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Effects of pidotimod and bifidobacteria mixture on clinical symptoms and urinary metabolomic profile of children with recurrent respiratory infections: a randomized placebo-controlled trial

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ABSTRACT

Background: Many preschool children develop recurrent respiratory tract infections (RRI). Strategies to prevent RRI include the use of immunomodulators as pidotimod or probiotics, but there is limited evidence of their efficacy on clinical features or on urine metabolic profile.
Objective: To evaluate whether pidotimod and/or bifidobacteria can reduce RRI morbidity and influence the urine metabolic profile in preschool children.
Materials and methods: Children aged 3–6 years with RRI were enrolled in a four-arm, exploratory, prospective, randomized, double-blinded, placebo-controlled trial. Patients were randomly assigned to receive pidotimod *plus* bifidobacteria, pidotimod *plus* placebo, bifidobacteria *plus* placebo or double placebo for the first 10 days of each month over 4 consecutive months. Respiratory symptoms and infections were recorded with a daily diary by parents during the study. Metabolomic analyses on urine samples collected before and after treatment were performed.
Results: Compared to placebo, children receiving pidotimod, alone or with bifidobacteria, had more symptom-free days (69 *versus* 44, $p = 0.003$; and 65 *versus* 44, $p = 0.02$, respectively) and a lower percentage of days with common cold (17% *versus* 37%, $p = 0.005$; and 15% *versus* 37%, $p = 0.004$, respectively). The metabolomic analysis showed that children treated with Pidotimod (alone or in combination with bifidobacteria) present, respect to children treated with placebo, a biochemical profile characterized by compounds related to the pathway of steroids hormones, hippuric acid and tryptophan. No significant difference in the metabolic profile was found between children receiving bifidobacteria alone and controls.
Conclusions: Preschool children with RRI treated with pidotimod have better clinical outcomes and a different urine metabolomic profile than subjects receiving placebo. Further investigations are needed to clarify the connection between pidotimod and gut microbiome.

1. Introduction

Recurrent respiratory infections (RRI) represent a widespread condition that largely contributes to pediatric morbidity and has also considerable economic and social impact [1]. In patients with RRI antibiotics may be overused, and increased bacterial resistance has

Abbreviations: RRI, Recurrent respiratory tract infections; RCTs, Randomized controlled trials; FA, Formic acid; UPLC, Ultra-performance liquid chromatography; MS, Mass spectrometry; QC, Quality control samples; POS, Positive-ionization mode; NEG, Negative-ionization mode; PCA, Principal component analysis; ptPLS2-DA, Post-transformation of projection to latent structures discriminant analysis; AUC, Area under the receiver operating characteristic curve; SUS-plot, Shared and unique structure plot

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become an important matter of concern worldwide [2]. Interestingly, RRI in the first years of life is a possible co-factor in the development of chronic lung disorders, namely asthma and chronic obstructive pulmonary disease [3]. In view of the early impact of RRI on human health and of its social burden, limiting the condition in the pediatric population shows remarkable promise to prevent chronic lung diseases in adulthood.

Pidotimod is an immunostimulant with proved clinical efficacy in RRI prevention both in open [4–7] or randomized controlled trials (RCTs) [8–10], albeit the mechanism of action has been elucidated only in part [11–13]. A recent study of children with RRI demonstrated that pidotimod can partially "restore" the altered metabolic profile found in these children, even though some metabolites originating from the microbiota were still partially altered after treatment [14]. Based upon these findings, we hypothesized a potential interactive effect of immunostimulants and probiotics on preventing RRI in children and also wondered if they had any influence on the metabolomic profiling. Also probiotics were suggested to have a preventive effect on respiratory infections, but evidences are quite limited [15]. Hence, we conducted an exploratory prospective RCT to determine if the treatment with pidotimod and/or bifidobacteria can reduce the morbidity of RRI and modify the urine metabolomic profile of preschool children with RRI.

2. Methods

2.1. Study design and participants

The exploratory study, designed as a four-arm, prospective, randomized, double-blinded, placebo-controlled clinical trial, was conducted at the Department of Translational Medical Sciences, Section of Pediatrics, Federico II University, Naples, Italy. We recruited all 3-to-6 year-old children with RRI consecutively seen at the Pediatric Pulmonology Unit, Federico II University, and at the office of 22 primary care pediatricians uniformly distributed across the urban and suburban areas of the city of Naples. Metabolomic analysis was performed at the Department of Women's and Children's Health, Mass Spectrometry Laboratory, Fondazione Istituto di Ricerca Pediatrica Città della Speranza, University of Padua, Italy.

