UNIVERSITÀ
DEGLI STUDI
DI PADOVA

Sede Amministrativa: Università degli Studi di Padova

Dipartimento di PEDIATRIA

SCUOLA DI DOTTORATO DI RICERCA IN :
MEDICINA DELLO SVILUPPO E SCIENZE DELLA PROGRAMMAZIONE
INDIRIZZO: MALATTIE RARE
CICLO: XXII

IL CONDENSATO DELL'ARIA ESPIRATA NELLO STUDIO DI PATOLOGIE RESPIRATORIE PEDIATRICHE

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RIASSUNTO

Background e obiettivi.

Negli ultimi 10 anni, nel campo della pneumologia pediatrica, c'è stato un crescente interesse verso lo sviluppo di metodiche non invasive per lo studio dell'infiammazione delle vie aeree. Infatti, sebbene la metodica gold standard sia rappresentata dalla broncoscopia con il broncolavaggio e le biopsie bronchiali, l'invasività di tale procedura ne limita l'uso a selezionate indicazioni cliniche non permettendo l'applicazione ad un'ampia popolazione, in particolare nell'ambito pediatrico.

Sono state pertanto sviluppate metodiche non invasive tra le quali la più studiata è la misura dell'ossido nitrico nell'aria esalata (FE_{NO}), marker di infiammazione eosinofilica delle vie aeree. Altra promettente metodica non invasiva è rappresentata dall'analisi del condensato dell'aria espirata (EBC). Il condensato è un biofluido ottenuto mediante il raffreddamento dell'aria espirata. La composizione di tale biofluido rispecchia le caratteristiche del liquido di superficie delle vie aeree. Caratteristica fondamentale del condensato è che permette il dosaggio di diversi biomarkers, consentendo di indagare sui vari processi patogenetici coinvolti nelle malattie respiratorie. Recentemente, accanto alla possibilità di dosare singoli mediatori, è stata proposta la possibilità di analizzare il condensato mediante un approccio innovativo: l'analisi metabolomica. Questa biotecnologia si basa sull'applicazione di tecniche spettroscopiche (generalmente la spettroscopia basata sulla RNM e la spettrometria di massa) combinate con un'analisi statistica multivariata e permette di identificare profili metabolici caratteristici di un gruppo di soggetti,

consentendo quindi la discriminazione sia tra sani e malati che tra soggetti con diversi fenotipi di malattia.

Obbiettivo del presente progetto di ricerca, sviluppato attraverso 3 studi distinti, è stato l'applicazione della metodica non invasiva del condensato dell'EBC nello studio di patologie respiratorie di interesse pediatrico.

Metodi e Risultati

1) Nel primo studio ("*Exhaled leukotriene B4 in children with community acquired pneumonia")* l'EBC è stato utilizzato per la prima volta nella valutazione di bambini con polmonite acquisita in comunità (CAP). Il leucotriene B4 (LTB4), un potente agente chemiotattico dei neutrofili attivati, è stato dosato nell'EBC di 18 bambini con CAP. La concentrazione dell'LTB4 era aumentata in questi bambini rispetto a 17 controlli sani ed andava incontro a normalizzazione dopo una settimana di terapia antibiotica. Lo studio ha dimostrato che mediante la metodica dell'EBC è possibile monitorare in modo non invasivo l'andamento di un marcatore della risposta biologica del polmone alle infezioni respiratorie nei bambini. Lo studio è stato pubblicato sulla rivista **Pediatric Pulmonology** (*Carraro S, et al. Exhaled leukotriene B4 in children with community acquired pneumonia. Pediatr Pulmonol. 2008;43:982-6*).

2) Nel secondo studio *("EIA and GC-MS analysis of 8-isoprostane in EBC of children with problematic asthma"*) abbiamo valutato l'8-isoprostano nell'EBC di bambini con asma ben controllato e con asma "problematico", dimostrando concentrazioni più elevate di questo marker di stress ossidativo nei bambini con asma problematico. Tale dato apre la strada

allo sviluppo di nuove strategie terapeutiche, mirate al controllo dello stress ossidativo, che potrebbero migliorare la gestione dell'asma problematico.Questo studio comprendeva anche una parte metodologica che consisteva nel confronto del dosaggio dell'8-isoprostano effettuato con metodica immunoenzimatica, che costituisce la tecnica più frequentemente utilizzata negli studi, e con gas comatografia accoppiata alla spettrometria di massa (GC-MS), che rappresenta una metodica analitica di riferimento. Le due metodiche di analisi hanno dimostrato una riproducibilità accettabile, sebbene la CG-MS sia caratterizzata da maggiore accuratezza. Lo studio è stato pubblicato sulla rivista **European Repiratory Journal** (*Carraro S, et al. EIA and GC-MS analysis of 8-isoprostane in EBC of children with problematic asthma. Eur Respir J. 2009 Nov 6. [Epub ahead of print]* doi:10.1183/09031936.00074909*)*

