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Invited Perspective

Metabolomic profile of children with recurrent respiratory infections

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ABSTRACT

Recurrent respiratory infections (RRI) represent a widespread condition which has a severe social and economic impact. Immunostimulants are used for their prevention. It is crucial to better characterize children with RRI to refine their diagnosis and identify effective personalized prevention strategies. Metabolomics is a high-dimensional biological method that can be used for hypothesis-free biomarker profiling, examining a large number of metabolites in a given sample using spectroscopic techniques. Multivariate statistical data analysis then enables us to infer which metabolic information is relevant to the biological characterization of a given physiological or pathological condition. This can lead to the emergence of new, sometimes unexpected metabolites, and hitherto unknown metabolic pathways, enabling the formulation of new pathogenetic hypotheses, and the identification of new therapeutic targets.

The aim of our pilot study was to apply mass-spectrometry-based metabolomics to the analysis of urine samples from children with RRI, comparing these children's biochemical metabolic profiles with those of healthy peers. We also compared the RRI children's and healthy controls' metabolomic urinary profiles after the former had received pidotimod treatment for 3 months to see whether this immunostimulant was associated with biochemical changes in the RRI children's metabolic profile.

13 children (age range 3–6 yeas) with RRI and 15 matched per age healthy peers with no history of respiratory diseases or allergies were enrolled. Their metabolomic urine samples were compared before and after the RRI children had been treated with pidotimod for a period of 3 months. Metabolomic analyses on the urine samples were done using mass spectrometry combined with ultra-performance liquid chromatography (UPLC–MS). The resulting spectroscopic data then underwent multivariate statistical analysis and the most relevant variables characterizing the two groups were identified.

Data modeling with post-transformation of PLS2-Discriminant Analysis (ptPLS2-DA) generated a robust model capable of discriminating the urine samples from children with RRI from those of healthy controls ($R^2 = 0.92, Q^2_{CV7\text{-}fold} = 0.75$, $p\text{-value} < 0.001$). The dataset included 1502 time per mass variables, and 138 of them characterized the difference between the two groups. Thirty-five of these distinctive 138 variables persisted in the profiles of the children with RRI after pidotimod treatment.

Metabolomics can discriminate children with RRI from healthy controls, suggesting that the former have a dysregulated metabolic profile. Among the variables characterizing children with RRI there are metabolites that may reflect the presence of a different microbiome.

After pidotimod treatment, the metabolic profile of the children with RRI was no longer very different from that of the healthy controls, except for the persistence of some microbiome-related variables. We surmise that pidotimod partially "restores" the altered metabolic profile of children with RRI, without modifying the metabolites related to the composition of the gut microbiota. In the light of these results, we hypothesize a potential synergic effect of the combined use of immunostimulants and probiotics for the purpose of prevention in children with RRI.

Abbreviations: RRI, recurrent respiratory infection; PLS-DA, projection to latent structures discriminant analysis; ptPLS2-DA, post-transformation of PLS2-DA discriminant analysis; QC, quality control; PCA, principal component analysis.

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1. Introduction

Recurrent respiratory infections (RRI) are among the most common complaints in the pediatric population and can cause severe morbidity. It has been estimated that at least 6% of children under 6 years old present RRI. This clinical category poses a considerable "challenge" to pediatricians [1]. The criteria for defining RRI include the absence of any underlying pathological condition (primary or secondary immunodeficiency, cystic fibrosis, airway malformations, primary ciliary dyskinesia) and the presence of at least one of the following: 1) six or more diseases due to respiratory infections in a year; 2) one or more diseases due to respiratory infections in a month, from October to February; 3) three or more diseases due to lower airway respiratory infections in a year [1]. RRI are the consequence of a greater exposure to infectious agents (particularly with early attendance at day nurseries) associated with exposure to environmental risk factors in the first years of life, when the immune system is still relatively immature. RRI have a significant economic and social impact. In addition, recent evidence has implicated common viral respiratory infections in childhood in the pathogenesis of asthma and chronic obstructive pulmonary disease in adulthood [2]. A better characterization of children with RRI is crucial to the identification of effective personalized prevention strategies and new therapeutic approaches.

