UNIVERSITA' DEGLI STUDI DI PADOVA

UNIVERSITÀ
DEGLI STUDI
DI PADOVA

DIPARTIMENTO DELLA SALUTE DELLA DONNA E DEL BAMBINO

DOTTORATO IN MEDICINA DELLO SVILUPPO E SCIENZE DELLA PROGRAMMAZIONE SANITARIA

EFFECTS OF PIDOTIMOD AND BIFIDOBACTERIA MIXTURE ON CLINICAL SYMPTOMS AND METABOLOMIC PROFILE OF CHILDREN WITH RECURRENT RESPIRATORY INFECTIONS: A RANDOMIZED PLACEBO-CONTROLLED TRIAL

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Anno Accademico: 2017/2018

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RIASSUNTO

PRESUPPOSTI DELLO STUDIO: Le infezioni respiratorie ricorrenti (IRR) rappresentano una condizione molto diffusa; contribuiscono in modo sostanziale alla morbilità pediatrica ed hanno un costo economico e sociale elevato. Gli immunostimolanti, come il pidotimod, sono utilizzati per la loro prevenzione. Recenti evidenze suggeriscono che anche i probiotici possano avere un ruolo preventivo nelle IRR.

OBIETTIVO DELLO STUDIO: Valutare se il trattamento con pidotimod e/o bifidobatteri si associ a 1) riduzione della morbilità correlata alle IRR e 2) differenze nel profilo metabolomico urinario pre e post terapia

MATERIALI E METODI: Si tratta di uno studio a 4 braccia, esplorativo, prospettico, randomizzato e controllato, in doppio cieco e versus placebo condotto durante gli stessi 3 mesi autunnali in due anni consecutivi. Sono stati arruolati bambini dai 3 ai 6 anni con diagnosi di IRR che frequentavano la scuola materna e sono stati assegnati in modo randomizzato a ricevere il trattamento attivo (pidotimod e/o bifidobatterio) o il placebo per i primi 10 giorni di ciascun mese per 4 mesi. L'analisi metabolomica sui campioni di urine raccolti prima e dopo il trattamento è stata eseguita mediante spettroscopia di massa accoppiata con cromatografia liquida ad alta performance (UPLC-MS).

RISULTATI: Confrontati con il gruppo placebo, i bambini trattati con pidotimod, con o senza bifidobatteri, presentavano una proporzione di giorni liberi da sintomi significativamente più alta ($p=0.02$ and $p=0.003$, rispettivamente) e una percentuale più bassa di giorni con rinite ($p=0.004$ and $p=0.005$, rispettivamente). Dal punto di vista metabolomico questi bambini presentavano un profilo significativamente diverso rispetto a quelli trattati con placebo. I bambini trattati con solo pidotimod dimostravano un profilo metabolico urinario ancora diverso rispetto a quelli trattati con la combinazione pidotimod e bifidobatterio. Dall'altro lato i bambini trattati con solo bifidobatterio non dimostravano differenze significative se confrontati con il gruppo placebo né per quanto riguarda gli outcome clinici né nel profilo metabolomico.

CONCLUSIONI: lo studio dimostra che i bambini con IRR trattati con pidotimod hanno un outcome clinico migliore e un profilo metabolomico urinario diverso rispetto ai bambini trattati con placebo, mentre i bambini trattati con il solo bifidobatterio non hanno dimostrato differenze né negli outcome clinici né nel profilo metabolomico se confrontati con quelli che hanno assunto placebo. L'associazione del bifidobatterio al pidotimod non modifica l'outcome clinico, ma l'analisi metabolomica è stata in grado di dimostrare, andando oltre la clinica, che questi due gruppi presentano delle differenze a cui la composizione del microbiota intestinale potrebbe contribuire.

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 on the clinical effects of the treatment, alone or combined, as well as on the changes of urine metabolic profile following it.

OBJECTIVES. To investigate whether the treatment with pidotimod and/or bifidobacteria were associated with 1) a reduced morbidity related to RRI and 2) differences in the urine metabolic profile.

MATERIALS AND METHODS: The study is a four-arm, exploratory, prospective, randomized, double-blinded, placebo-controlled clinical trial conducted during 2 autumn seasons, over the same three-months periods in 2 consecutive years. Children aged 3-6 years with RRI who attended the nursery school were enrolled and randomly assigned to one of the 4 arms to receive active medications or placebos for the first 10 days of each month for 4 consecutive months. Metabolomic analyses on urine samples collected before and after treatment were performed using mass spectrometry combined with ultra-performance liquid chromatography (UPLC-MS).

RESULTS: Compared to the placebo group, children receiving pidotimod, alone or combined with bifidobacteria, had a significantly higher proportion of symptom-free days ($p=0.02$ and $p=0.003$, respectively) and a significantly lower percentage of days

with common cold (p=0.004 and p=0.005, respectively). In those children, we also found relevant changes in the urine metabolomic profile compared to children receiving placebo. Furthermore, children treated with pidotimod alone showed a different metabolic profile compared to children treated with pidotimod plus bifidobacteria. On the other side, children receiving bifidobacteria alone did not show differences with respect to the placebo group in clinical outcomes or metabolomic profiles.

CONCLUSIONS: this study shows that children with RRI treated with pidotimod have a better clinical outcome and a different metabolomics profile after treatment, compared to subject 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. The combined treatment (pidotimod plus bifidobacterium) did not modify the clinical outcome, but metabolomic analysis was able to reveal, going beyond the clinic, a different behavior for these two groups, suggesting a possible role for the microbiota composition in the underlying physiopathologic mechanism.

1.INTRODUCTION

1.1 Metabolomics

1.1.1 Global System biology and metabolomics

Studying a biological system, a major challenge of molecular biology is to unravel the organization and interactions of cellular networks that enable complex processes. The underlying complexity of the biological system itself arises from intertwined nonlinear and dynamic interactions among large numbers of cellular constituents[1]. The reductionist approach, is based on the assumption that complex problems are solvable by dividing them into smaller, simpler and thus more tractable units[2]. Through this approach, most of the components of biological systems and many of their links have been identified. However, the pluralism of causes and effects in biological networks can be also addressed by observing multiple components simultaneously. This system-wide perspective has emerged in recent years to move toward the comprehension of the system as a whole, and has been defined as *Systems Biology*[1]. This approach appreciates how a system works altogether, in an holistic view of the biological organism[3]. The strength of *Systems Biology* lies in a comprehensive investigation of the "omic cascade", with a combination of data deriving from different analytical platforms, such as genomics, transcriptomics, proteomics and metabolomics[3]. The term "omics" refers to approaches capable of studying entities in aggregate. *Genomic*s is the science that studies the genome. *Transcriptomics* is the study of gene expression and *transcriptome* describes the full set of mRNAs present in a cell or tissue at any one time. Similarly, the study of protein translation is called *proteomics*[4]. *Metabolomics*, instead, one of the most recent "omic" sciences, has been suggested to provide the most "functional" information of the omics technologies[5]. The

term “metabolome” has been first used by Olivier et al. in 1998 to describe the quantitative complement of all the low molecular weight molecules (< 1kDa) present in cells in a particular physiological or developmental state[5]. The word *Metabolomics* was first coined by Fiehn and later defined as the comprehensive quantitative analysis of all metabolites of an organism or specified biological sample, which represents the multiparametric time-related metabolic responses of a complex system to a pathophysiological intervention or genetic modification[6]. Metabolomics is considered to overcome the limits of other “omic” sciences, as metabolites represent the final “omic” level in a biological system[5,7]. With its non-selective analytical approach, not driven by a-priori hypothesis, metabolomics is the “omic” science closest to phenotype expression [8]. Metabolites reflect the surrounding environment, and the metabolic profile of a biological sample or system derives from the interaction of both genetic and exogenous factors[9]. Metabolomics reflects the holistic vision of systems biology, as it offers a picture of all the biochemical processes ongoing in a complex organism over time and space[10,11] and is emerging as a promising tool in disease diagnosis and prognosis, drug discovery and personalized pharmacology[7].

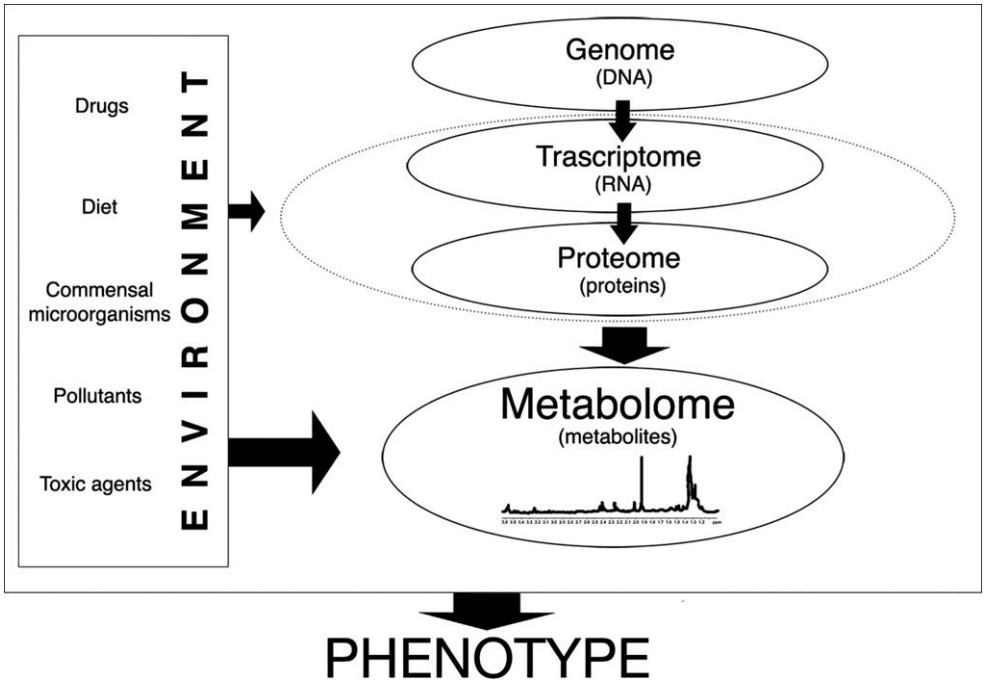

Figure 1.“Omics” sciences and their relationship with environment and phenotype. Carraro et al. (3)

1.1.2 The study of metabolites

The exact number of human metabolites is still not known. Since its first release in 2007, the Human Metabolome Database (HMDB) (the world’s largest and most comprehensive organism-specific metabolomics database) has incremented the number of annotated metabolite entries from 2180 (HMDB version 1.0) to 41828 (HMDB version 3.6) in July 2014 (www.hmdb.ca). The human metabolome is currently estimated to contain many thousands of metabolites, as defined by metabolic reconstruction and HMDB. However, these are under-estimates of the actual number of metabolites expected to be defined in the future[12].