3) Il terzo studio ("*Metabolomic analysis of breath condensate in the characterization of asthma phenotypes in children"*) si è basato sull'applicazione dell'analisi metabolomica all'EBC per caratterizzare dal punto di vista metabolico diversi fenotipi di asma. L'analisi metabolomica, grazie alla sua natura non selettiva, permette il dosaggio contemporaneo di un numero molto elevato di metaboliti consentendo di individuare cluster di biomolecole coinvolte nella caratterizzazione di specifici gruppi di pazienti. Abbiamo dimostrato che l'analisi metabolomica è in grado di discriminare nettamente i bambini con asma lieve (trattati o meno con steroidi inalatori) da quelli con asma severo, suggerendo che un diverso profilo biochimico-infiammatorio sottende questi 2 fenotipi di asma. Nella caratterizzazione dei bambini con asma lieve, in particolare, è emersa

come importante una variabile che identifica un metabolita appartenente alla famiglia dei prostanoidi. Per i bambini con asma severo, pur non essendo stata identificata una singola variabile caratterizzante, è emerso un profilo metabolico che nel suo insieme li distingue nettamente dagli altri gruppi. Studi ulteriori potrebbero confermare il ruolo di tali profili metabolici dell'EBC nella caratterizzazione precoce del fenotipo asmatico nei bambini.

Conclusioni

Il presente progetto di ricerca dimostra nel suo complesso che la metodica del condensato dell'aria espirata può essere applicata con successo in diverse malattie respiratorie pediatriche sia acute, quali la polmonite, che croniche, quali l'asma.

Lo studio dell'8-isoprostano fornisce inoltre interessanti elementi metodologici, dimostrando una accettabile riproducibilità tra la metodica immunoenzimatica e la GC-MS, sebbene quest'ultima abbia una maggiore accuratezza.

Infine lo studio metabolomico rappresenta un significativo passo avanti nella caratterizzazione dei fenotipi di asma da un punto di vista biochimico-infiammatorio e apre la strada a nuovi studi che sfruttino questo approccio innovativo.

SUMMARY

Background and aims.

In the field of pediatric pulmonology, in the past 10 years there has been a growing interest toward the study of the airway inflammation by means of non-invasive techniques. In fact, although bronchoscopy with bronchoalveolar lavage and bronchial biopsies still represents the gold standard technique for the study of the lung, its invasiveness prevents a diffuse use, particularly when working with children.

Non-invasive techniques have therefore been developed, the most studied of which is the measurement of the exhaled nitric oxide (FE_{NO}), a marker of eosinophilic inflammation. A second promising non-invasive technique is represented by the analysis of exhaled breath condesate (EBC). This is a biofluid collected by cooling the exhaled air, the composition of which is believed to reflect that of airway lining fluid. The main advantage of this technique is that it allows the study of a wide range of biomarkers, enabling the study of different pathogenetic pathways in different respiratory diseases.

Recently, beside the measure of single biomarkers of disease, a new approach for the analysis of EBC has been proposed: the metabolomic approach. The metabolomic analysis is based on spectroscopic techniques (usually NMR-spectroscopy and mass spectrometry) combined with a multivariate statistical analysis, and it leads to the identification of metabolite profiles that characterize groups of subjects, enabling the

discrimination between healthy and ill subjects or between subjects with different disease phenotypes.

Aim of the present research project, which has been developed through 3 different studies, was the application of the EBC technique in the study of pediatric respiratory diseases.

Methods and Results.

1) In the first study ("*Exhaled leukotriene B4 in children with community acquired pneumonia")* the EBC was applied for the first time in the evaluation of children with community acquired pneumonia. Leukotriene B4 (LTB4), a strong chemotactic agent for activated neutrophils, has been measured in the EBC of 18 children with CAP. LTB4 concentration was higher in the EBC of these children than in the EBC of 17 healthy controls and normalized after one week of antibiotic therapy. The study demonstrated that by means of the EBC technique it is possible to non-invasively monitor a marker of the lung's biological response to infections in children. The study has been published in **Pediatric Pulmonology** (*Carraro S, et al. Exhaled leukotriene B4 in children with community acquired pneumonia. Pediatr Pulmonol. 2008;43:982-6*).

2) In the second study *("EIA and GC-MS analysis of 8-isoprostane in EBC of children with problematic asthma"*) we evaluated 8-isoprostanein the EBC of children with well-controlled and problematic asthma, finding increased levels of this biomarker of oxidative stress in the problematic asthma group. This finding paves the way to the development of new therapies - targeted at the control of oxidative stress - which could improve

the management of problematic asthma. This study had also a methodological objective: the comparison between the measurements of 8-isoprostane in EBC performed by enzymatic immunoassay and those performed by gas chromatography-mass spectrometry (GC-MS), a reference analytical technique. We found an acceptable reproducibility between the two methods, but the latter had higher accuracy. The study has been published in the **European Repiratory Journal** (*Carraro S, et al. EIA and GC-MS analysis of 8-isoprostane in EBC of children with problematic asthma. Eur Respir J. 2009 Nov 6. [Epub ahead of print]* doi:10.1183/09031936.00074909*)*

3) In the third study ("*Metabolomic analysis of breath condensate in the characterization of asthma phenotypes in children"*) the metabolomic analysis of EBC was applied to characterize from a metabolic standpoint different asthma phenotypes. Because of its non-selective nature, the metabolomic analysis considers a great number of metabolites and can identify clusters of bimolecules involved in the characterization of specific groups of patients. We found that the metabolomic analysis enables a clear discrimination between children with mild asthma (either regularly treated with inhaled steroids or steroid naive) and children with severe asthma. In the characterization of children with mild asthma a metabolite emerged as important, belonging to the family of prostanoids. In severe asthma, although no single variables were identified, there was an overall metabolic fingerprint that clearly characterizes this group. These results suggest that a different biochemical-inflammatory profile underlies these two asthma phenotypes. Further study could confirm the role of the EBC

metabolic profile in the early characterization of asthma phenotype in children.