Inclusion criteria were: (a) age 3–6 years; (b) attendance to nursery school/kindergarten; (c) diagnosis of RRI [16]. Exclusion criteria were: (a) not meeting inclusion criteria; (b) presence of chronic medical conditions, including cardiovascular or any systemic disease, neurological disorders, primary or secondary immunodeficiency, cystic fibrosis, or primary ciliary dyskinesia; (c) Down syndrome; (d) airways malformation; (e) recurrent wheezing [17]; (f) administration of immunomodulators or systemic steroids in the previous 4 weeks; (g) current acute respiratory and/or any other infection requiring hospital admission.

We generated a randomization list and subsequently balanced between treatments. Patients were randomly assigned to one of the 4 arms to receive active medications or placebos as follows:

- group A (pidotimod + bifidobacteria group): pidotimod as liquid suspension in 400 mg vial (one vial/day) + bifidobacteria mixture (B longum BB536, 3×10^9 CFU; B infantis M-63, $1x10^9$ 105 CFU; B breve M-16 V, $1x10^9$ 106 CFU) as powder in 3 g sachet (one sachet/day);
- group B (pidotimod group): pidotimod as liquid suspension in 400 mg vial (one vial/day) + identical-looking and -tasting placebo of bifidobacteria mixture sachet (one sachet/day);
- group C (bifidobacteria group): identical-looking and -tasting pidotimod placebo as liquid suspension in a vial (one vial/day) + bifidobacteria mixture (B longum BB536, 3×10^9 CFU; B infantis M-63, $1x10^9$ 105 CFU; B breve M-16 V, $1x10^9$ 106 CFU) as powder in 3 g sachet (one sachet/day);
- group D (placebo group): identical-looking and -tasting pidotimod placebo as liquid suspension in a vial (one vial/day) + identical-looking and -tasting placebo of bifidobacteria mixture sachet (one sachet/day).

The study protocol required that patients received oral active medications and/or placebos for the first 10 days of each month for 4 months, and were subsequently followed-up for an additional period of 2 months. The compounds were provided in identical vials and sachets, and the placebo and active drugs did not differ in smell or color. Neither study personnel nor parents were aware of the nature of the product.

The study had primary and secondary endpoints. The primary clinical endpoint included the number of symptom-free days and the number of days with common cold per participant. The secondary endpoint was to determine any change in the urine metabolic profile before and after treatment.

This RCT was conducted during 2 autumn seasons, over the same three-months periods in 2 consecutive years. In the first study period (October, November, December 2015), we enrolled patients with the aim of seeking out any clinically evident effect of the treatment with pidotimod and/or bifidobacteria. In the second study period (October, November, December 2016), we enrolled another cohort of patients who satisfied the same study inclusion criteria with the dual aim of evaluating the clinical effects of the treatments and of characterizing the metabolic profiles of patients' urine samples by mass-spectrometry-based metabolomics, in order to fulfill the secondary aim of the study, i.e. to determine whether there is any difference in the urine metabolic profile before and after treatment among the 4 groups. The study started on October 2015 and the follow-up of the last child was completed in May 2017. The schedule of the 4 visits (at the recruitment and after 8, 16, and 24 weeks) and data collection points are summarized in Table 1. The daily diary included questions about the occurrence of body temperature superior to 37 °C, cough, sore throat, common cold, ear pain, hoarseness, and/or a physician-made diagnosis of tracheobronchitis or pneumonia. Any additional therapy was allowed with the exclusion of immunomodulators, probiotics and systemic steroids. Over the entire study, patients were monitored by telephone calls every month to remind the study procedure to the parents and monitor participants' adherence to the protocol. Personal history was 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.

All study procedures were performed in accordance with the declaration of Helsinki and approved by the Ethical Committee, Federico II University, Naples (protocol no. 173/2015). Study participants and parents were informed about the study procedure in detail and written informed consent was obtained.

Table 1
Summary of visits and measurements for the trial.