Historically, two complementary approaches allow the metabolite analysis: metabolite profiling and metabolite fingerprinting[5,13].

Metabolite Profiling

Metabolite profiling involves the identification and quantitation of a predefined set of metabolites belonging to a selected metabolic pathway or a class of compounds[5]. *Target analysis* is even a more specific and selective approach, constrained exclusively to the qualitative and quantitative identification of a particular metabolite or metabolites. By their nature, these approaches provide a restrictive non-comprehensive view of the metabolome, as they are hypothesis-driven. As a result, only a very small fraction of the metabolome is focused upon and signals from all other components are ignored[5,13].

Metabolite Fingerprinting

Metabolite fingerprinting aims to analyze patterns or "fingerprints" of metabolites[13]. It can be simultaneously applied to a wide range of metabolites and can rapidly classify numerous samples on the basis of their metabolic characteristics. This truly comprehensive methodology is not driven by research's hypothesis. Unexpected or unknown metabolites may turn out to be important in characterizing specific groups of subjects, and new pathophysiological hypothesis may be formulated[3]. The identification of each observed metabolite is not mandatory and metabolite fingerprinting can potentially be used as a diagnostic tool. Nonetheless, qualitative and quantitative analysis of the discriminant matabolites strengthen the results[13] .At the moment, human metabolome is not completely mapped and known. The metabolome identification process is rapidly expanding; the Human Metabolome Database, the most complete available database, had 6500 metabolites in the 2.0 version of 2009; and more than 40000 in the 3.5 version of 2013; reaching the 74.461 of the last version 3.6 and data are continuously incrementing.

1.1.3 The "Metabolomic Workflow"

The analytical process in the field of metabolomics requires different specific steps. Sample acquisition is primarily driven by the experimental design. After an appropriate sample preparation, two main analytical techniques are used for the detection of a wide range of metabolites in a single measurement: nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). These techniques produce the spectra where the intensities of peaks represent the amount of the corresponding metabolites. Subsequently, multivariate statistical analysis approaches extract the information obtained from the spectra to generate multidimensional plots of metabolic activity. In this way, patterns of metabolites characterizing specific groups of subjects

can be recognized. The characteristics of the emerged discriminant molecules are compared with those of known metabolites through the use of Metabolite databases (HMDB, METLIN and others), to identify potential putative biomarkers with biological significance[13-15]. The final step is represented by the structural identification of the analytes that have emerged as possible biomarkers.

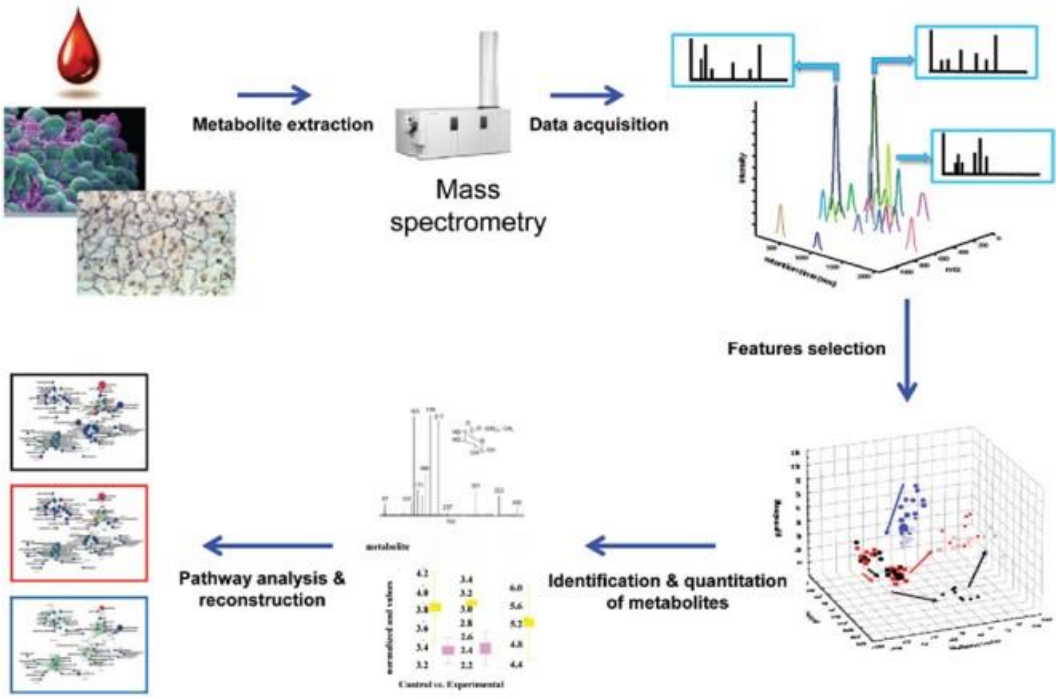

Figure 2. The characteristic "Metabolomic Worflow"

Sample acquisition and preparation

Metabolites can be detected in a wide range of biological matrices, such as plasma, whole blood, saliva, cerebrospinal fluid, urine, amniotic fluid, tissue homogenates and cell pellets. Before any metabolome measurements are taken, it is essential to stop metabolism as quickly as possible, in order to minimize the formation or degradation of metabolites after sampling due to remaining enzymatic activity or oxidation processes. Metabolism can be inhibited by several techniques, such as freezing in liquid nitrogen,

acid treatment or quenching in cold buffered methanol. The subsequent sample preparation is very important for the success of the analytical procedure. Sample preparation typically entails metabolite extraction and enrichment, depletion of proteins and removal of sample matrix, meaning all the matrix components that may interfere with the analysis. In a true metabolomics approach, all small molecules are the targets and only salts and macromolecules (such as proteins and larger peptides) can be considered as matrix. Depending on the matrices phase and on the type of metabolites studied, numerous sample preparation protocols have been developed for the metabolites extraction[13,14,16]. These protocols aim at limiting the loss of metabolites, although any kind of sample preparation will cause a certain degree of analyte loss[13].

Analytical platforms

Nuclear magnetic resonance (NMR) spectroscopy and Mass spectrometry (MS) (often combined with chromatographic separation techniques) are widely used platforms for performing metabolomic analysis and profiling. These two spectroscopic techniques are complementary and play a central part in the "omic" world[3]. Both can be performed on very small quantity of sample[3,16]. The choice of the analytical tool is based on the level of chemical information required about the metabolites[14].

NMR spectroscopy

In NMR spectroscopy, a compound is placed in a magnetic field. Isotopes within the compound absorb the radiation and resonate at a frequency which is dependent on its location in the small molecule[8]. The resultant NMR spectrum is a collection of peaks at different positions and intensities and each compound has a unique pattern[16]. NMR spectroscopy provides detailed information on molecular structures of compounds, both pure and in complex mixture. NMR spectroscopy is particularly useful for studying

metabolite levels in intact tissues (such as biopsy samples), that can thus be used in further experiments.

Mass Spectrometry

Mass spectrometry is a powerful method for identifying and quantifying positively or negatively charged metabolites and it is considered to have high sensitivity (<1nmol/L) compared to ^{1}H-NMR spectroscopy. Combination with a separation technique reduces the complexity of the mass spectra due to metabolite separation in a time dimension, provides isobar separation and delivers additional information on the physical and chemical properties of the metabolites[13]. Biomolecules derived from the sample are separated by liquid chromatography (LC) or gas chromatography (GC), and subsequently ionized. Nowadays, the “soft” ionization techniques are widely used, such as electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI)[17]. The majority of ionized metabolites is singularly charged because of their low molecular weight capable of carrying single charges only[12]. Then the mass analyzer separates the obtained ions according to their mass-to-charge ratios (m/z). Finally, the electric charged molecules are detected in the spectra as peaks proportional to the abundance of each species. In many configurations, additional tandem MS analysis (MS/MS or MS^{n}) are feasible[3,16,17].

The advantages of MS are its speed, high selectivity, and high sensitivity.

Figure 3.High resolution mass spectrometer (Synapt G2, Waters) at the Laboratory of Mass Spectrometry of the Research Tower. Women's and Children's Health Department, University of Padova.

Recent applications of MS in metabolomics are based on instruments like Quadrupole Time-of-Flight (Q-ToF) and Fourier Transform Mass Spectrometry (FT-MS)[3].

Data Mining, Extraction and Analysis

NMR spectroscopy and MS are powerful spectroscopic methods for generating multivariate datasets. Several commercial and free specialized software packages are available to properly interpret the data and convert them into a uniform format. This process includes the reduction of background spectral noise, appropriate peak assignment for the same compound, peak alignment across multiple samples and peak normalization[13,16].

As spectra derived from NMR spectroscopy and MS are highly complex, the biological information they contain can only be extracted using appropriate multivariate statistical approaches, the so-called *pattern recognition* methods. These computer-based procedures can be classified as *unsupervised* or *supervised*[3].

The *unsupervised* methods reduce the complexity of the data contained in the spectra and represent them by means of plots that the human eye can interpret. These methods are useful when prior information about the sample identity is unknown and when the aim of the analysis is sample classification. In fact, this approach enables to identify sample clustering to see whether different groups of subjects can be discriminated by their spectra characteristics[3,13]. An example of these unsupervised methods is Principal Component Analysis (PCA). This method reduces the data dimension and devises the most informative descriptors in the data set. Afterwards, the aim is to verify if these principal components are capable of distinguishing the different classes of samples[18].

On the other hand, when sample identity is known and the aim of the study is to discover characteristic biomarkers, the *supervised* methods are more appropriate. With this approach, a training set of samples of known classification is used to build a mathematical model, which is then evaluated using an independent dataset. In this way, the supervised methods enable to predict which group a new sample belongs to on the basis of its spectra characteristics[3,13]. Partial Least Square Analysis combined with Discriminant Analysis (PLS-DA) is a very popular example of these supervised methods. It allows to find the relationships between different blocks of data using "latent variables", and to calculate a discriminant function for the classes separation[18].

These bioinformatic methods can not only enable the discrimination between groups, but also lead to the identification of the more important metabolites involved in the sample classification.

Identification of Metabolites

The last step of the "Metabolomic Work-flow" is the structural identification of the analytes that have emerged as possible biomarkers. Working with mass spectrometry,

the basic strategy behind the exact compound identification consists of different passages. First, a mass spectrum is recorded at high resolution to define the accurate mass (<5ppm) of the ion of interest and its isotopic pattern. This will lead to a minimum list of possible elemental formula of the compound. An additional step is the ion fragmentation by MS/MS. This technique consists of two mass analyzers put in series in space or in time and separated by a collision cell. This enables either to select a precursor-ion and obtain the resultant product-ions m/z ratios with the second analyzer ("product-ion" scan), or to select the ions that fragment in the collision cell in order to find a predefined product-ion with known m/z ratio ("precursor-ion" scan)[19]. As each compound has its own specific fragmentation pattern, the MS/MS analysis can provide further important information for the structural identification of the molecule in question.