Conclusions

In conclusion the research project demonstrated EBC can be applied with success in different respiratory diseases of childhood, both acute, as CAP, and chronic, as asthma.

The study on 8-isoprostane also provides important methodological information inasmuch as it demonstrates an acceptable reproducibility between the immunoenzymatic method (which is the most commonly used) and the reference analytical method GC-MS, though the latter is more accurate.

Eventually the metabolomic study represents a step forward the characterization of different asthma sub-phenotypes from a biochemical-inflammatory standpoint and it paves the way to further studies applying this innovative approach.

INTRODUZIONE

Approccio non invasivo nello studio delle vie aeree

La broncoscopia con l'ausilio del lavaggio broncoalveolare e della biopsia bronchiale costituisce la metodica gold standard per lo studio dei processi infiammatori a carico di vie aeree e polmone (1). Tuttavia tale metodica ha il limite intrinseco dell'invasività e, per tale motivo, il suo uso deve essere guidato dalla presenza di specifiche indicazioni cliniche. La necessità di avere informazioni sui processi patologici polmonari in modo più semplice ha spinto la ricerca verso lo sviluppo di metodiche non invasive. La non invasività è infatti una caratteristica di cruciale importanza in quanto consente di utilizzare tali tecniche in una popolazione più ampia di soggetti e permette campionamenti ripetuti nel tempo, rendendo possibile anche un monitoraggio longitudinale dei processi biochimico-infiammatori nelle patologie respiratorie croniche. Va inoltre sottolineato cha la non invasività è particolarmente importante quando la metodica deve essere applicata in ambito pediatrico.

Tra le metodiche non invasive quelle più studiate sono la misura dell'ossido nitrico nell'aria esalata (FE_{NO}) e la raccolta e analisi del condensato dell'aria espirata (EBC).

Il FE_{NO} è un marker che correla con la presenza di infiammazione eosinofilica nelle vie aeree ed il suo significato è stato studiato soprattutto in relazione alla malattia asmatica (2,3). L'American Thoracic Society e la European Respiratory Society hanno pubblicato delle linee guida per la misurazione standardizzata del FE_{NO} sia negli adulti che in età pediatrica, indicando come metodica gold standard la tecnica di misurazione online

ad espirio singolo con un flusso costante di 50 ml/sec (4,5). Nella pratica clinica la misura del FE_{NO} può avere diverse applicazioni nell'asma, sia nella fase diagnostica che nel monitoraggio dell'andamento della malattia e della risposta alla terapia (6,7).

Il condensato dell'aria espirata (EBC)

La tecnica del condensato dell'aria espirata (EBC) rappresenta un approccio innovativo che ha grandi potenzialità per la comprensione dei meccanismi biochimico-metabolici alla base delle malattie respiratorie (8,9). La raccolta del condensato è completamente non invasiva e di facile esecuzione tanto da essere possibile anche in bambini a partire dai 4 anni di età (10). La metodica consiste nel far respirare il soggetto a volume corrente attraverso un apparecchio (condensatore) che raffredda l'aria espirata permettendo la raccolta del condensato, un biofluido la cui composizione rispecchia quella del liquido di superficie delle vie aeree (8). Nel 2005 una Task Force congiunta dell'American Thoracic Society e della European Respiratory Society ha redatto un documento nel quale sono riportate le raccomandazioni per la raccolta e analisi del condensato e sono discussi punti di forza e criticità di tale approccio (9). La standardizzazione di questa metodica non è tuttavia ancora completa, e sono molto importanti gli studi metodologici che confrontano diversi approcci nella raccolta e nell'analisi di questo biofluido.

Nel condensato (EBC) sono state dosati numerosi mediatori che hanno permesso di indagare aspetti fisiopatologici relativi a varie patologie respiratorie (asma, fibrosi cistica, BPCO) (11). Tra questi meritano di

essere menzionati i leucotrieni, metaboliti dell'acido arachidonico che hanno un ruolo attivo in diversi processi infiammatori. Ad esempio, un significativo aumento di cisteinil-leucotrieni è stato dimostrato nell'EBC dei bambini asmatici (12) e, in particolare, di quelli con asma da sforzo (13). Il leucotriene B4 (LTB4) invece, marker di infiammazione neutrofilica, è stato riscontrato in concentrazioni elevate nell'EBC dei pazienti con BPCO (14).

Nell'EBC sono stati misurati anche numerosi marker di stress ossidativo. Nei pazienti asmatici, in particolare, è stato dimostrato un significativo aumento di 8-isoprostano (15), malondialdeide (16), nitrotirosina (17), tutti markers che indicano un aumento dello stress ossidativo nelle vie aeree dei pazienti affetti da asma.

L'analisi metabolomica

Recentemente è stato proposto un approccio innovativo allo studio dell'EBC: l'analisi metabolomica (18). Tale approccio permette di cambiare prospettiva spostandosi dallo studio del singolo biomarker, che non può riflettere da solo la complessità patogenetica di una malattia, allo studio di profili di biomarkers.