	Visit 1	Visit 2	Visit 3	Visit 4
	T_0	8 weeks after	16 weeks after	24 weeks after
Written informed consent	✓			
Demographics	✓			
Inclusion/exclusion criteria verification	✓			
Randomization	✓			
Medications/placebos delivery/collection	✓	✓	✓	
Adverse events registration		✓	✓	✓
Daily diary delivery/collection/supervision	✓	✓	✓	✓
Urine for metabolomics analysis	✓		✓	

2.2. Untargeted metabolomics analysis

In the second study period (October, November, December 2016), all children who had been recruited underwent the same clinical protocol of the first study period and, in addition, were asked to collect at least 6 mL of urine for the metabolomic analysis at visit 1, before any active drug or placebo administration, and at visit 3 (4 months after enrollment), respectively. The urine samples were immediately stored at −80 °C until metabolomic analysis was performed. The urine samples were thawed at room temperature, stirred for 30 s in a vortex mixer, and then centrifuged at 6000 g for 10 min to remove the sediment present in the urine. We transferred 100 µL of the supernatant into a test tube and added 400 µl of H_2O containing 0.1% formic acid (FA) to obtain a 1:5 dilution. Each diluted sample was transferred into a glass vial, placed in the autosampler and kept at 5 °C.

2.3. Ultra-performance liquid chromatography (UPLC)-Mass spectrometry (MS) analysis

All urine samples were analyzed using a Waters Acquity UPLC system coupled to a Waters Q-TOF Synapt G2 mass spectrometer (Waters Corp., Milford, MA, USA). We injected 5 µL of each diluted sample into a Waters Acquity HSS T3 2.1 × 100 mm column packed with 1.7 µm beads kept at 50 °C. The mobile phase for elution was composed of solvent A (H_2O, 0.1% FA) and solvent B [methanol/acetonitrile (MeOH/CH3CN) 90:10/0.1% FA, v/v]. The gradient elution started with 5% B isocratically for 1 min, followed by a linear gradient to 30% B in 2.5 min, then to 95% B in other 2.5 min. The composition was kept at 95% B for 2 min to clean the column and then changed to 5% B to equilibrate to the initial conditions for 3 min, for a total run time of 11 min. The flow rate was 500 µL/min. The electrospray source of Q-TOF was operated in positive (ESI+) and negative (ESI-) ionization mode with a capillary voltage set at 3 kV and 1.5 kV respectively. Data were collected in continuum mode, with a mass scan range of 20–1200 *m/z*, a resolution of 20.000. A leucine-enkephalin solution was used as lock-mass. All UPLC–ESI-TOF-MS operations were controlled with MassLynx 4.1 (Waters, Milford, MA, USA).

In order to assess the reproducibility and accuracy during the analysis and to evaluate the metabolite content of the samples, Quality Control samples (QC) and Standards Solution Samples (Mix) were used. The QCs were prepared mixing together an aliquot of each sample and then diluting the mixture to 1:2, 1:3 and 1:5 (H_2O, 0.1% FA), obtaining three type of QCs. The standards solution consisted of a mix of nine compounds, whereof the exact mass and retention time are known.

The QCs and Mix samples were injected at regular intervals during the sequence, together with blank samples (H2O, 0.1% FA), to determine specific ions from the mobile phase and to find out any contaminants. The analysis was performed in triplicate. The samples were injected randomly to prevent any spurious classification deriving from the samples position in the sequence.

2.4. Data pre-processing and pre-treatment

UPLC-MS data were processed by the software Progenesis (Waters) and two data sets were generated, one for the positive-ionization mode (POS data set) and the other for the negative-ionization mode (NEG data set). The parameters used for data extraction were optimized through the preliminary analysis processing of the QC samples. As a result, the so called Rt_mass variables (where Rt is the retention time and mass is the mass to charge ratio *m/z* of the chemical compound) were generated.

Variables with a coefficient of variation in the QCs greater than 15% or a ratio of the 5th percentile measured in the QCs and the 95th percentile measured in the blank samples less than 5 were excluded. Missing data were imputed by generating a random number between zero and the minimum value measured for the variable. For each type of QCs, linear regression models were generated to estimate the variable level as a function of the run order. Then, the level of each variable in the samples was calculated regressing the intensity of the variable obtained by data extraction on the linear model built using the level of the variable in the three QCs estimated at the same run order of the sample as response and the dilution factors as independent variables.

After probabilistic quotient normalization [18], median was applied to each variable of the triplicates. The differences between the urine metabolite content after 16 weeks of treatment (Visit 3) and at the baseline (Visit 1) were used to obtained the sample representation useful for data analysis.

2.5. Data analysis

In the analysis of the primary outcome, homogeneity of baseline values was carried out by using appropriate statistical test (ANOVA or Chi Square test). Inferential statistics to compare treatment groups was performed using ANOVA followed by post–hoc Dunnett's test multiple comparison *versus* placebo. Significance level was set to $\alpha = 0.05$.