A fundamental support for molecular identification comes from various on-line databases, which are in increasing expansion and carry a wide range of information on the already-known metabolites, such as their physico-chemical properties, their spectroscopic characteristics and the biochemical pathways in which they are involved. *The Human Metabolome Database* is currently the largest and most comprehensive organism-specific metabolomics database. It contains spectroscopic, quantitative, analytic and physiological information about human metabolites, their associated enzymes or transporters, their abundance and their disease-related properties[20]. The latest release of the HMDB now has detailed information on more than 41000 metabolites (www.hmdb.ca). Other important databases are KEGG (http://www.genome.jp/kegg), BioCyc (http://biocyc.org), and METLIN (http://metlin.scripps.edu/). LIPID maps (http://www.lipidmaps.org/data/index.html) provides an useful database to search for lipid metabolites. Once the m/z ratio, the retention time, and the fragmentation pattern of a compound are known, these data can be compared with those of know metabolites in the just-mentioned databases. When the molecule is presumptively identified with this process, the final proof and

confirmation of its identity is a comparison of LC retention time and MS/MS spectrum with that of an authentic standard. However, it is not always possible to identify all metabolites of interest by MS alone, either because the compound has not been characterized in the databases yet, or because of the absence of a reference standard[20].

1.1.4 Metabolomics: clinical applications

Over the last few years, there has been a rapidly growing number of metabolomics applications aimed at finding biomarkers[7]. Biomarkers have been defined by the National Institute of Health as “biological characteristics that are objectively measured and evaluated as indicators of normal biological processes, pathological processes or pharmacologic responses to a therapeutic intervention” (NIH, Biomarkers Definitions Working Group 2001). Sets of metabolic biomarkers, may be appropriate in the future, especially in the cases of multifactorial and complex diseases[21].

From a clinical standpoint, the metabolomic analysis has three major potential applications. First, metabolic profiling of populations could allow the development of “molecular epidemiology”, where the susceptibilities of specific groups to a disease is recognized. In this sense, metabolites could be identified as risk biomarkers of diseases, with implications for health screening programmes[15]. The second application concerns the early diagnosis and characterization of disease phenotypes[22]. The third potential clinical application consists in the identification of individual metabolomic characteristics able to predict drug effectiveness and/or toxicity, referred to as pharmacometabolomics [22].

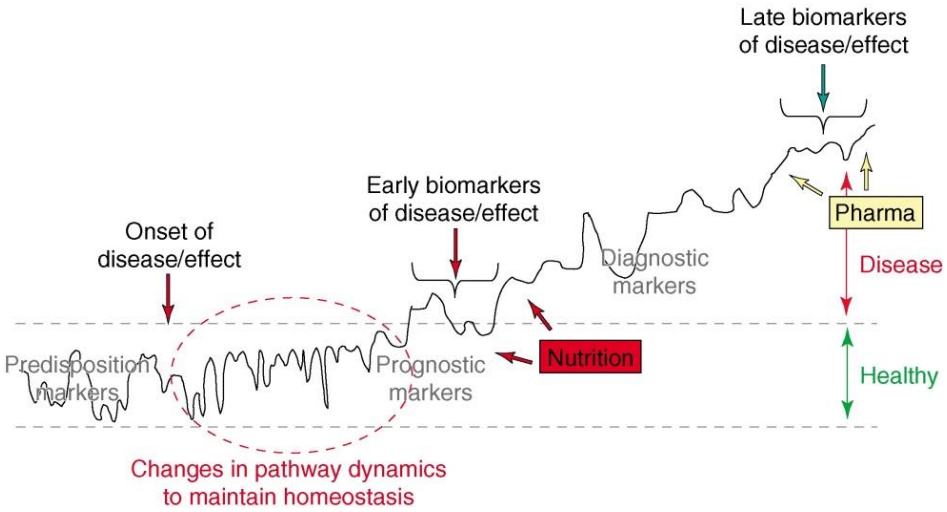

Figure 4.The development of disease from healthy (homeostasis within black dotted lines) to sub-optimal health and eventually an overt disease state. Biomarker patterns (for graphical reasons represented as a single line) are essential to describe the changes from normality to dysfunction. Van der Greef et al. (23)

In respect to the systems biology perspective, the results in metabolomics should be integrated with those obtained from the other omics platforms in a multi-omic approach to achieve a holistic picture of a given disease[24]. This approach correlates well also with the essence of the emerging P4 medicine, which consists of predictive, preventive, personalized and participatory medicine using the enormous amount of data from the omic sciences to focus on the individual[25]. The clinician will then appear as the connector in the omics-era: making true the 4Ps of precision medicine.

Metabolomics: Pediatric clinical studies

Metabolomic analysis can be applied to the study of biological fluids collected in non-invasive (urine, exhaled breath condensate) or minimally-invasive ways (blood). What is more, NMR and MS techniques can be performed on very small quantity of sample. These characteristics make the metabolomic approach suitable and particularly

promising in the field of pediatric medicine[3,22]. A number of recently-published studies have applied the metabolomic approach to the pediatric population.

Some of these studies evaluated how physiological variables can affect children's metabolic profiles[22]. Metabolomic analysis was able to differentiate urinary metabolic profiles of neonates with different gestational ages, suggesting that the neonate's metabolic status at birth is strictly dependent on the time of delivery[26]. In the same way, age seems to have an effect on the urinary metabolite profile in children[27]. Diet is another important factor that may affect metabolic composition of biofluids, in particular urine[28]. Finally, gender too has been shown to influence the metabolic plasma composition[29].

All these studies prove the potential of metabolomic technologies in detecting specific patterns of biomarkers of physiological states. However, it is especially in the understanding of pathological mechanisms that metabolomics approaches have been applied recently, with an increasing number of publications in the last few years. Here we will report the most significant.

Metabolic biomarkers of diagnosis and characterization of metabolic phenotypes in different settings

The neonatal screening of inborn errors of metabolism is a common practice in many pediatric hospitals and is based on the detection of known appropriate metabolic biomarkers using MS techniques[30].

In neurological diseases, the urinary metabolic phenotypes in 3 groups of children aged 3 to 9 years (39 affected by autism, 28 non-autistic siblings and 34 aged-matched healthy controls) were characterized using ^{1}H-NMR spectroscopy and pattern recognition methods. Some biochemical changes were found in the urine of autistic children reflecting sulfur and amino acid metabolism, and were consistent with some abnormalities of gut microbiota[31].

In regards to kidney diseases, ^{1}H-NMR based-metabolomics was able to discriminate the urinary metabolic profiles of children with nephrouropathies (such as renal dysplasia, vesico-ureteral reflux, urinary tract infections) from those of healthy controls[32].

As far as respiratory diseases are concerned, the metabolomics approach has been applied also on exhaled breath condensate (EBC) in order to discriminate asthmatic patients from healthy children and to characterize asthma subphenotypes. The analysis of this particular biofluid through metabolomics is called *breathomics,* and the matrix is collected in a totally non-invasive way by cooling down the exhaled air. Its composition is believed to mirror that of the airway lining fluid. The LC-MS-based metabolomics profiling of EBC could clearly distinguish the severe asthmatic patients from the healthy subjects, and it could even discriminate severe and non-severe asthma metabolic phenotypes[33]. A previous study had demonstrated that asthma phenotypes in childhood could be differentiated through the LC-MS analysis of urine samples as well, also with the identification of relevant metabolites[34]. These important findings demonstrate the potential role of the metabolomic approach in eventually leading to tailored therapies depending on the severity of asthma. To remain in the field of respiratory diseases, MS-based metabolomic analysis was applied in the investigation of the volatile organic compounds (VOCs) in the exhaled air of subjects affected by several chronic different diseases[35].

The metabolomics approach has concerned pediatric oncology as well. Zhang et al. have applied GC-MS in serum and urine of healthy subjects and young adults affected by osteosarcoma and benign bone tumor. The analysis discriminated patients with osteosarcoma from healthy controls and patients with benign bone tumor. What is more, some metabolites were identified both in serum and in urine in association with osteosarcoma morbidity[36].

Prognostic profiles and predictive biomarkers

Over the last decade, metabolomics has introduced new insights into the pathology of diabetes as well as methods to predict disease onset and timing of complications by discovering new biomarkers[37]. These results, suggesting that metabolic dysregulation precedes the impairment of the immune system in type 1 diabetes, offer good perspectives for a future and efficient method of primary prevention and intervention in this condition[38].

In perinatology, metabolomic analysis has been increasingly used in recent years[39]. An example is represented by perinatal asphyxia, one of the major causes of brain injury and neurological sequelae. Chu et al. analyzed through the use of MS the urinary metabolite profiles of 256 newborns with severe birth asphyxia. In particular, eight organic acids were recognized as potential discriminators between good and poor neonatal outcome, thus representing possible prognostic markers of neurological consequences[40].

Another relevant disease occurring especially in preterm infants is patent ductus arteriosus. A preliminary study using ^{1}H-NMR spectroscopy was carried out in order to assess the possibility of identifying in advance the persistence of PDA. A PLS-DA model was able to distinguish infants born at term, preterm infants with PDA and preterm infants without PDA on the basis of different urine metabolic patterns at birth[39].

In the respiratory field, the application of metabolomics to the amniotic fluid has demonstrated that infants who will or will not experience wheezing in their first year of life have distinct amniotic fluid metabolomic profiles at birth[41]. It has also been demonstrated that metabolomic urinary profile can discriminate preschoolers with recurrent wheezing who will outgrow their symptoms from those who have early-onset asthma. These results may pave the way to the characterization of early non-invasive biomarkers capable of predicting asthma development[42].

Pharmacometabolomics

Metabolomic analysis can be applied in the study of xenobiotic effects on an organism, potentially leading to the prediction of effectiveness or toxicity of a drug based on the individual's pre-treatment characteristics[3]. This is of great importance in pediatrics and neonatology, since pharmacokinetic of almost all the drugs is completely different from adults, especially in the neonatal age and over the first years of life.

An elegant study by Clayton et al. provided proof-of-principle of the pharmacometabolomics approach, with the administration of paracetamol (acetaminophen) to rats. In the study, it was possible to determine an association between pre-dose urinary metabolite composition and the extent of liver damage sustained after paracetamol administration[43]. Later, the authors have confirmed that even in humans the pre-dose urinary metabolic profiles could predict subsequent acetaminophen metabolism and excretion[44].

A relevant study was performed by Price et al. in order to differentiate the urine metabolic profiles of healthy subjects and children affected by seizures undergoing treatment with carbamazepine or with valproic acid. The group treated with valproic acid appeared to have a distinct urinary metabolic pattern from each of the control groups[45].