La metabolomica, ultima delle cosiddette scienze "omiche" (genomica, proteomica, transcriptomica), si caratterizza come metodica finalizzata alla descrizione biologica globale di un sistema mediante un approccio non selettivo che prende in considerazione simultaneamente un numero elevato di metaboliti in un campione biologico (19) . Essa comporta l'analisi e l'interpretazione dei dati metabolici di un sistema vivente, considerati nel loro complesso, come espressione della risposta

metabolica dell'organismo a stimoli patofisiologici o modificazioni genetiche (20).

Tra le scienze "omiche" la metabolomica è quella più vicina all'espressione fenotipica. I metaboliti, infatti, non sono semplicemente i prodotti dell'espressione genica ma sono il risultato dell'interazione tra il genoma e l'ambiente e comprendono in maniera integrata anche l'attività dell'apparato regolatorio (20). Questo significa che il quadro tracciato dall'insieme dei metaboliti presenti in un biofluido è quello che maggiormente può contribuire alla comprensione del fenotipo e delle sue modificazioni in relazione alle alterazioni genetiche ma anche agli stimoli patologici e alle influenze ambientali (ad es nutrizione, effetto di tossici, esposizione ad inquinanti) (21).

L'analisi metabolomica si basa sull'utilizzo di metodiche spettroscopiche (principalmente spettrometria di massa e spettroscopia RMN) e sulla successiva interpretazione dei dati analitici mediante approcci statistici multivariati che utilizzano strumenti bioinformatici (22).

Va sottolineato che il processo di caratterizzazione del profilo metabolico mediante l'approccio metabolomico ha come obiettivo non tanto l'identificazione di ogni singolo metabolita rappresentato nello spettro, quanto piuttosto l'individuazione di cluster di segnali. Tali cluster possono consentire di distinguere il sano dal patologico e di monitorare la risposta alla terapia seguendo l'evoluzione del profilo metabolico che da uno stato patologico torna, dopo il trattamento, al profilo di un soggetto sano (21). La metabolomica infatti permette di prendere in considerazione un grande numero di metaboliti, sia noti che non noti, e di individuare come questi si

associano, raggruppandosi in profili caratteristici che permettono la classificazione e discriminazione dei campioni (20,21).

La seconda fase dell'analisi metabolomica è rappresentata dal tentativo di identificare i singoli metaboliti che caratterizzano i diversi gruppi di soggetti in studio, aprendo la strada alla comprensione dei meccanismi patogenetici coinvolti nella malattia oggetto di studio e alla possibile identificazione di nuovi target terapeutici.

L'analisi metabolomica, potendo essere utilizzata nello studio di biofluidi raccolti in modo non invasivo (es. urina e condensato), ha interessanti prospettive di applicazione nell'ambito pediatrico (23). L'approccio metabolomico nello studio dell'EBC in patologie respiratorie croniche pediatriche è particolarmente promettente. Infatti, rispetto a biofluidi sistemici quali il sangue e l'urina, il condensato è "direttamente" raccolto dalle vie aeree ed è meno influenzato dal metabolismo di altri organi essendo pertanto in grado di rispecchiare in modo più fedele la fisiopatologia del sistema respiratorio.

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OBIETTIVI DEL PROGETTO DI RICERCA E PRINCIPALI RISULTATI

Obiettivo generale del progetto di ricerca è stato l'applicazione della metodica del condensato dell'aria espirata (EBC) nello studio di patologie respiratorie dell'età pediatrica.

Il progetto di ricerca si è articolato nei seguenti 3 studi:

1) **Leucotriene B4 nel condensato di bambini con polmonite acquisita in comunità (CAP)**

   Questo studio è stato volto a dosare il leucotriene B4 (LTB4), marker di infiammazione neutrofilica, nell'EBC dei bambini con polmonite acquisita in comunità (CAP). Abbiamo dimostrato che i bambini con CAP in fase acuta hanno un significativo aumento dei livelli di LTB4 nell'EBC, con normalizzazione di tale biomarker dopo una settimana di terapia antibiotica. Diverso è l'andamento della spirometria che in fase acuta mostra un pattern di tipo restrittivo e si normalizza solo a distanza di un mese.

   Lo studio ha dimostrato che mediante la metodica dell'EBC è possibile monitorare in modo non invasivo l'andamento di un marcatore della risposta biologica del polmone alle infezioni respiratorie nei bambini

Pubblicazione relativa a tale studio:

Carraro S, Andreola B, Alinovi R, Corradi M, Freo L, DaDalt L, Baraldi E. Exhaled leukotriene B4 in children with community acquired pneumonia. Ped Pulm 2008;43:982-986.

2) **Analisi immunoenzimatica (EIA) e spettrometria di massa accoppiata alla gas cromatografia (GC-MS) nel dosaggio dell'8-isoprostano nel condensato dell'aria espirata di bambini con asma problematica.**

Questo studio è stato condotto per valutare i livelli di 8-isoprostano, un prodotto di perossidazione lipidica considerato un valido marker di stress ossidativo, nell'EBC dei bambini con asma ben controllata e con asma "problematica".