In the analysis of the secondary outcome, multivariate data analysis based on projection methods and univariate data analysis were applied to investigate the differences in the metabolomic profile of the 4 groups of interest. Specifically, the group D (i.e. placebo) was considered as a control group and the other three groups were independently compared to it. Exploratory data analysis was performed by Principal Component Analysis (PCA), whereas post-transformation of Projection to Latent Structures Discriminant Analysis (ptPLS2-DA) [19] was applied to evaluate if differences exist between the group under investigation and group D. The predictive performance of the ptPLS2-DA models was estimated by means of the Area Under the Receiver Operating Characteristic curve (AUC) of the outcomes of the predictions during 5-fold cross-validation (i.e. AUC_{CV}). Permutation test on the group response was performed to avoid over-fitting. Since multivariate data analysis explores the correlation structure of the collected data, while univariate data analysis investigates the properties of single variables, we performed also univariate data analysis by *t*-test and ROC curve analysis with False Discovery Rate in order to complete the results of the multivariate data analysis. We selected the variables with q-value less than 0.05 for both *t*-test and AUC. We performed PCA, ptPLS2-DA, *t*-test and ROC analysis with False Discovery Rate by the R 3.3.2 platform (R Foundation for Statistical Computing).

The main available metabolomic databases (Human Metabolome DataBase and METLIN) were searched to annotate the selected variables characterizing each group. We considered a mass tolerance of 10 ppm.

3. Results

3.1. Clinical results

A total of 55 children were enrolled (30 in the first and 25 in the second study period), were randomized to the 4 groups, and completed the study (Table 2).

No significant differences in age, gender, body weight, height, and number of upper or lower respiratory tract infections in the previous year were found among the groups. Twenty-five out of 55 children underwent the metabolomic analysis of urine samples. When compared to the data from the 55 patients, no significant differences in gender, age, weight and height were found in this subgroup (Table S1 in Supplementary material). In Table 3 we report the results of the whole 6-month study period for the 55 enrolled children.

Compared to group D ("placebo group"), groups A ("pidotimod + bifidobacteria group") and B ("pidotimod group") showed a significantly higher proportion of symptom-free days (69 *versus* 44, $p = 0.003$; and 65 *versus* 44, $p = 0.02$, respectively) and a lower percentage of days with common cold (17% *versus* 37%, $p = 0.005$; and

Table 2
Characteristics of the 55 children with recurrent respiratory infections.

	Group A (Pidotimod + bifidobacteria) (n = 13)	Group B (Pidotimod + placebo of bifidobacteria) (n = 13)	Group C (Placebo of pidotimod + bifidobacteria) (n = 13)	Group D (Double placebo) (n = 16)
Males/Females	9/4	11/2	6/7	8/8
Age at the study (yrs)	4 (3-6)	3.5 (3-6)	3.5 (3-6)	4.2 (3-6)
Body weight at the study (Kg)	18.0 ± 1.0	18.2 ± 1.0	18.0 ± 0.7	20.5 ± 1.6
Height at the study (cm)	106.8 ± 1.3	106.5 ± 2.2	106.2 ± 2.0	110.2 ± 2.8
URTI in the previous year	6.8 (1-13)	5.9 (1-8)	6.9 (5-9)	7.5 (5-12)
LRTI in the previous year	1.4 (0-9)	1.5 (0-6)	0.6 (0-4)	0.6 (0-3)

Values are expressed as mean and range or standard error of the mean.
Abbreviations: URTI, upper respiratory tract infections; LRTI, lower respiratory tract infections.

15% *versus* 37%, $p = 0.004$, respectively). No differences in treatment adherence were found among the groups. All treatments were well tolerated and no adverse events associated to treatments were reported in any patient. No significant differences in the proportion of children treated with antibiotics and in the number of days of therapy were found among the groups. Similar clinical results were found in the subset of 25 children enrolled in the second study period (Table S2 in Supplementary material).

3.2. Metabolomic analysis

The urine metabolomic analysis was performed in 25 patients enrolled in the second study period. The UPLC-MS analysis of the urine samples led to the generation of two data sets: the POS data set, obtained in positive ionization mode including 1329 Rt_mass variables, and the NEG data set, in negative ionization mode comprising 1346 Rt_mass variables. A preliminary exploratory data analysis was performed by PCA on each data set to identify outliers and specific patterns in the data collected. No outliers were detected on the basis of the DModX test and Hotelling's T2 test ($\alpha = 0.05$) performed on the PCA models of each group. Moreover, no differences in the metabolic profile were observed among the 4 groups at the baseline.