A more recent study examined the metabolic profiles of paired bone marrow and peripheral blood samples from 10 children with acute lymphoblastic leukemia (ALL). Samples were collected from the same patient at the time of diagnosis and after 29 days of induction therapy with the standard protocol PEG-I-asparaginase, vincristine and a glucocorticoid. A peripheral blood sample was collected from each patient also after 8 days of treatment. The analysis showed different metabolic profiles between the two biofluids, both at time of diagnosis and after 29 days of therapy. In addition, the treatment significantly altered the metabolite composition of peripheral blood samples collected at different time. These findings confirm how a specific drug can alter the

metabolite composition of the targeted tissue, and offer the basis for further researches on how metabolic profile of pretreatment samples can predict sensitivity to a chemoterapy[46].

Pharmacometabonomics approach might provide the basis for the identification of new biomarkers with which individuals could be selected according to their suitability for treatment with particular drugs, drug classes or drug doses. Adverse drug reactions could potentially be avoided, and dose levels could be targeted more effectively according to the metabolic characteristics of each individual[43].

1.2 Recurrent respiratory infections: problem entity and definition

At the end of 1990 World Hearth Organization considered acute respiratory tract infections (ARTIs) to be the “forgotten pandemic” with a wide difference between industrialized and developing countries in mortility and morbility. In 2000 we know that 1.9 million children died from ARTIs worldwide, with 70% of the deaths in Africa and South-East Asia[47]. Also in high-income countries (USA, Canada, Western Europe) ARTIs are the leading cause of morbidity and account for 20% of medical consultations, 30% of days lost from work and 75% of antibiotic prescriptions[48]. ARTIs are responsible for most sick days amongst school children[49] and parental absenteeism from work[50]. Today the introduction of new antibiotics and vaccines has contributed to the reduction of serious infections, but no effect on prevalence and treatment of viral respiratory infections in children has been noted.

In particular, the recurrence of respiratory infections is one of the major complaint in the paediatric population and represents a great cause of morbidity. It has been estimated that at least 6% of Italian children with less than 6 years present recurrent respiratory infections (RRI)[51]. RRI increase the use of useless antibiotic therapies and

contribute to the development of resistances.

The definition of RRI was formulated in the 1970s by the Immunology Study Group of the Italian Pediatric Society based on epidemiological studies in Italy. The criteria are the absence of any pathological underlying condition (primary or secondary immunodeficiency, cystic fibrosis, airways malformations, primary ciliary dyskinesia) justifying the recurrence of infections and the presence of at least one of the following conditions: 1) six or more annual diseases due to respiratory infections; 2) one or more monthly diseases due to respiratory infection from October to February; 3) three or more annual diseases due to lower airway respiratory infections[51]. Other definitions of RRI distinguish the number of infections according to the age of the child: > 6 ARTI per year if age is > 3 years, and > 8 ARTI per year if age is < 3 years[52].

RRI represent essentially the consequence of an increased exposure to infectious agents associated to the exposure to environmental risk factors during the first years of age and the relative immaturity of the immune system. Social and environmental factors, particularly daycare attendance, but also family size, air pollution, parental smoking, and home dampness, represent important risk factors for airway diseases and may contribute in various degrees to determine the incidence of RRI. Approximately 75% of children attending day-care centres start to suffer from RRI during the first year at child-care facilities. Early enrolment in day-care centres influences the prevalence of respiratory infections and accelerates the acquisition of immunological experiences but this occurs with the cost of the disease (because of the naivety of the immune system). The younger the child, the greater is the risk of developing a symptomatic disease, rather than an unapparent infection, after the contact with an infectious agent[49]. The postponed enrolment of children at day-care centres may prevent this excess risk. Environmental factors such as parental smoking and pollution can influence the risk of RRI. Second hand smoke and air pollution have been demonstrated to be associated with the development of cough, wheezing and

asthma in children and to a reduction in lung function[53-55]. A recent Italian study demonstrated that there is a significant association between traffic-related pollution and the development of asthma exacerbations and respiratory infections in children born to atopic parents and in those suffering from recurrent wheezing or asthma. These findings suggest that environmental control and reduction in parental smoking may be crucial for respiratory health in children with underlying respiratory disease[56].

Recent studies demonstrated that also intrauterine exposure to fine particulate matter is a risk factor for increased susceptibility to acute broncho-pulmonary infections in early childhood[57-59].

The complete maturity of the immune system is not reached until the child is 5-6 years old and in the first years of life, the immune system is still developing with a demonstrated alteration in the cytochine levels[51]. When immune functions are still largely immature, the airway epithelium plays a primary defensive role since, besides providing a physical barrier, it is also involved in the innate and the adaptive immune responses. The epithelial cell actually is the principal site of viral infection in the airways and plays a central role in viral modulation of airway inflammation. The mechanisms by which viral infections modulate epithelial function, therefore, is a topic of intense investigation[60].

The recurrence of RI represent a great “challenge” for the paediatricians on the preventive and therapeutic point of view. First of all because they have to discriminate between children that present RRI associated with an increase in environmental risk factors exposure and with a physiological immaturity of the immune system, from children that have an underlying pathologic condition (immunological or not). The annual infection number cannot be the only parameter considered to this purpose, but it is more significant to analyse respiratory infections’ characteristics, severity and duration in particular to individuate children requiring deeper investigation[61]. The

majority of children with RRI do not have recognised immunodeficiency, but in some children we may find low levels of certain immunological parameters, such as reduced levels of immunoglobulin isotypes. However, a number of the observed immunological alterations are of questionable significance and may not be related to the increased susceptibility to respiratory infections. Several viral infections may influence immune response, altering cytokine responses or macrophage phagocytosis and it is possible consequently, that children with RRI develop a sort of deeper virus-induced immune depression[51].

It is of note that substantial and recent evidences implicate common respiratory viral infections also in the pathogenesis of asthma and chronic obstructive pulmonary disease (COPD), the mechanisms by which viruses predispose to these diseases remain poorly understood. It is clear that viral infections lead to enhanced airway inflammation and can cause airways hyper-responsiveness. RRI remain a social problem for both their pharmaco-economic impact and the burden for the family. Although current therapies may help combat virally induced disease exacerbations, they are less than ideal. To this purpose, a better characterization of these patients is crucial to identify effective preventive and personalized and new therapeutic approaches.

1.3 Immunostimulants

Immunostimulants (IS) are heterogeneous compounds that seem to be effective in modulating the innate immunity and improving defences against infections. They can come from a synthetic source (levamisole, isoprenaline, pidotimod); or are of biological origin such as Klebsiella extracts containing lipopolysaccharide and mixtures of bacterial extracts and bacterial lysates, prebiotics and probiotics. When a diagnosis of RRI has been formulated in a child, accurate environmental prophylaxis (reduction of

second-hand smoke, postponed enrolment in at day-care centres) is crucial[51] but evidences suggest a benefit regarding the prevention of ARTI coming also from the use of immunostimulants. The recent discovery of Toll-like-receptors (TLRs) in 1990s supported the possible mechanism of action of immunostimulants[62]. It may be postulated that products with IS properties activate the immune cells using the receptors that recognise common bacterial products or receptors that provide additional stimulation for activation. For instance, TLRs recognise components common to a range of bacteria, so-called pathogen-associated molecular patterns such as lipopolysaccharide, peptidoglycan, lipoteichoic acid, lipoarabinomannan, un-methylated DNA with CpG motif and bacterial lipoproteins which activate the innate immune responses. The innate immune response is responsible for the early mechanisms of defence against infection; for instance the phagocytosis and neutralisation of bacteria entering the body. The mechanisms that enhance the innate immune responses (cytokines and chemokines) also stimulate the adaptive immune response (production of specific antibodies and reproduction of specific T cells)[63,64].

Immunostimulants mimic the immune response normally evoked by a pathogen in the host system (first with the activation of the innate immune system and then of the adaptive response).

There are many natural products, such as Vitamin D, Resveratrol, Zinc, Echinacea, β-Glucans and bacterial lysates for which it has been described an immunostimulant effect. As part of the NHLBI 2013 workshop on the primary prevention of chronic lung diseases, the asthma group underlines the relevance of prebiotics, probiotics, and bacterial lysates and states that immunmodulation using this compounds is a field that should be investigated in future clinical trials in high-risk infants for asthma prevention[65].

Vitamin D has a demonstrated action against respiratory infections and it has recently been re-discovered as an important immunostimulant, in particular during the winter

season[66].

Resveratrol (trans-3,4,5-trihydroxystilbene) is a natural non-flavonoid polyphenol and belongs to a subclass of stilbenes. It is found in various fruits and vegetables and abundant in grape skin, it functions as a phytoalexin (a class of vegetal antibiotics) so protecting the plant from environmental stress or infections. Resveratrol exerts anti-infective and anti-inflammatory activities. The anti-inflammatory effects of resveratrol depend on the inhibition of the transcription factor NF-kB, mainly inhibiting Ik-B kinase. Moreover, resveratrol inhibits viral replication. A recent study provided evidence that resveratrol inhibits the replication of rhinovirus, the etiologic agent of common cold, on nasal epithelial cells and the rhinovirus-dependent expression of ICAM-1, that is the main rhinovirus receptor. Resveratrol has also demonstrated anti-inflammatory and anti-asthmatic effects in mouse model of allergic asthma, diminishing IL-4 and IL-5 in plasma and bronchoalveolar lavage fluid, and suppressing bronchial hyperactivity, lung eosinophilia, and mucus hypersecretion[67].

Zinc is an essential micronutrient important for growth and for normal function of the immune system. Studies suggest that zinc administration could reduces the risk of new episodes of acute respiratory illnesses, while no effect in the acute phase has been observed[68].

Echinacea is a widely used herbal remedy for treatment of upper respiratory tract infections. A recent met-analysis concludes that the use of echinacea extracts is associated with reduced risk of recurrent respiratory infections[69].

Metabolites and components of medicinal mushrooms have been used in medicine for many centuries. β-Glucans, a heterogeneous group of glucose polymers, are biologically active polysaccharides that are responsible for the observed clinical efficacy of mushroom extracts. β-Glucans appear to be an interesting group of natural immunomodulating substances, which are associated with a low risk of side effects. A recent review supports, based on published studies, the preventive use of beta-

glucans in managing RRIs. Preventive application of beta-glucans may decrease the frequency of various forms of respiratory tract infection, supporting a protective immune mechanisms[70].

Broncho-Vaxom is an orally administered immunomodulator containing the lyophilized bacterial lysate of eight pathogenic bacteria of the respiratory tract. Broncho-Vaxom stimulates immune defences and the production of salivary and bronchoalveolar IgA as well as serum IgA and IgG. It has been administered since the 1980s to adults and children in order to prevent recurrences of respiratory tract infections. A very recent review showed a significant reduction in RRIs, a decrease in the duration of the course of antibiotics, infections, fever, cough, and wheezing in children with RRIs who were treated with Broncho-Vaxom in comparison to controls[71].