Lo studio ha dimostrato che l'8-isoprostano ha concentrazioni più elevate nel condensato dei bambini con asma "problematica" rispetto a quelli con asma in buon controllo, suggerendo che lo stress ossidativo possa avere un ruolo centrale nella patogenesi di questo particolare fenotipo di asma. Il ruolo centrale dello stress ossidativo potrebbe anche spiegare la scarsa risposta alle terapia antiasmatica basata sugli steroidi inalatori e potrebbe aprire la strada allo sviluppo di nuove e più mirate strategie terapeutiche.

Dal punto di vista metodologico lo studio ha dimostrato che i dosaggi di 8-isoprostano effettuati con le due metodiche (EIA e GC-MS) hanno una riproducibilità accettabile, anche se la GC-MS ha una maggiore accuratezza, con una migliore riproducibilità intraindividuale.

Pubblicazione relativa a tale studio:

Carraro S, Cogo P, Isak I, Simonato M, Corradi M, Carnielli V, Baraldi E. EIA and GC-MS analysis of 8-isoprostane in EBC of children with problematic asthma. Eur Respir J published online before print November 6, 2009. doi:10.1183/09031936.00074909.

3) **Analisi metabolomica del condensato nella caratterizzazione dei diversi fenotipi di asma in età pediatrica.**

Scopo di questo studio è stata applicare l'analisi metabolomica all'EBC per caratterizzare dal punto di vista biochimico-metabolico bambini asmatici con diverso grado di severità della malattia. L'analisi metabolomica basata sulla spettrometria di massa ha permesso di discriminare chiaramente i bambini can asma lieve (caratterizzati da un buon controllo della malattia ottenuto mediante steroidi inalatori a basso dosaggio o mantenuto senza necessità di una terapia di fondo) dai bambini con asma severo (caratterizzati da scarso controllo della malattia nonostante l'uso di cortisonici inalatori ad alto dosaggio in combinazione con altri farmaci antiasmatici). Nella caratterizzazione dell'asma lieve è emersa l'importanza di una variabile che identifica un metabolita della famiglia dei prostanoidi. Per l'asma severo, sebbene non siano emerse singole variabili, è stata comunque possibile una chiara caratterizzazione del gruppo sulla base del profilo metabolomico complessivo. L'individuazione di tali profili apre la strada a studi futuri che valutino l'utilità del modello analitico costruito nella identificazione precoce dei fenotipi asmatici.

Tale lavoro è stato inviato come abstract al Congresso della European Respiratory Society che si terrà a Barcellona (18-22 Settembre 2010)

EXHALED LEUKOTRIENE B4 IN CHILDREN

WITH COMMUNITY ACQUIRED PNEUMONIA

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The paper has been published in *Pediatric Pulmonology* :

Carraro S, Andreola B, Alinovi R, Corradi M, Freo L, Da Dalt L, Baraldi E.
Exhaled leukotriene B4 in children with community acquired pneumonia.
Pediatr Pulmonol. 2008 Oct;43(10):982-6

ABSTRACT

Background: The infiltrate in pneumonia is characterized by a large number of activated neutrophils, for which leukotriene B4 (LTB4) is a strong chemotactic agent. Exhaled breath condensate (EBC) is a non-invasive technique for studying the lower airways. The present study was conducted to measure EBC LTB4 as a potential non-invasive marker of inflammatory response in community acquired pneumonia (CAP).

Methods: 18 children with CAP and 17 healthy children were recruited (age 5-13). The CAP children underwent physical examination, chest X-ray, leukocyte count and C-reactive protein measurement. The CAP and the control children performed spirometry, exhaled nitric oxide measurement (FE_{NO}) and EBC collection for LTB4 assessment. In the CAP children spirometry, FE_{NO} and EBC collection were repeated twice over a one-month follow-up.

Results: LTB4 EBC concentrations were higher in children with CAP than in healthy controls (10 pg/ml [7.0-15.3] vs 3 pg/ml [3.0-6.9], p=0.001) and decreased after one week (3 pg/ml [3.0-7.2], p<0.01) with no further change a month later. In the acute phase spirometry demonstrated a restrictive pattern that gradually improved later. No difference in FE_{NO} levels was found between children with CAP and healthy controls.

Conclusion: Exhaled LTB4 levels increase in CAP and returns to normal after one week. EBC collection is feasible in children with CAP and may represent a new way to non-invasively monitor the lung's biological response to infections.

INTRODUCTION

Community-acquired pneumonia (CAP) is still a potentially serious infection (1). It is quite common in children (2,3), being the third reason for hospitalization in the USA for people under 18 years old, exceeded only by injuries and asthma (4).

The pathophysiological processes involved in the occurrence and regression of CAP are known from the histopathological standpoint, but very few data are available on the in vivo measurement of CAP-related biological products. Systemic inflammatory markers are easy to measure in blood, but they correlate poorly with proven pneumonia (5). Bronchoalveolar lavage enables the direct collection of samples from the lung in which several inflammatory biomarkers can be measured (6), but bronchoscopy is an invasive procedure that is unacceptable for routine use in children. In the last few years, ~~a~~ growing interest has focused on non-invasive methods for studying the lung, one of which involves analyzing exhaled breath condensates (EBC). The EBC technique is entirely non-invasive, safe and easy to perform, even in young children (7-9).