3.2.1. Group D ("placebo group") versus group C ("bifidobacteria group")

No significant differences were highlighted by multi- and univariate data analysis for both the NEG data set and the POS data set. Specifically, ptPLS2-DA models did not pass the permutation test on the group response and q-values resulted to be greater than 0.20 for all the variables.

3.2.2. Group D ("placebo group") versus group B ("pidotimod group")

Significant differences between group D and group B were detected by multi- and univariate data analysis. ptPLS2-DA models (mean centering and Pareto scaling, 1 predictive latent variables) showed $AUC_{CV} = 0.85$ (p-value = 0.041) for the POS data set and $AUC_{CV} = 0.81$ (p-value = 0.049) for the NEG data set. The score bar plot of the model for the POS data set is reported in Fig. 1.

A similar plot was obtained for the NEG data set (data not shown). We selected 384 variables by univariate data analysis, 357 from the POS data set and 27 from the NEG data set. Variable annotation is reported in Table S3 in Supplementary material.

3.2.3. Group D ("placebo group") versus group A ("pidotimod + bifidobacteria group")

Significant differences between group D and group A were highlighted by multi- and univariate data analysis. ptPLS2-DA models (mean centering and Pareto scaling, 1 predictive latent variables) showed $AUC_{CV} = 0.98$ (p-value = 0.016) for the POS data set and $AUC_{CV} = 0.99$ (p-value = 0.002) for the NEG data set. The score bar plot of the model for the POS data set is reported in Fig. 2.

A similar plot was obtained for the NEG data set (data not shown). We selected 647 variables by univariate data analysis, 356 from the POS data set and 291 from the NEG data set. Variable annotation is reported in Table S4 in Supplementary material.

The results of data analysis are summarized in the Shared and Unique Structure plot (SUS-plot) showed in Fig. 3 [20], where the predictive correlation loadings of each variable calculated for the ptPLS2-DA model distinguishing group D and group A and for the model separating group D and group B are reported in the same plot.

Many variables are close to the diagonal of the plot, and therefore it can be assumed that the variables distinguishing group D from group A likely separate also group D from group B ("shared variables"), this suggesting that the effect might be attributed to the presence of pidotimod. Among them, we found variables that could be ascribable to some metabolites of steroid hormones or those deriving from the vitamin B pathway, and some amino acid derivatives. On the other hand,

Table 3
Clinical outcomes in the 55 children with recurrent respiratory infections.

	Group A (Pidotimod + bifidobacteria) (n = 13)	Group B (Pidotimod + placebo of bifidobacteria) (n = 13)	Group C (Placebo of pidotimod + bifidobacteria) (n = 13)	Group D (Double placebo) (n = 16)
Number of URTI	39	53	64	58
Number of LRTI	2	4	1	6
Number of days with URTI	21 (0-66)[a]	25 (0-72)[a]	37 (7-72)[a]	32 (0-82)[a]
Number of days with LRTI	7 (7-7)[a]	5 (2-7)[a]	10 (10-10)[a]	10 (4-17)[a]
Symptom-free days (%)	65[b]	69[c]	59	44
Days with common cold (%)	15[d]	17[e]	26	37
Days with fever (%)	1	4	4	3
Days with cough (%)	21	18	27	32
Days with sore throat (%)	8	3	3	10
Days with hoarseness (%)	2	1	2	6
Days with ear pain (%)	1	1	1	1
Workdays lost by the parents (%)	2	7	5	14

Abbreviations: URTI, upper respiratory tract infections; LRTI, lower respiratory tract infections.
[a] Values are expressed as mean (range).
[b] $p = 0.02$ *versus* Group D.
[c] $p = 0.003$ *versus* Group D.
[d] $p = 0.004$ *versus* Group D.
[e] $p = 0.005$ *versus* Group D.