Immunostimulants are used for the prevention of RRI [3,4], and they have also been suggested in the National Heart, Lung, and Blood Institute roadmap as a topic of future research focusing on the primary prevention of chronic lung diseases [5]. Pidotimod (3-L-pyroglutamyl-L-thiaziolidine-4carboxylic acid) is a synthetic dipeptide molecule with immunomodulatory properties. Animal and human studies have shown that it takes effect on both innate and adaptive immune responses [6,7], it has a preventive action against respiratory infections, and it helps to combat pneumonia and improve vaccine response [8–11]. However further studies are still needed to confirm its efficacy and better elucidate its mechanisms of action.

Metabolomics is one of the core disciplines of systems biology, a high-dimensional biological method that allows for hypothesis-free biomarker profiling (instead of the traditional hypothesis-driven approach). The metabolomic approach can simultaneously consider a large number of metabolites in a given sample, and – with the aid of bioinformatic tools – it generates metabolite profiles that are capable of discriminating between different groups of individuals [12,13], providing a snapshot of all the biochemical processes underway in a given biological system. Metabolomics may reveal new or unexpected metabolites, and may lead to the characterization of hitherto unknown metabolic pathways, prompting the formulation of new pathogenetic hypotheses and enabling new treatment targets to be identified [14].

Considering the burden and complexity of RRI and the comprehensive picture that metabolomics might offer, we used this approach to investigate the metabolic profile of a group of children with RRI.

In detail, our aim was to apply the mass-spectrometry-based metabolomic approach to analyzing urine samples from children with RRI, in order to characterize their biochemical metabolic profiles by comparison with those of their healthy peers. This was done before and after the children with RRI received treatment with pidotimod for 3 months to see whether this treatment was associated with any biochemical changes in their metabolic profile.

Table 1
Clinical and demographic characteristics of children included in the study: age and body mass index (BMI) were reported as mean with minimum and maximum values.

	RRI group	Control group
Number of subjects	13	15
Sex	F:M 6:7	F:M 11:4
Mean age (range)	4,6 (3,2–5,9)	5,3 (3,1–7,3)
Mean BMI (range)	17 (14–20)	16 (14–19)
Positive skin prick tests	3/13 23%	0/15
Wheezing	8/13 61%	0/15

2. Materials and methods

2.1. Study design

This pilot study was conducted at the Department of Women's and Children's Health of the University of Padova (Italy). We enrolled 13 Italian Caucasian children with RRI between 3 and 6 years old [1]. Table 1 shows the children's demographic and clinical characteristics. Children with chronic respiratory diseases or demonstrated immunodeficiency were ruled out. The sample of children with RRI was compared with 15 healthy children of comparable age with no history of respiratory problems or allergies.

A second urine sample was obtained from the children with RRI 1 month after they had completed a 3-month period of treatment with the immunostimulant pidotimod (400 mg twice a day, for 10 days a month) and their metabolomic profiles after pidotimod treatment were compared with those of the healthy controls. For all the considered subjects urine samples were collected at least a week before/after any episodes of acute respiratory infections or an antibiotic treatment to avoid any bias. Enrolled children did not regularly assume probiotics and they were not fasting at the time of urine collection. Personal history was detailed collected for every patient and none of the children enrolled followed special diets or an elimination diet. We therefore assumed that children considered in the study had common diet habits and common lifestyles according to their age and living country.

All the parents gave their informed consent to the children's participation in the study, which was approved by the local Ethical Committee.

2.2. Sample preparation and metabolomic analysis

All urine samples were collected in plastic bottles previously washed with methanol, and were stored at −80 °C until use to limit the metabolite degradation. The urine samples were analyzed at the mass spectrometry and metabolomics laboratory of the Department of Women's and Children's Health at the University of Padova. After thawing at room temperature, the urine samples were centrifuged at 6000 *g* for 10 min to remove any possible cellular debris and insoluble sediments following the recommended protocols for metabolomic analysis of urine. Then 0.2 mL of each sample were diluted with 0.8 mL of a 0.1% formic acid solution (H_2O-0.1% FA), obtaining a 1:5 dilution, and transferred to a vial for injection. A quality control (QC) sample and a test mix sample were prepared for the assessment in parallel of mass accuracy, retention time deviation, and intensity along the run.

To prepare the test mix, nine compounds (sulfaguanidine, acetaminophen, hippuric acid, caffeine, leucine-enkephalin, sulfadimethoxine, verapamil, terfenadine and cholic acid) of known mass-to-charge ratio and retention time under our analytical conditions were dissolved in H_2O-0.1% FA and transferred to a vial for injection. The QC sample was prepared by mixing together 50 μL of each urine specimen and diluting the mix with the appropriate