A number of studies have investigated the immunostimulants' effect on cellular and innate immunity, and their clinical efficacy. The majority of them have shown that the number of infections decreases after immunostimulants treatment but most studies in meta-analyses are often heterogeneous and of poor quality because of methodological biases. That is why caution is needed when considering apparently positive results and this is the reason why, to date, there is no consensus as to their real usefulness. A 2011 Cochrane meta-analysis, including randomised controlled trials (RCTs), comparing immunostimulants, administered by any method, to placebo to prevent ARTIs in children younger than 18 years concluded that immunostimulants seem to be able to reduce the incidence of ARTIs by 40% on average in susceptible children. The main limitations of this review were the poor methodological quality and diverse trial results. They concluded that ARTI-susceptible children may benefit from immunostimulants (not specifying the type), but more high-quality studies are needed[72].

1.3.1 Probiotics

Oral probiotics are non-pathogenic live microbes that have a role in the prevention and treatment of a wide range of disorders. The most widely studied probiotics are Lactobacillus and Bifidobacterium. Albeit with large differences from one microorganism to another, it is now established that probiotics can produce antimicrobial products capable of eliminating bacterial pathogens[73], blocking toxin-mediated responses[74], interfering with bacteria limiting their presence and virulence[75], and modulating systemic immune responses by enhancing humoral and cellular immunity[76]. Most of the data regarding probiotic use in children have been collected in studies of gastrointestinal disorders, such as infectious and antibiotic-associated diarrhea, travellers' diarrhea, necrotizing enterocolitis, and Helicobacter pylori infection[77-79].

Routine use of probiotics as an additive therapy in subjects with RTIs has been poorly studied and evidences about this topic are still insufficient and are mainly related to the field of upper respiratory tract infections. A recent Cochrane meta-analysis of 10 clinical trials found that probiotics were more beneficial than placebo in terms of infection prevention, and reduced the rate of acute upper respiratory tract infections and frequency of antibiotic use, but did not decrease the duration of each single episode[80]. A recent review and meta-analysis by Laursen et al concluded that Lactobacillus rhamnosus GG is modestly effective in decreasing the duration of RTIs, but underlined that more RCTs investigating specific probiotic strains or their combinations in prevention of RTIs are needed. Further research to establish the role of probiotics in the treatment and prevention of RTIs, including those involving the lower respiratory tract, are then required[81].

1.3.2 Pidotimod

Pidotimod (3-L-pyroglutamyl-L-thiaziolidine-4carboxylic acid) is a synthetic dipeptide molecule with immunomodulatory properties. It is a highly purified molecule with high reproducibility among batches. It is rapidly absorbed by the gastrointestinal tract, with a bioavailability of 45 % not influenced by food and it is eliminated unmodified via renal excretory mechanisms. Half-life of the compound did never exceed 8-9 hours. The safety profile of pidotimod is good, no serious adverse event were reported except for one case of suspected Schonlein-Henoch purpura.

In vitro studies, in both animal and human specimens have shown that this product has an immunomodulatory activity on both innate and adaptive immune responses[82]. In detail pidotimod induces dendritic cell maturation, up-regulates the expression of HLA-DR and co-stimulatory molecules CD83 and CD 86, stimulates dendritic cells to release pro inflammatory molecules, driving T cell proliferation and differentiation towards a Th1 phenotype, enhances natural killer cell functions, inhibits thymocyte apoptosis and promotes phagocytosis[83,84].

A recent study conducted by Carta et al showed that pidotimod, in vitro, through different effects on ERK1/2 and NF-kB was able to increase the expression of TLR-2 proteins, surface molecules involved in the initiation of the innate response to infectious stimuli. It had no effect on ICAM-1 expression, the receptor for rhinovirus, and on IL-8 release, the potent chemotactic factor for neutrophils (that are already present in sites of infection) and this may represent a protective function from infections if confirmed in vivo. The Authors concluded that, in children, pidotimod seems to modulate airway epithelial cells functions involved in host-virus interactions, possibly through NF-kBactivation[83]. Recently it has been demonstrated that pidotimod facilitatesM2 macrophage (activated macrophage) polarization that are able to tune inflammatory responses, enhance phagocytosis, scavenge debris, and promote tissue remodelling and repair[85].A study was conducted in 1994 in CD-1 mice who were

treated with methylprednisolone in order to achieve an immunosuppressed state. The study demonstrated that immunosuppressed mice who were treated also with pidotimod were able to recover the capacity to produce TNF-alpha and NO in response to different stimuli (lipopolysaccharide, IFN-gamma or conidia from an opportunistic fungus, Aspergillus fumigatus) in contrast with mice treated only with metilprednisolone (that completely inhibit the antimicrobial activity)[86]. Furthermore, it has been demonstrated that pidotimod can modulate the inflammatory cascade triggered by TLR ligands through the up-regulation of the nucleotide binding and oligomerization domain receptor (NOD-like receptor) NLRP12[87].

In vitro and in vivo studies using experimental model systems are essential for identifying the biological mechanisms of action of pidotmod. These assumptions anyway cannot precisely predict human responses and encouraging results from in vitro and in vivo studies have to be confirmed with clinical trials. The first clinical trials investigating pidotimod efficacy appeared in 1990s and showed that this compound seems to have a beneficial effect in children, reducing the number of ARTI, the number of days of fever and the severity of the signs and symptoms of acute episodes. A significant reduction in use of antibiotics, antipyretics drugs, and symptomatic drugs, and absence from school and caregiver absenteeism was also observed. Some studies focused on the prevention of recurrent tonsillitis showing that pidotimod reduces the incidence of upper respiratory tract infections[88].

In recent years, with the increasing interest towards this product, many studies have been conducted with the aim of demonstrating its effects and trying to bridge the gap between preclinical and clinical research. A clinical randomized prospective Russian study enrolled 157 children (age range 3-6 years) with RRI assigned to two arms, a group treated with pidotimod (78 children, dose of 400 mg/die) for 30 days and one control group (79 children, 50% of them with demonstrated allergies). Changes in serum immunological markers were evaluated at baseline and 30 days after treatment

discontinuation. A statistically significant reduction in the number of ARI was observed in the treated group ($p<0,05$). After 6 months ARTI developed in 92,3% in the treated group versus 100% of the control group. An interesting finding was that, concerning the immunological markers considered (IL-8), the treatment group showed a better profile of normalization compared to the control group[89]. A study in children with Down Syndrome, that represent a particular class of patients more susceptible to infectious agents, showed that pidotimod (given a dose of 400 mg a day for 90 days) potentiate innate and adaptive immune response increasing the efficacy of influenza vaccine. Particularly this compound can induce the up-regulation of a number of genes involved in the activation of immune response and in the antimicrobial activity. Authors concluded suggesting that this synthetic immunostimulant can potentiate the beneficial effect of immunization, resulting in a stronger immune response[90].

On the other side a recent Italian double-blinded randomized placebo-controlled trial study assessed the efficacy of pidotimod in a population of 3-year-old healthy children who just entered kindergarten concluding that it did not prove to be statistically superior to placebo for the prevention of ARTI. However, pidotimod showed some potential as a means for reducing antibiotic usage in these children. The limit of the study is the number of patients enrolled (24 children who received pidotimod and 25 who received placebo) [91].

It has been hypothesized that pidotimod could have a role also in Th1-Th2 balance with a possible “antiallergic” activity[84].through the down-regulation of CD30 in asthmatic and healthy children[92]. Moreover there are studies that confirm a role of pidotimod on IL-4 and IFNgamma and also on IgE levels[93].

In a recently published metabolomic study by our group, pidotimod has shown the possibility to partially "restores" the altered metabolic profile found in children with RRI compared to healthy controls. After pidotimod treatment some metabolic differences between RRI children and the healthy controls persisted; and among them emerged

some metabolites, that appeared to be related to the microbiota composition. In the light of these results, Authors hypothesize a potential synergic effect of the combined use of immunostimulants and probiotics for the purpose of prevention in children with RRI[95].

Considering available studies, it seems that pidotimod has a beneficial effect and a good tolerability in children, but further studies are still needed to confirm its efficacy and to better understand its mechanism of action.

2 RATIONALE AND OBJECTIVES OF THE STUDY

Recurrent respiratory infections (RRI) represent a widespread condition that largely contributes to pediatric morbidity and has considerable economic and social impact[50]. In patients with RRI antibiotics may be overused, and increased bacterial resistance has become an important matter of concern worldwide. Interestingly, RRI in the first years of life has been indicated as a possible co-factor in the development of adult respiratory disorders, namely asthma and chronic obstructive pulmonary disease (COPD)[94]. 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[82], albeit the mechanism of action has been elucidated only in part[87, 83, 84]. In a recent study of children with RRI, we showed that pidotimod can partially "restore" the altered metabolic profile found in these children, even though some metabolites possibly originating from the microbiota were persistently altered[95]. Based upon these findings, we hypothesized a potential interactive effect of immunostimulants and probiotics on preventing RRI in children and wondered if they had any influence on the metabolomic profiling. Actually, also probiotics were suggested to have a preventive effect on respiratory infections, but evidences are quite limited[81]. Hence, we conducted an exploratory prospective, randomized controlled trial (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.

3 MATERIALS AND METHODS

3.1 Study design

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. 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[96]; 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 as liquid suspension in 400 mg vial (one vial/day) + bifidobacteria mixture (B longum BB536, 3x109 CFU; B infantis M-63, 1x109 105 CFU; B breve M-16V, 1x109 106 CFU) as powder in 3 g sachet (one sachet/day);

- group B: 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: identical-looking and -tasting pidotimod placebo as liquid suspension in a vial (one vial/day) + bifidobacteria mixture (B longum BB536, 3x109 CFU; B infantis M-63, 1x109 105 CFU; B breve M-16V, 1x109 106 CFU) as powder in 3 g sachet (one sachet/day);
- group D: 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, taste, 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. According to data from the Italian National Institute of Health, there was no significant difference between the 2 study seasons in frequency, distribution and virulence of respiratory pathogens. 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	✓		✓	

3.2 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 metabolomics 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 metabolomics analysis was performed.

The urine samples were thawed at room temperature, stirred for 30 seconds in a vortex mixer, and then centrifuged at 6000 g for 10 minutes to remove the sediment present in the urine. We transferred 100 µL of the supernatant into a test tube and added 400 µl of H2O 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.

3.3 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). 5µL of each diluted sample were injected into a Waters Acquity HSS T3 2.1 x 100mm column packed with 1.7µm beads kept at 50ºC. The mobile phase for elution was composed of solvent A (H2O, 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 2min 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 500µL min-1. The electrospray source of Q-TOF was operated in positive (ESI+) and negative (ESI-) ionization mode with a capillary voltage set at 3kV and 1.5kV 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 (H2O, 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.