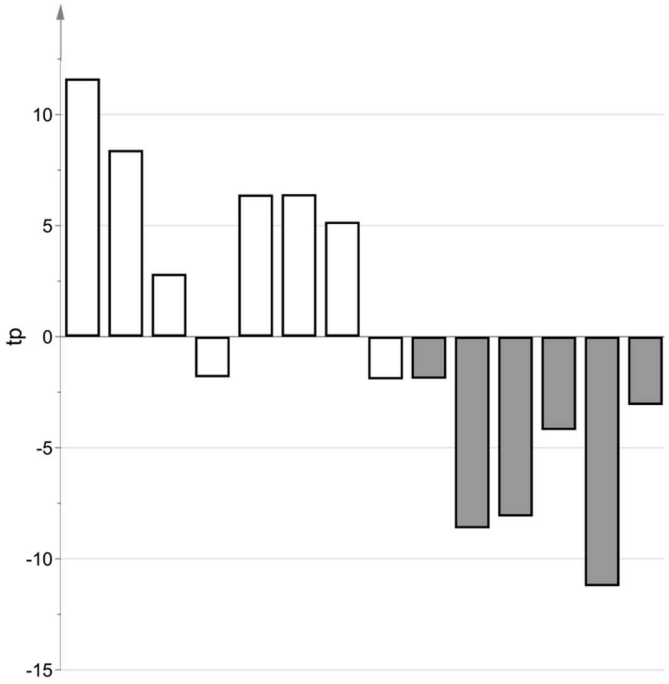

Fig. 1. Group D ("placebo group") *versus* group B ("pidotimod group"): score bar plot of the ptPLS2-DA model for the POS data set. White bars indicate children from group D, grey bars refer to group B children. tp is the predictive score of the model.

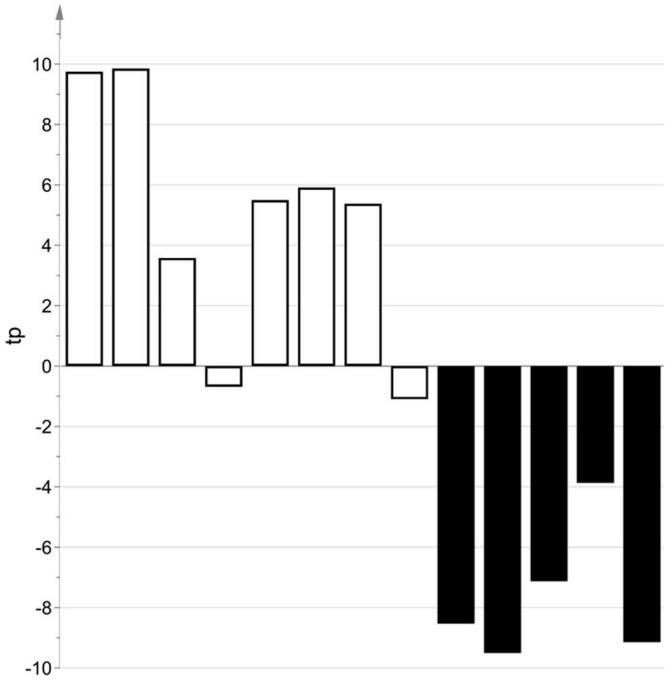

Fig. 2. Group D ("placebo group") *versus* group A ("pidotimod + bifidobacteria group"): score bar plot of the ptPLS2-DA model for the POS data set. White bars indicate children from group D, black bars refer to children from group A. tp is the predictive score of the model.

some variables lie on the extreme regions of the horizontal/vertical axis (close to −0.8 or 0.8) suggesting that some metabolites can be related to interactive effects of pidotimod and bifidobacteria. Among them, we annotated variables that could be ascribable to a derivative of hippuric acid (Methylhippuric acid) and to tryptophan metabolites (L-Kynurenine), to deoxycholic acid 3-glucuronide (a metabolite of bile acid), to oxoglutaric acid, to metabolites of tryptamine (trace amines) and to some metabolites belonging to the ubiquinone family.

4. Discussion

This RCT enrolled preschool children who attended nursery school/kindergarten and had RRI, a condition representing an early life troublesome event typically cared for in primary care settings. We demonstrated that pidotimod, alone or combined with bifidobacteria, is effective in significantly decreasing the days of illness along with reducing symptoms due to common cold, while there were no significant differences when patients treated with bifidobacteria alone were compared to the placebo group.