Neutrophils are the most important effector-cells of innate immune response during lung infection (10,11). Activated neutrophils are recruited into the lung, where they accumulate moving up the chemotactic gradient created by several inflammatory mediators released at the site of infection (10).

LTB4 has an important role among the mediators involved in the inflammatory response to lung infections, it is released by macrophages

and neutrophils and, in turn, it stimulates neutrophil chemotaxis, enhances neutrophil-endothelial interactions and stimulates neutrophil activation, leading to degranulation and release of mediators, enzymes, and superoxides (12). LTB4 is also involved in the innate immune response itself, being able to enhance phagocytosis and killing of bacteria (13).

The aim of the present study was to evaluate the possible role of EBC LTB4 as a biological marker of the evolution of CAP in children. In addition we proposed to assess the trend of exhaled air levels of nitric oxide - a mediator of innate immune response to infections - and lung function parameters in relation to the evolution of CAP.

MATERIALS AND METHODS

Subjects and study design

We recruited 18 children consecutively admitted to our hospital's Emergency Room for community-acquired pneumonia (CAP). They all had fever, respiratory symptoms and lung infiltrates on chest X-ray.

At the time of their recruitment, the children underwent physical examination and FE_{NO} measurement, spirometry and EBC collection. A blood sample was also drawn to measure leukocyte count and C-reactive protein (CRP). Then a course of antibiotic therapy was administered, according to the BTS guidelines (14). EBC collection, FE_{NO} measurement and spirometry were repeated twice during the follow-up (one week and one month after recruitment). One child was lost to the one-week and 6 to the one-month follow up.

The CAP patients were otherwise healthy children, with no personal history of atopic diseases.

As a control group, 17 age-matched healthy children with no history of atopy, respiratory diseases or any other chronic disease were recruited from among the relatives of doctors and nurses at our hospital.

The Ethics Committee of our hospital reviewed and approved the protocol and all parents gave their informed consent.

Table 1. Characteristics of subjects enrolled in the study

	CAP children	**Healthy children**
Number (males)	18 (10)	17 (7)
Age (years)	8.5 (range 5-13)	8.5 (range 6-13)
White blood cell count (number/ml)*	10705 (7550-16290)	NA
PMN leukocytes (number/ml)*	5925 (4440-13850)	NA
C reactive protein (mg/L)*	49.8 (26.5-173)	NA
EBC LTB4 (pg/ml) *	10 (7.0-15.3)	3 (3.0-6.9)
FEV1 (%pred) *	75 (62-78)	95 (92-106)
FVC (%pred) *	73 (61-78)	95 (90-108)

*Data are expressed as median and interquartile range.

CAP= community acquired pneumonia; EBC= exhaled breath condensate; FEV1= forced expiratory volume in one second; FVC= forced vital capacity; LTB4= leukotriene B4; NA= not applicable; PMN= polymorphonucleate;

EBC collection

EBC was collected and processed according to recent ATS/ERS recommendations (8). EBC was collected using the TURBO-DECCS (transportable unit for research on biomarkers obtained from disposable exhaled condensate collection systems) (ItalChill, Parma, Italy).

As previously reported (15), TURBO is a refrigerating system that relies on a thermo electrical module giving rise to a Peltier effect. The cold side of the Peltier module is connected to an aluminium support shaped to house the test tube. TURBO is supplied with DECCS, a disposable respiratory system that consists of a mouthpiece equipped with a one-way valve and a reliable saliva trap, connected to a collecting vial (50 ml) by means of a tube. The children breathed tidally through the mouth for 15 minutes, while sitting comfortably and wearing a nose clip. They kept their mouths dry during EBC collection by periodically swallowing excess saliva. EBC samples were stored at –80°C in polypropylene tubes until assay.

Leukotriene measurements

LTB_4 concentrations were measured in the EBC using a specific enzyme immunoassay kit (Cayman Chemical Milan, Italy), according to the manufacturer's instructions and as previously described (16,17). Amylase concentrations were checked in duplicate in each sample to exclude salivary contamination.

Fractional exhaled nitric oxide (FE_{NO}) measurement

FE_{NO} was measured with the NIOX system (Aerocrine, Stockholm, Sweden) using a single-breath on-line method according to the ERS/ATS

guidelines for measuring FE_{NO} in children (18). Children inhaled NO-free air to total lung capacity and exhaled through a dynamic flow restrictor with a target flow of 50 ml/sec for at least 6-7 seconds. No nose clip was used. The NIOX system was calibrated using a 200 ppb NO tank (Lindegas Hoek Loos Speciality gases, Amsterdam, Netherlands) according to the manufacturer's instructions.

Lung function test

Lung function parameters were measured with a 10-liter bell spirometer (Biomedin, Padova, Italy) and the best of three maneuvers was expressed as a percentage (%) of the predicted reference values according to Polgar and Promadhat (19).