3.4 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 more than 95% of missing values in the blank samples or with a ratio between the 5th percentile measured in the QCs and the 95th percentile measured in the blank samples greater than 5 were included. Moreover, variables with a coefficient of variation in the QCs greater than 15% 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 [97], median was applied to each variable of the triplicates. The differences between the urine metabolite content after 16 weeks of treatment and at the baseline were used to obtained the sample representation useful

for data analysis.

3.5 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 □=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 metabolome 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) [98] 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. AUCCV). 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.

4 RESULTS

4.1 Patients' characteristics

A total of 55 children were enrolled, 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. No significant differences in gender, age, weight and height were found.

Table 2. Characteristics of the 55 children with recurrent respiratory infections.
Values are expressed as mean and range or standard error of the mean.
Abbreviations: URTI, upper respiratory tract infections; LRTI, lower respiratory tract infections.

	Group A (n = 13)	**Group B (n = 13)**	**Group C (n = 13)**	**Group D (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)

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 + Bifidobacterium group") and B ("pidotimod group") showed a significantly higher

proportion of symptom-free days (p=0.02 and p=0.003, respectively) and a lower percentage of days with common cold (p=0.004 and p=0.005, 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.

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

	Group A (n = 13)	**Group B (n = 13)**	**Group C (n = 13)**	**Group D (n = 16)**
Number of URTI	39	53	64	58
Number of LRTI	2	4	1	6
Number of days with URTI	21 (0-66)§	25 (0-72)§	37 (7-72)§	32 (0-82)§
Number of days with LRTI	7 (7-7)§	5 (2-7)§	10 (10-10)§	10 (4-17)§
Symptom-free days (%)	65*	69**	59	44
Days with common cold (%)	15≠	17#	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.

§ Values are expressed as mean (range)

* p = 0.02 versus Group D

** p = 0.003 versus Group D

≠ p = 0.004 versus Group D

p = 0.005 versus Group D

4.2 Metabolomic analysis

The urine metabolomics 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 (□=0.05) performed on the PCA models of each group. Moreover, no differences in the metabolic profile were observed between the 4 groups at the baseline.

Group D ("placebo group") versus group C ("bifidibacterium group")

The variations in the metabolite content of the urines from the 8 children of the control group D were compared to those from the 6 children of group C. 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.

Group D (“placebo group”) versus group B (“pidotimod group”)

Urine samples were available for 6 children of group B. 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 AUCCV=0.85 (p-value=0.041) for the POS data set and AUCCV=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 figure 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 S1 in Supplementary Information. Among them some variables could be ascribable to a derivative of hippuric acid and to triptophan metabolites (L-Kynurenine and indolacetic acid).

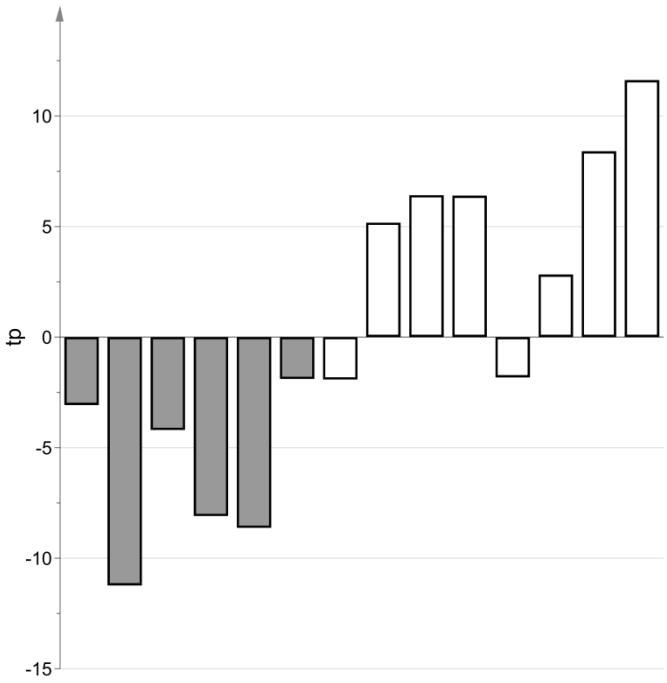

Figure 1. Group D (“placebo group”) *versus* group B (“pidotimod group”): score bar plot of the ptPLS2-DA model for the POS data set. White bars indicate group D children, grey bars refer to group B children. tp is the predictive score of the model.

Group D (“placebo group”) *versus* group A (“pidotimod + bifidobacterium group”)

Urine samples were available for 5 children of group A. 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 figure 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 S2 in Supplementary Information. Among them, we identified a metabolite of bile acid (deoxycholic acid 3-glucuronide), oxoglutaric acid, metabolites of tryptamine (trace amines) and some metabolites belonging to the ubiquinone family.

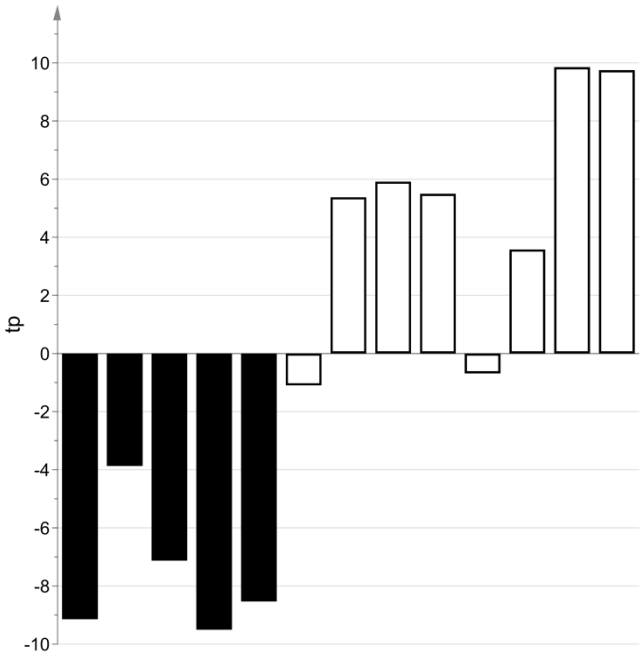

Figure 2. Group D (“placebo group”) *versus* group A (“pidotimod + Bifidobacterium group”): score bar plot of the PLS-DA model for the POS data set. White bars indicate group D children, black bars refer to group A children. tp is the predictive score of the model.

The results of data analysis can be summarized in the Shared and Unique Structure plot (SUS-plot) of figure 3 [99], 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 which distinguish group D and group A likely separate also group D and 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 metabolite of steroid hormones, metabolites of vitamin B metabolism and aminoacid derivatives. On the other hand, 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 the interaction effects of pidotimod and bifidobacteria.

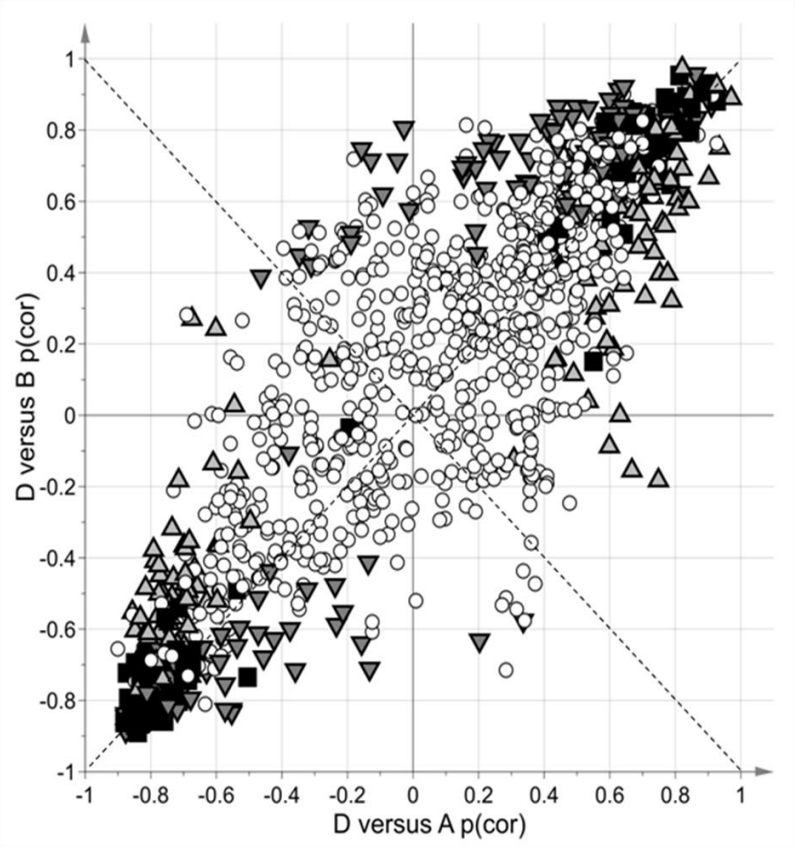

Figure 3. SUS-plot for the POS data set: variables distinguishing group D and group B are reported as grey inverted triangle, variables separating group D and group A as light grey triangles, whereas black boxes indicate variables distinguishing both group D and group A, and group D and group B. Open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

5 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 found that pidotimod, administered alone or combined with bifidobacteria, is effective in significantly decreasing the days of illness along with reducing symptoms due to common cold. In contrast, in the group treated with bifidobacteria mixture alone no significant differences in clinical outcomes compared to the placebo group were found.

A major novel finding from this study is provided by the metabolomic analysis. For the first time, we demonstrated that children with RRI receiving pidotimod have a metabolomic profiling of urine significantly different from the placebo group. These differences were observed regardless children were taking pidotimod alone (group B) or in combination with bifidobacterium mixture (group A) and concern steroid hormones, metabolites of vitamin B metabolism and aminoacid derivatives.

On the other hand children treated with bifidobacterium mixture alone did not showed variations in the metabolomic profile compared to those of the placebo group.

Steroid hormones exert immuno-regulatory effects both in vivo and in vitro and they could represent the activation of the hypothalamus-pituitary-adrenal axis in the interaction between neuroendocrine and immune systems[100]. Vitamin B is mostly derives from diet and bacteria can synthesize it. Recent studies have demonstrated that our immune system can uses vitamin B as a point of difference to recognize infection through mucosal associated invariant T cells that lie in mucosal and other surfaces (intestine, mouth, lungs) [101,102].

