patients to define the applicability of currently employed diagnostic biomarkers as monitoring biomarkers. Particularly, this is the first study to present >12-month follow-up data on both serum C5 and urine 2MBG values in SBCADD patients. All enrolled subjects were diagnosed by NBS, allowing the first estimation of the incidence of SBCADD within Europe (1:17,780 newborns). Previous studies reported an incidence between 1:132 in the Hmong population (12) and 1:540,780 in non-Hmong groups (12, 25), indicating wide regional and ethnic differences.

Although serum C5 values stayed above the reference range in all subjects, a relative stabilization was observed in 7/9 SBCADD patients after starting oral L-carnitine supplementation, even with no dietary protein restriction. An increase in serum C5 values occurred in 2 SBCADD patients upon L-carnitine discontinuation (P6) and intercurrent illness (P5), respectively (Figure 1). Urine 2MBG values showed an inconsistent trend with larger variability among (and within) patients; no apparent relation with medical data could be found except in 2 patients (P6, P9) in whom L-carnitine discontinuation was followed by an increase in urine 2MBG excretion (Figure 2). Although neither concurrent increase in dietary protein intake nor clinical symptoms were detected, temporary subclinical (protein) catabolism cannot be ruled out in these 2 patients. Collective data suggest a possible role for serum C5 in patient (treatment) monitoring and may support the benefit of L-carnitine supplementation in SBCADD. Conversely, the role of urine 2MBG in patient monitoring remains unclear.

Few previous studies have presented biochemical data collected over time in SBCADD patients. However, data were either limited to DBS and/or serum C5 (23, 26) or scarce and/or with no defined time-point evaluations or not comparable to one another (5, 13, 22, 27) and always with a shorter follow-up as compared to the present study. In one study the follow-up duration was not mentioned (6). One SBCADD patient with a 4-year follow-up has also been reported for whom neither serum C5 nor urine 2MBG values were available (16).

As none of the patients enrolled in the present study developed symptoms related to SBCADD, no association between biochemical parameters and clinical phenotype could be investigated. Indeed, most of the patients showed *ACADSB* variants which have been previously found in asymptomatic patients (5, 8, 26–28). However, some variants (e.g., c.443C>T, c.908G>C, and c.1102T>C) found in the present study have been formerly reported in symptomatic SBCADD patients (23). Although specific variants have been most commonly found in symptomatic patients, no clear genotype-phenotype correlation exist in SBCADD and its clinical course cannot be easily predicted based on the genotype only. In fact, two siblings carrying the same ACADSB genotype (i.e., c.443C>T/c.1145C>T) have been reported, who presented with different clinical phenotypes (namely one being symptomatic and the other being asymptomatic, respectively) (23). Although the homozygous c.443C>T *ACADSB* variant has been previously described in symptomatic patients (22), one of the patients included in the present study carried the same genotype and was asymptomatic. Whether this patient will develop symptoms in the future or upon metabolic stress conditions (e.g., intercurrent illness, fasting) is unknown. Also, whether early diagnosis (by NBS) and treatment could have prevented the occurrence of symptoms in this patient remains to be determined.

Although no clear genotype-clinical phenotype exists, an association between *ACADSB* variants and biochemical phenotype has been reported. Consistently with previous observations showing serum C5 values up to up to 3 μmol/l in SBCADD patients carrying the homozygous variant c.443C>T

(5, 22), P5 displayed the highest serum C5 values in the present study (Figure 1). In addition, an overlapping trend in serum C5 and urine 2MBG values was observed between the two twin subjects carrying the same *ACDSB* variants (P3 and P4). As the mechanism underlying these observations remains unresolved, future investigation is warranted.

Besides 9 SBCADD patients, 1 subject was also enrolled in the present study who only carried one pathogenic variant in the *ACADSB* gene and could not be conclusively diagnosed with SBCADD. Although below the median values found in SBCADD patients, serum C5 and urine 2MBG values were above the reference range in this subject. Despite the short follow-up data, serum C5 values found in P10 were comparable to individual SBCADD patients. Thus, SBCADD could not be ruled out in this subject. Interestingly, in the present study serum C5 values were found between 0.5 and 2.8 μmol/l in SBCADD patients and 0.8-0.9 μmol/l in the subject with no conclusive diagnosis of SBCADD. We speculate about a potential role of serum C5 values in subjects' stratification. In principle, a possible role of serum C5 value in clarifying the (biochemical) impact of (novel) *ACADSB* variants may be hypothesized. On the other hand, the circumstance of increased urine 2MBG excretion in conditions other than SBCADD appears intriguing. Future studies addressing these issues are worthy.