Statistical analysis

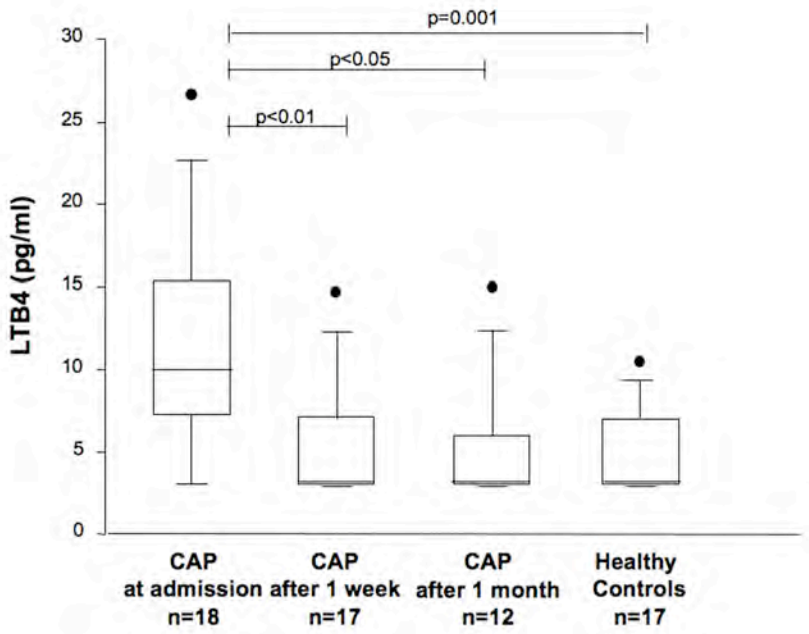
Since data were not normally distributed, they are given as medians and interquartiles ranges, and analyzed using non-parametric methods. The Mann-Whitney U test was used for comparisons between groups. Repeated-measures (RM) ANOVA on ranks was used, with an appropriate post-hoc test (Dunn's method), to compare data between the acute phase and the one-week and one-month follow-up visits. Correlations were evaluated using Spearman's test. Results were considered significant at a value of $p< 0.05$.

RESULTS

LTB4

We found significantly higher EBC LTB4 levels in children with CAP than in healthy controls (10 pg/ml [7.0-15.3] vs 3 pg/ml [3.0-6.9], p=0.001) (figure 1). LTB4 concentrations returned to normal after a one-week course of antibiotic therapy (3 pg/ml [3.0-7.2], p<0.01 vs acute phase, p=0.84 vs healthy controls), with no further change a month later (3 pg/ml [3-5.9]) (figure 1).

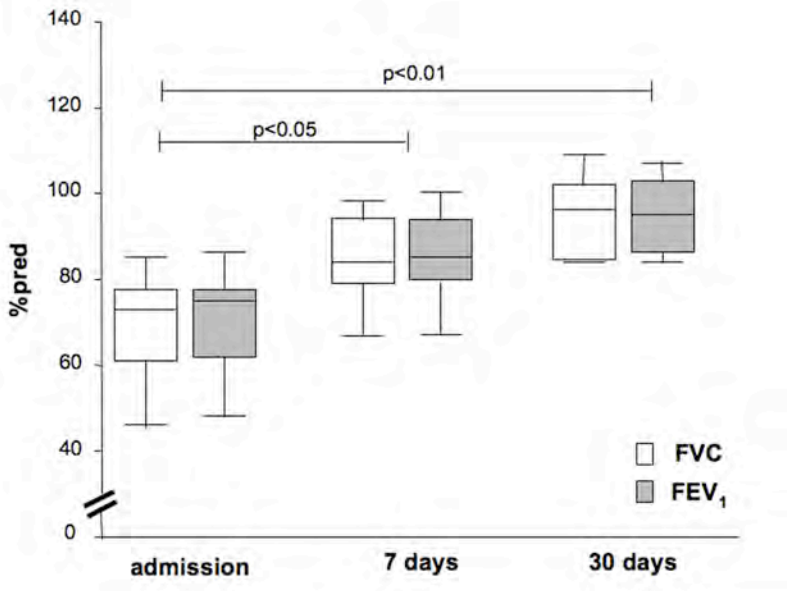
Figure 1



Lung function parameters

Children with CAP showed a restrictive spirometric pattern, with a reduced FEV_1 and FVC and a normal FEV_1/FVC ratio. Lung function parameters were significantly worse in the acute phase of CAP (FEV_1 75% pred [62-78], FVC 73% pred [61-78]) and they improved significantly after one week (FEV_1 85% pred [80-94], FVC 84% pred [79-94], $p<0.05$), showing a further improvement after one month (figure 2), by which time they had returned to normal and were no different between CAP cases and healthy children (FEV1 95% pred [86-103] vs FEV_1 95% [92-106], $p=0.43$; FVC 96% pred [89-102] vs FVC 95% pred [90-108], $p=0.55$) (table 1).

Figure 2



FE_{NO}

No differences emerged in FE_{NO} levels between children with CAP at the time of hospital admission and healthy controls (7.9 ppb [5.0-18.3] vs 8.3 ppb [6.2-14.0], p=0.98). The FE_{NO} levels measured during the follow up in the children with CAP were also no different from the baseline levels (1 week: 8.5 ppb [6.3-11.8]; 1 month 15 ppb [9.1-18.0], p=ns).

Correlations

We found no significant correlation between EBC LTB4 levels and lung function measures, nor was there any apparent correlation with total leukocyte count, PMN count or CRP blood levels (table 1).