From a clinical point of view, no differences were found in children treated with pidotimod alone and children taking pidotimod plus bifidobacterium mixture, and these

two groups seem to behave in the same way. However, the metabolomics analysis revealed a different biochemical behavior. The interaction between bifidobacterium and pidotimod produces different metabolic perturbations related to a number of products such as metabolites of bile acid, a derivative of hippuric acid, triptophan metabolites, the oxoglutaric acid, a metabolite of tryptamine and a metabolites belonging to the ubiquinone family. Actually hippuric acid and bile acid metabolites are likely associated with the microbiota composition and they emerged as discriminant metabolites also in a recent metabolomic study by our group considering, in that case, children with RRI treated with pidotimod compared to healthy controls[95]. These metabolites, previously described in children with RRI, point toward a role of the microbiota in the immune regulation, even though we still do not know how they can precisely interact and respond to a possible probiotics supplementation.

Moreover, triptophan metabolites L-Kynurenine and indolacetic acid could derive from enteric biotransformation and thus be related to the microbiota composition[103]. A recent review also underlined in this respect the crucial role of tryptophan and its metabolites in the balance between intestinal immune tolerance and gut microbiota maintenance[104]. The metabolites of oxoglutaric acid may be related to the microbiota composition as well[105], 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[106].

Our findings are not due to the bifidobacterium 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 bifidobacterium) on the metabolomic arrangement.

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 accuracy of the study is warranted by the 4 arms design with a comparison not only versus the subjects receiving placebo, but also versus each active medication group. In this way, we could appropriately assess the role that each treatment, alone or in combination, played in the trial in order to achieve the final effect. The inclusion criteria we set were also very strict, thus avoiding as many confounding factors as possible. Moreover, we administered a mixture of bifidobacteria instead of a single strain. Indeed, multistrain probiotics seem to be more effective than single strains[107]. 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, we acknowledge that our population size was small. Future RCTs including a larger number of subjects and a healthy group of participants are warranted to further understand the immunomodulatory effects of pidotimod and bifidobacteria in terms of RRI prevention.

6 CONCLUSIONS

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. Group A ("pidotimod + bifidobacteria group") and B ("pidotimod group") did not differ from the clinical point of view, but the metabolomic analysis was able to reveal, going beyond the clinic, a different behaviour for these two groups, suggesting a possible role for the microbiota composition in the underlying physiopathologic mechanism. We believe that the study of metabolome in pediatric or adult RRI is promising to uncover the possible connection between the gut microbiome, the role of immunostimulants and their combined relationship to RRI.

7 SUPPLEMENTARY INFORMATIONS

Compound	m/z	ESI	Compound ID	Charge	Urine	Adducts	Formula	Description	Biological class/pathway	Mass Error	Isotope Similarity
0,87_990,4973m/z	990.4973	ESI+	HMDB06902	1	x	M+H	C48H72CoN11O8+2	Cobinamide	intermediate in porphyrin and chlorophyll metabolism	0.2	53.4
0,90_162,1120m/z	162.112	ESI+	HMDB00062	1	x	M+H	C7H15NO3	L-Carnitine	fatty acid metabolism/acylcarnitine	-2.9	95.9
0,96_90,0550m/z	90.05502	ESI+	HMDB00161	1	x	M+H	C3H7NO2	L-Alanine	aminoacid	0.7	99.6
1,01_160,1329m/z	160.1329	ESI+	HMDB00991	1	x	M+H	C8H17NO2	DL-2-Aminooctanoic acid	amino compound	-2.0	99.3
1,05_204,1235m/z	204.1235	ESI+	HMDB00201	1	x	M+H	C9H17NO4	L-Acetylcarnitine	fatty acid metabolism/acylcarnitine	2.3	98.5
1,07_254,0879m/z	254.0879	ESI+	HMDB00727	1	x	M+H	C9H11N5O4	L-Threoneopterin	catabolism of nucleotide	-1.8	61.2
1,18_250,0379m/z	250.0379	ESI+	HMDB02062	1	x	M+H	C8H11NO6S	Norepinephrine sulfate	Tyr derivative	-0.2	93.8
1,40_170,0920m/z	170.092	ESI+	HMDB00001	1	x	M+H	C7H11N3O2	1-Methylhistidine	His derivative	-2.5	56.7
1,47_259,0920m/z	259.092	ESI+	HMDB04813	1	x	M+H	C10H14N2O6	3-Methyluridine	nucleoside derivative	-1.7	90.1
1,52_218,1385m/z	218.1385	ESI+	HMDB00824	1	x	M+H	C10H19NO4	Propionylcarnitine	fatty acid metabolism/acylcarnitine	-0.7	96.9
1,78_153,0655m/z	153.0655	ESI+	HMDB04194	1	x	M+H	C7H8N2O2	N1-Methyl-4-pyridone-3-carboxamide	derivative of dinucleotide (NAD)	-2.2	98.8
2,57_283,1037m/z	283.1037	ESI+	HMDB02721	1	x	M+H	C11H14N4O5	1-Methylinosine	Modified nucleotide	0.1	95.1
2,66_173,0805m/z	173.0805	ESI+	HMDB00341	1	x	M+H	C8H12O4	2-Octenedioic acid	fatty acid	-1.8	95.8
2,66_250,0935m/z	250.0935	ESI+	HMDB01526	1	x	M+H	C10H19NO2S2	S-Acetyldihydrolipoamide	intermediate in alanine, aspartate and pyruvate metabolism	1.9	87.3
2,83_286,1041m/z	286.1041	ESI+	HMDB05923	1	x	M+H	C11H15N3O6	N4-Acetylcytidine	Modified nucleotide	2.4	94.7
2,84_367,0651m/z	367.0651	ESI+	HMDB01439	1	x	M+H	C10H15N4O9P	Phosphoribosyl formamidocarboxamide	purine metabolism	0.3	90.4
2,86_209,0919m/z	209.0919	ESI+	HMDB00684	1	x	M+H	C10H12N2O3	L-Kynurenine	Trp metabolism	-0.9	94.5
3,40_185,0800m/z	185.08	ESI+	HMDB01565	1	x	M+H	C5H15NO4P+	Phosphorylcholine	precursor metabolite of choline	-6.1	93.2
3,58_111,0440m/z	111.044	ESI+	HMDB00957	1	x	M+H	C6H6O2	Pyrocatechol	catechol	-0.4	97.9
3,58_246,1698m/z	246.1698	ESI+	HMDB00378	1	x	M+H	C12H23NO4	2-Methylbutyroylcarnitine	fatty acid metabolism/acylcarnitine	-0.8	97.3
4,58_369,1284m/z	369.1284	ESI+	HMDB01204	1	x	M+H	C16H20N2O8	trans-3-Hydroxycotinine glucuronide	nicotine metabolite	-2.4	85.3
4,66_459,1294m/z	459.1294	ESI+	HMDB01142	1	x	M+H	C17H23N4O9P	FMNH	vit B2 metabolism	4.0	94.6
4,82_195,0641m/z	195.0641	ESI+	HMDB00954	1	x	M+H	C10H10O4	trans-Ferulic acid	phenolic compound	-5.6	93.6
4,83_137,0595m/z	137.0595	ESI+	HMDB01326	1	x	M+H	C8H8O2	Phenyl acetate	organic acid derivative	-1.3	96.6
5,07_361,1394m/z	361.1394	ESI+	HMDB06045	1	x	M+H	C18H20N2O6	Dityrosine	Tyr derivative	-0.1	95.9
5,18_197,0800m/z	197.08	ESI+	HMDB00434	1	x	M+H	C10H12O4	Homoveratric acid	metabolite of 3,4-dimethoxyphenylethylamine	-4.2	96.6
5,33_304,1571m/z	304.1571	ESI+	HMDB03573	1	x	M+H	C17H21NO4	Scopolamine	alkaloid	9.1	82.7
5,45_288,2166m/z	288.2166	ESI+	HMDB00791	1	x	M+H	C15H29NO4	L-Octanoylcarnitine	fatty acid metabolism/acylcarnitine	-1.1	96.8
5,79_302,2324m/z	302.2324	ESI+	HMDB06320	1	x	M+H	C16H31NO4	2,6 dimethylheptanoyl carnitine	fatty acid metabolism/acylcarnitine	-0.5	99.3
5,79_361,1990m/z	361.199	ESI+	HMDB02802	1	x	M+H	C21H28O5	Cortisone	steroids	-5.4	81.2
5,99_163,0751m/z	163.0751	ESI+	HMDB02333	1	x	M+H	C10H10O2	Safrole	food flavour	-1.5	99.1
6,15_389,1637m/z	389.1637	ESI+	HMDB05032	1	x	M+H	C21H25ClN2O3	Cetirizine	medication	2.6	73.3
6,22_379,2105m/z	379.2105	ESI+	HMDB00418	1	x	M+H	C21H30O6	18-Hydroxycortisol	steroids	-2.8	58.9
1.38_180.0656m/z	180.0656	ESI-	HMDB00158	1	x	M-H	C9H11NO3	L-Tyrosine	aminoacid	-5.4	99.1
4.59_174.0553m/z	174.0553	ESI-	HMDB00197	1	x	M-H	C10H9NO2	Indoleacetic acid	Trp metabolism	-4.0	94.9
3.00_218.1025m/z	218.1025	ESI-	HMDB00210	1	x	M-H	C9H17NO5	Pantothenic acid	vitamin B5	-3.9	99.0
3.54_189.0763m/z	189.0763	ESI-	HMDB00325	1	x	M-H	C8H14O5	3-Hydroxysuberic acid	fatty acids	-2.9	92.4
3.26_145.0501m/z	145.0501	ESI-	HMDB00448	1	x	M-H	C6H10O4	Adipic acid	dicarboxylic acid	-3.7	95.3
3.47_157.0499m/z	157.0499	ESI-	HMDB00555	1	x	M-H	C7H10O4-2	3-Methyladipic acid	catabolism of phytanic acid.	-4.4	99.2
4.59_174.0553m/z	174.0553	ESI-	HMDB00859	1	x	M-H2O-H	C10H11NO3	Methylhippuric acid	metabolites of fatty acids	-3.7	94.7
1.83_138.0556m/z	138.0556	ESI-	HMDB00875	1	x	M-H	C7H9NO2	Trigonelline	alkaloid	-3.5	99.3
1.83_138.0556m/z	138.0556	ESI-	HMDB00959	1	x	M-H2O-H	C7H11NO3	Tiglylglycine	metabolites of fatty acids	-3.1	99.1
1.38_180.0656m/z	180.0656	ESI-	HMDB01119	1	x	M-H	C9H11NO3	4-Hydroxy-4-(3-pyridyl)-butanoic acid	nicotine metabolite	-5.4	99.1
4.67_457.1146m/z	457.1146	ESI-	HMDB01142	1	x	M-H	C17H23N4O9P	FMNH	vit B2 metabolism	3.5	87.7
4.65_150.0555m/z	150.0555	ESI-	HMDB01537	1	x	M-H2O-H	C8H11NO3	6-Hydroxydopamine	amine	-3.5	98.2
1.60_162.0233m/z	162.0233	ESI-	HMDB01890	1	x	M-H	C5H9NO3S	Acetylcysteine	Cys derivative	1.3	91.9
3.47_157.0499m/z	157.0499	ESI-	HMDB02025	1	x	M-H2O-H	C7H12O5	2,3-Dimethyl-3-hydroxyglutaric acid	fatty acid	-3.9	99.0
4.65_150.0555m/z	150.0555	ESI-	HMDB02210	1	x	M-H	C8H9NO2	2-Phenylglycine	alpha amino acids	-3.9	98.4
3.26_145.0501m/z	145.0501	ESI-	HMDB02712	1	x	M-H2O-H	C6H12O5	1,5-Anhydrosorbitol	polyol	-3.3	95.0