Potential limitations of this study include the retrospective design and the absence of functional studies. In addition, it is unknown whether higher L-carnitine dose and/or dietary protein restriction could have resulted in normalization of serum C5 and/or urine 2MBG values in the enrolled subjects. No direct correlation between serum C0 and C5 values was found in the present study. Indeed, P1 and P5 showed stable serum C5 values despite displaying supraphysiological serum C0 values at several time points (Figure 1). Yet, it cannot be ruled out that supraphysiological serum C0 values may have concurred to C5 accumulation in P2 (Supplementary Figure 2). Conversely, P6 and P9 displayed a decreasing trend in serum C0 levels after L-carnitine supplementation discontinuation. Although all patients showed (supra)normal serum C0 values in the present study, longer follow-up is required to assess whether untreated SBCADD patients are at risk of developing carnitine deficiency. Whether serum C5 and urine 2MBG values accurately reflected their tissue concentrations in SBCADD also remains to be ascertained.

In conclusion, the clinical course of SBCADD remains poorly predictable. Therefore, regular clinical monitoring (including serum glucose, ammonia and CK concentrations, liver function tests, blood gases, heart ultrasound, electrocardiogram, weight, height, and psychomotor development assessment) together with general advice on how to prevent catabolism is recommended (15, 23, 29). As serum C5 appears as a potential long-term biomarker for patients' (treatment) monitoring we suggest including regular serum C5 assessment in patients' monitoring. Conversely, available data do not support a role for urine 2MBG beyond the diagnosis. Although previous untreated cases have been reported that remained asymptomatic over a variable period of time (5, 14, 26), L-carnitine supplementation may be beneficial (at least) on the "biochemical phenotype" in SBCADD. Yet, appropriate dose titration is recommended in order to avoid supraphysiological free carnitine levels potentially concurring to C5 accumulation. The clinical relevance of such "biochemical benefit" remains unclear. On the other hand, the role of dietary protein restriction in SBCADD remains to be established. Longer follow-up studies are warranted to identify accurate monitoring (and prognostic) biomarkers and define the optimal management approach in SBCADD.

## Data availability statement

The raw data supporting the conclusions of this article are available from the corresponding author upon reasonable request.

## Ethics statement

The studies involving human participants were reviewed and approved by the Medical Ethical Committee of the University of Naples "Federico II". Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

## Author contributions

AR and MT wrote the first version of the manuscript. GF, GV, PS, GP, and MR critically reviewed the manuscript. All authors substantially contributed to the work and were involved in conception and design of the study and/or analysis and interpretation of data, and revising the article critically for important intellectual content. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work. All authors confirm the absence of previous similar or simultaneous publications.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2022.895921/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Regional Operative Procedure for newborns suspected with an inherited metabolic disease at newborn screening.

SUPPLEMENTARY FIGURE 2
Individual serum free carnitine (C0, black circles, top section of the graph) and 2-methylbutyryl carnitine (C5, gray squares, bottom section of the graph) values in SBCADD patients. For each subject the first time point shows the serum C5 and C0 values detected before starting the L-carnitine treatment. Subsequent time points show serum C5 value after starting with L-carnitine treatment, unless stated otherwise. C0 reference range (10–45 μmol/L) is shown (dashed lines). #Samples collected after carnitine discontinuation.

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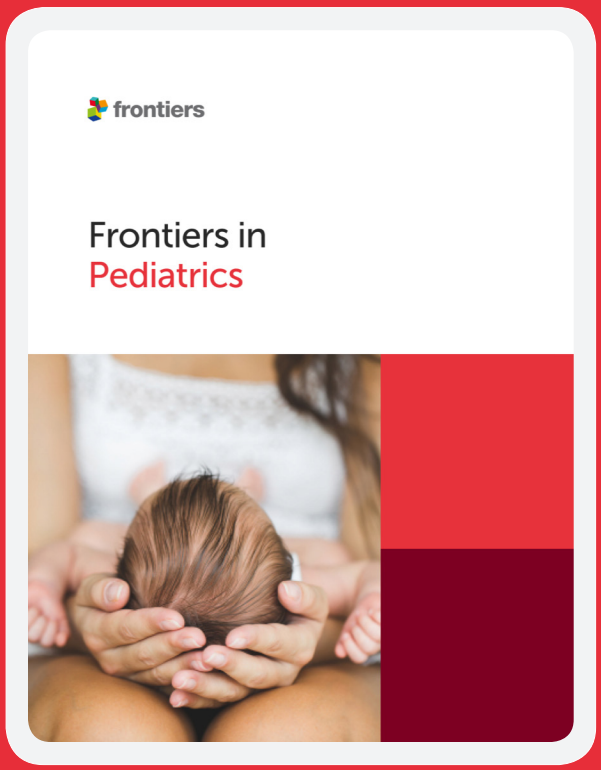


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