In the last decades several studies on the immunostimulant pidotimod have been published to prove its clinical efficacy in pediatric RRI [4–6,8,9,21], but only few of these were RCTs. Interestingly, one of the first document reviewing the pediatric literature encouraged national health authorities to conduct large, multicentre, double-blind, placebo-controlled trials on the effects of pidotimod [22]. A recent clinical RCT found that 3-year-old children receiving pidotimod had less than 44%

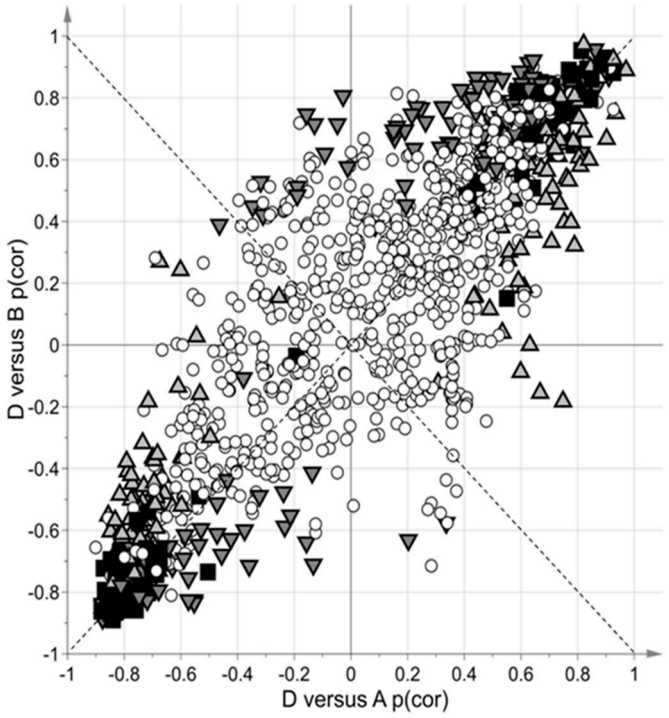

Fig. 3. SUS-plot for the POS data set: variables distinguishing group D and group B are reported as grey inverted triangle, variables separating group D and group A as light grey triangles, whereas black boxes indicate variables distinguishing both group D from group A and group D from group B. Open circles indicate variables that did not result significant in the analysis.

of relative prescription of antibiotics when compared to the placebo group [10]. Two additional studies have shown that, when administered together with standard antibiotic treatment, pidotimod is associated with a favorable persistent immunomodulatory effect in children or adults with pneumonia, suggesting a decrease in the risk of early recurrences of the episodes [7,23]. Finally, a recent meta-analysis of trials conducted in children with RRI has demonstrated that the treatment with pidotimod results in a significant decrease in the duration of cough and fever without increasing the risk of adverse events of any cause [24]. The findings from the current RCT confirm and extend these results, highlighting that pidotimod significantly improves the clinical outcome of children with RRI.

A major novel finding from the present study is provided by the metabolomic analysis. Metabolomics is a high-dimensional biological method that can be used for hypothesis-free biomarker discovery, examining a large number of metabolites in a given sample using spectroscopic techniques [25]. Statistical data analysis then enables us to infer which metabolic information is relevant to the biological characterization of a given condition. This can lead to the discovery of new metabolites, and hitherto unknown metabolic pathways, enabling the formulation of new pathogenetic hypotheses and novel therapeutic targets [26]. The metabolomic analysis has been largely applied in the field of pediatric infectious disorders [27–31]. For the first time, we demonstrated that children with RRI receiving pidotimod have a metabolomic profile of urine significantly different from the placebo group. These differences were observed regardless children were taking pidotimod alone (group B) or in combination with bifidobacteria (group A), and concern steroid hormones, metabolites of vitamin B metabolism and amino acid derivatives. Steroid hormones exert immuno-regulatory effects both *in vivo* and *in vitro* and could represent the activation of the hypothalamus-pituitary-adrenal axis in the interaction between neuroendocrine and immune systems [32]. Vitamin B mostly derives from diet, but also bacteria can synthesize it, and recent studies have demonstrated that human immune system can use vitamin B as a point of difference to recognize infection through mucosal-associated invariant T cells in mucosal surfaces (intestine, mouth, and lungs) [33,34]. From a clinical point of view, no differences were found between children treated with pidotimod alone and children taking pidotimod *plus* bifidobacteria mixture, and these two groups seem to behave in the same way. However, the metabolomic analysis revealed a different biochemical behavior. The interaction between bifidobacteria and pidotimod produces different metabolic changes related to a number of products such as the bile acid metabolites, a derivative of hippuric acid, the tryptophan metabolites, the oxoglutaric acid, the metabolite of tryptamine and the metabolites belonging to the ubiquinone family. Actually, hippuric acid and bile acid metabolites are likely associated with the microbiota composition and emerged as discriminant metabolites in a recent trial of children with RRI receiving pidotimod compared to healthy controls [14]. The findings from that study point toward a role of the microbiota in the immune regulation, even though we do not know how they can precisely interact and/or are influenced by the probiotic supplementation [14].