Table S1: B versus D. The table reported the ID of the variables , the mass of the ion, the type of ESI, the HMDB ID, the type of adduct, the chemical formula, the putative marker, the pathway involved or the biological class of the compounds, the mass error of the m/z extracted respect to the matched compound and the isotopic similarity between the spectrum of the ion and the matched compound (expressed in %)

Compound (rt_m/z)	m/z	ESI	HMDB ID	Charge	Urine	Adducts	Formula	Description	Biological class/pathway	Mass Error (ppm)	Isotope Similarity
0.87_990.4973m/z	990.4973	ESI+	HMDB06902	1	x	M+H	C48H72CoN11O8+2	Cobinamide	intermediate in porphyrin and chlorophyll metabolism	0.2	53.4
0.89_774.6082m/z	774.6082	ESI+	HMDB05331	1	x	M+H	C43H84NO8P	GPEtn(18:0/20:1(11Z))	glycerophospholipid	9.6	88.9
0.99_310.1132m/z	310.1132	ESI+	HMDB00773	1	x	M+H	C11H19NO9	N-Acetyl-a-neuraminic acid	sialic acid	-0.3	93.5
1.00_223.0742m/z	223.0742	ESI+	HMDB00099	1	x	M+H	C7H14N2O4S	L-Cystathionine	dipeptide	-2.1	42.5
1.13_231.0977m/z	231.0977	ESI+	HMDB02335	1	x	M+H	C9H14N2O5	Aspartyl-L-proline	dipeptide	0.6	94.7
1.18_250.0379m/z	250.0379	ESI+	HMDB02062	1	x	M+H	C8H11NO6S	Norepinephrine sulfate	Tyr derivative	-0.2	93.8
1.45_288.1196m/z	288.1196	ESI+	HMDB02089	1	x	M+H	C11H17N3O6	n-Ribosylhistidine	His derivative	2.1	98.7
2.66_250.0935m/z	250.0935	ESI+	HMDB01526	1	x	M+H	C10H19NO2S2	S-Acetyldihydrolipoamide	intermediate in alanine, aspartate and pyruvate metabolism	1.9	87.3
2.83_286.1041m/z	286.1041	ESI+	HMDB05923	1	x	M+H	C11H15N3O6	N4-Acetylcytidine	nucleoside	2.4	94.7
2.84_367.0651m/z	367.0651	ESI+	HMDB01439	1	x	M+H	C10H15N4O9P	Phosphoribosyl formamidocarboxamide	purine metabolism	0.3	90.4
3.13_175.1227m/z	175.1227	ESI+	HMDB04370	1	x	M+H	C11H14N2	N-Methyltryptamine	alkaloids	-1.5	92.8
3.18_125.0598m/z	125.0598	ESI+	HMDB00873	1	x	M+H	C7H8O2	4-Methylcatechol	catechol	1.0	94.5
4.66_459.1294m/z	459.1294	ESI+	HMDB01142	1	x	M+H	C17H23N4O9P	FMNH	vit B2 metabolism	4.0	94.6
4.77_377.1450m/z	377.145	ESI+	HMDB00244	1	x	M+H	C17H20N4O6	Riboflavin	vit B2	-1.6	66.3
4.83_137.0595m/z	137.0595	ESI+	HMDB01326	1	x	M+H	C8H8O2	Phenyl acetate	organic acid derivative	-1.3	96.6
5.07_361.1394m/z	361.1394	ESI+	HMDB06045	1	x	M+H	C18H20N2O6	Dityrosine	Tyr derivative	-0.1	95.9
5.46_251.1279m/z	251.1279	ESI+	HMDB02012	1	x	M+H	C14H18O4	Ubiquinone	intermediate of Coenzyme Q	0.4	97.8
5.79_361.1990m/z	361.199	ESI+	HMDB02802	1	x	M+H	C21H28O5	Cortisone	steroids	-5.4	81.2
5.96_379.2086m/z	379.2086	ESI+	HMDB00418	1	x	M+H	C21H30O6	18-Hydroxycortisol	steroids	-7.7	92.0
6.45_319.1909m/z	319.1909	ESI+	HMDB06709	1	x	M+H	C19H26O4	Ubiquinone Q2	intermediate of Coenzyme Q	1.6	74.5
0.81_485.1979m/z	485.1979	ESI-	HMDB00585	1	x	M-H	C18H34N2O13	Glucosylgalactosyl hydroxylysine	Lys derivative	-1.8	85.1
0.89_1078.3541m/z	1078.354	ESI-	HMDB06513	1	x	M-H	C43H68N7O17P3S	docosa-4,7,10,13,16-pentaenoyl coenzyme A	unsaturated fatty acids	0.8	78.9
0.89_750.5486m/z	750.5486	ESI-	HMDB05779	1	x	M-H	C43H78NO7P	GPEtn(O-18:1(1Z)/20:4(5Z,8Z,11Z,14Z))	glycerophospholipid	5.6	81.8
0.97_267.0716m/z	267.0716	ESI-	HMDB00195	1	x	M-H	C10H12N4O5	Inosine	nucleoside	-7.1	93.0
1.04_117.0186m/z	117.0186	ESI-	HMDB00202	1	x	M-H	C4H6O4	Methylmalonic acid	metabolism of fat and protein	-6.0	96.0
1.04_85.0289m/z	85.02887	ESI-	HMDB00549	1	x	M-H	C4H6O2	gamma-Butyrolactone	precursor of gamma-hydroxybutyrate.	-7.4	97.6
1.05_240.0017m/z	240.0017	ESI-	HMDB06512	1	x	M-H	C6H11NO5S2	3-mercaptolactate-cysteine disulfide	Cys derivative	4.7	75.3
1.11_145.0136m/z	145.0136	ESI-	HMDB00208	1	x	M-H	C5H6O5	Oxoglutaric acid	TCA cycle	-4.7	96.1
1.21_67.0184m/z	67.01842	ESI-	HMDB06853	1	x	M-H	C4H4O	3-Butyn-1-al	butanoate metabolism	-7.6	96.2
1.38_180.0656m/z	180.0656	ESI-	HMDB00158	1	x	M-H	C9H11NO3	L-Tyrosine	aminoacid	-5.4	99.1
1.60_162.0233m/z	162.0233	ESI-	HMDB01890	1	x	M-H	C5H9NO3S	Acetylcysteine	Cys derivative	1.3	91.9
1.64_257.0774m/z	257.0774	ESI-	HMDB04813	1	x	M-H	C10H14N2O6	3-Methyluridine	nucleoside	-2.1	93.6
1.84_328.0447m/z	328.0447	ESI-	HMDB00058	1	x	M-H	C10H12N5O6P	Cyclic AMP	nucleotide	-1.7	98.0
2.01_197.0452m/z	197.0452	ESI-	HMDB00291	1	x	M-H	C9H10O5	Vanillylmandelic acid	catabolism of catecholamines	-1.7	97.7
3.00_218.1025m/z	218.1025	ESI-	HMDB00210	1	x	M-H	C9H17NO5	Pantothenic acid	vitamin B5	-3.9	99.0
3.00_88.0397m/z	88.03966	ESI-	HMDB00056	1	x	M-H	C3H7NO2	beta-Alanine or 3-aminopropanoate	aminoacid	-8.4	96.9
3.17_382.0997m/z	382.0997	ESI-	HMDB00912	1	x	M-H	C14H17N5O8	Succinyladenosine	nucleoside	-1.9	98.2
3.22_123.0446m/z	123.0446	ESI-	HMDB00873	1	x	M-H	C7H8O2	4-Methylcatechol	catechol	-4.4	99.2
3.54_189.0763m/z	189.0763	ESI-	HMDB00325	1	x	M-H	C8H14O5	3-Hydroxysuberic acid	fatty acids	-2.9	92.4
3.59_143.0707m/z	143.0707	ESI-	HMDB01988	1	x	M-H	C7H12O3	4-Hydroxycyclohexylcarboxylic acid	carboxylic acid	-4.6	96.6
3.86_333.0520m/z	333.052	ESI-	HMDB00229	1	x	M-H	C11H15N2O8P	Nicotinamide ribotide	vitamin B3	8.0	92.2
4.67_457.1146m/z	457.1146	ESI-	HMDB01142	1	x	M-H	C17H23N4O9P	FMNH	vit B2 metabolism	3.5	87.7
5.27_160.0761m/z	160.0761	ESI-	HMDB03447	1	x	M-H	C10H11NO	Tryptophanol	indole	-4.4	93.6
5.57_155.1072m/z	155.1072	ESI-	HMDB04362	1	x	M-H	C9H16O2	4-Hydroxynonenal	lipid peroxidation	-3.6	93.5
5.81_155.1072m/z	155.1072	ESI-	HMDB04362	1	x	M-H	C9H16O2	4-Hydroxynonenal	lipid peroxidation	-3.6	97.3
5.83_229.1436m/z	229.1436	ESI-	HMDB00623	1	x	M-H	C12H22O4	Dodecanedioic acid	fatty acids	-4.2	89.8
5.99_155.1072m/z	155.1072	ESI-	HMDB04362	1	x	M-H	C9H16O2	4-Hydroxynonenal	lipid peroxidation	-3.5	92.3
6.07_200.1285m/z	200.1285	ESI-	HMDB00832	1	x	M-H	C10H19NO3	Capryloylglycine	metabolites of fatty acids	-3.8	96.2
6.78_427.1792m/z	427.1792	ESI-	HMDB00851	1	x	M-H	C18H28N4O8	Pyridinoline	alpha-aminoacids	-9.8	85.2
6.82_567.3166m/z	567.3166	ESI-	HMDB02596	1	x	M-H	C30H48O10	Deoxycholic acid 3-glucuronide	glucuronide of bile acids	-1.5	89.6

Table S2: A versus D The table reported the ID of the variables , the mass of the ion, the type of ESI, the HMDB ID, the type of adduct, the chemical formula, the putative marker, the pathway involved or the biological class of the compounds, the mass error of the m/z extracted respect to the matched compound and the isotopic similarity between the spectrum of the ion and the matched compound (expressed in %)

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