Current metabolomic results deserve further comments. The tryptophan metabolite L-Kynurenine can derive from the enteric biotransformation and be associated with the microbiota composition, thus confirming the crucial role of tryptophan and its metabolites in the balance between intestinal immune tolerance and gut microbiota maintenance [35,36]. The metabolites of oxoglutaric acid may be related to the microbiota composition as well [37], while metabolites of tryptamine (trace amines) may be associated with microbiome growth. Considering the metabolites belonging to the ubiquinone family in the context of the microbiota, a recent study based on genomic analysis, provided further insight into the quinone biosynthesis by microorganisms from the human gut microbiota [38]. Our findings cannot be attributed to the bifidobacteria effect only because no differences in the metabolomic profile were found between placebo and group C (bifidobacteria group), and thus can be at least partially interpreted as the result of the combined effect of the two agents (pidotimod *plus* bifidobacteria) on the metabolomic arrangement. Gut microbiota helps to protect against pathogenic infections through several mechanisms, including competition, antimicrobial peptide secretion, antibody production, lymphoid tissue development, innate immune cell stimulation, and T cell differentiation [39]. However, there is emerging evidence that the role of gut microbiota on immunity extends beyond the gastrointestinal tract. In particular, it has been demonstrated to support immune functions that are critical for maintaining homeostasis in the respiratory tract against viruses and bacteria [40–45]. These findings point toward a critical role for gut microbes in respiratory host defense, as they provide microbial signals or determinants critical for immune priming and shaping of the response to infections. Hence, it seems reasonable that influencing the microflora may potentially modulate the immune response and eventually improve individuals' immune status. As in our study antibiotic assumption was allowed, if needed, and given that it is well known that antibiotics deeply affect microbiota composition and metabolomic profile, it is theoretically possible that this kind of treatment might have affected our results. However, no significant differences in the proportion of children treated with antibiotics and in the number of days of therapy were found among the groups, which significantly reduces the risk of bias due to this treatment.

Our study has strengths and limitations. The major strength is that this is the first prospective, randomized, double-blinded, placebo-controlled trial evaluating the clinical efficacy as well as the effects on the urine metabolomic profiling of the combined use of an immunostimulant and probiotics for preventing RRI in preschool children. The inclusion criteria we set were very strict, thus avoiding as many confounding factors as possible. Moreover, we administered a mixture rather than a single strain of bifidobacteria. Indeed, multistrain probiotics seem to be more effective than single strains [46]. Whether this

is due to synergistic interactions between strains or a consequence of the higher probiotic dose is at present unclear.

On the other hand, as limitation of the study, we acknowledge that our population size was small. However, the robustness of the discriminant models we found was guaranteed by the conservative thresholds we used to limit false discovery rate (q-value less than 0.05 for both *t*-test and AUC). Moreover, the fact that in this study children treated with bifidobacterium mixture alone did not show variations in the metabolomic profile *versus* the placebo group might be due to the design we adopted, that included a limited period of administration of the active medications or placebos in each group. Indeed, in previous publications the mixture has been continuously administered for 4–6 weeks [47,48], while our population has received all the medicines during the first 10 days of each month for 4 months in order to avoid loss of compliance due to different administration schedule among treatments. Thus, we cannot exclude that a prolonged treatment with probiotics might result in significant changes in the clinical outcomes and metabolomic findings associated with pediatric RRI. Finally, according to this study design, we did not enroll a group of healthy children in order to assess whether and to what extent the combined treatment and the monotherapies can "restore", at least in part, the metabolic profiles found in our patients with RRI. Future RCTs on populations receiving a more prolonged probiotic treatment and including also healthy controls are warranted to further understand the immunomodulatory effects of pidotimod and bifidobacteria in terms of RRI prevention.

In conclusion, the novel finding from this study is that children with RRI treated with pidotimod have better clinical outcomes and a different urine metabolomic profiling after treatment compared to subjects receiving placebo, while patients treated only with bifidobacteria did not show any difference in clinical outcomes and metabolomic profile in comparison to the placebo group. Although group A ("pidotimod + bifidobacteria group") and B ("pidotimod group") did not differ from the clinical point of view, the metabolomic analysis revealed a different behavior that went beyond the clinical effects. Some of the annotated metabolites suggest that a possible interaction between gut microbiota and pidotimod could explain the role of the immunostimulant in preventing RRI. Future studies will be designed to investigate that point.

Conflicts of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

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