DISCUSSION

In the present study, we applied EBC to the non-invasive study of acute lung infection, assessing the concentrations of exhaled LTB4 -a lipid mediator that induces leukocyte chemotaxis and activation- at various time points. We found that LTB4 levels were three times higher in children with CAP and that they returned to normal after one week (figure 1).

LTB4 has a strong chemotactic effect on neutrophils, activating them and increasing their capacity for the phagocytosis and killing of bacteria, playing a key part in immune response to lung infection (13).

A previous study conducted on rats, demonstrated that LTB4 plays an important role in inducing neutrophilic lung inflammation after exposure to staphylococcal enterotoxin, supporting the role of this mediator in response to lung infections (20). In keeping with our findings, Hopkins et al (6) demonstrated an increase in LTB4 levels in the bronchoalveolar lavage (BAL) of adult patients with pneumonia, suggesting that this mediator can contribute to the strong neutrophil chemotactic activity measured in these patients' lungs. The main novelty of our study, with respect to Hopkins' research on BAL, lies in our use of an entirely non-invasive method to investigate lung pathobiology. Few studies have sought markers of oxidative stress (21,22) and epithelial regeneration (23) in EBC of adult patients with CAP and, to our knowledge, there are no reports of this technique being applied to children with CAP. More in particular, although EBC LTB4 levels have been investigated in other conditions, such as asthma (24) and COPD (25), to our knowledge there are no previous

studies investigating exhaled LTB4 as a marker of leukocyte response in adults or children with CAP.

Breath condensate is obtained by cooling exhaled air and consists mainly of water vapor, but it also contains molecules coming from the respiratory tract (26). Though the technique has yet to be fully standardized and several factors, such as the effect of different collecting materials on the mediators measured (27), need to be considered when interpreting the results, EBC is a promising technique for investigating the lung (28). It is also easy to apply in children too because it requires only a minimal degree of cooperation.

LTB4 is more than just a generic marker of the inflammatory burst caused by an infection because it enhances macrophage phagocytosis, having an important role as a modulator of innate immune response (13, 29). Further studies investigating EBC LTB4 levels in children and adults might establish the expected concentrations of exhaled LTB4 as a marker of adequate immune response in patients with CAP. EBC could also be used to search for the etiological agent responsible for the infection, though further studies are needed to assess the feasibility of such a novel use of this technique.

Looking at the time-related trend of EBC LTB4 levels, we found they rose during the acute phase of the infection but returned to normal after a week of antibiotic therapy (figure 1). Biernacki et al (30) likewise reported higher EBC LTB4 levels in COPD patients during bacterial exacerbations, recording significant reductions after a course of antibiotic therapy. These observations support the role of LTB4 as a biological marker of acute

immune response to infection, which returns to normal once the microbial agent has been dealt with.

The present study identified a restrictive spirometry pattern in CAP patients, probably reflecting a reduction in lung volumes due to the infiltrate, with lung function parameters rising back to normal a month after the acute infection. Radiologically-confirmed resolution of infiltrate takes one or two months on average, as well (31,32). Compared to lung function measurements and radiological images, LTB4 thus reflects different aspects of CAP pathophysiology, acting as a biological marker of the evolution of the acute phase of CAP. In keeping with this hypothesis we found no correlation between lung function measurements and LTB4 concentrations.

Nor did any correlation emerge between LTB4 and systemic inflammatory markers, such as C-reactive protein, total white blood cells count, and neutrophils. A recent systematic review demonstrated that CRP is scarcely accurate in detecting radiologically-proven pneumonia in cases with symptoms of lower respiratory tract infection (5). It would be interesting to investigate whether LTB4 measured in EBC, being a direct marker of the lung's immune response, is more accurate than systemic markers of inflammation in identifying children with CAP.

Nitric oxide is part of the innate inflammatory response and its levels are expected to rise in acute lung infection (10) but, during the acute phase, our CAP cases had FE_{NO} levels no different from those detected in healthy subjects. There is a couple of possible explanations for our finding. NO output from expiratory flows of 50 mL/s mainly derives from airway NO

diffusion (18), so we can hypothesize that with higher flow rates, sampling deeper parts of the lung, higher NO levels might be found, as previously reported in lung allografts during pulmonary infection (33). Moreover, NO might react rapidly with reactive oxygen species, released by leukocytes at the site of infection, forming NO-metabolites (21).

A limit of this study is that we could not identify the etiological agents, so we cannot establish whether there is any correlation between the biological response in terms of EBC LTB4 levels and the microorganism involved in the disease. Another limit is that the immunoenzymatic method used to measure LTB4 works close to its detection limit when analyzing leukotrienes in EBC. Nevertheless the measurement of LTB4 in EBC has been validated by high performance liquid chromatography, which demonstrated that LTB4 is present in exhaled breath condensate and that it is specifically identified by the immunoenzymatic assays (34).

In conclusion, we have shown that children with CAP have increased LTB4 levels in EBC that return to normal after one week. The major novelty of our study lies in that it demonstrates the feasibility of using exhaled breath condensate to non invasively monitor markers of the lung's biological response to infections in children.

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EIA AND GC-MS ANALYSIS OF 8-ISOPROSTANE IN EBC OF CHILDREN WITH PROBLEMATIC ASTHMA

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The paper has been published in the *European Respiratory Journal* :

Carraro S, Cogo PE, Isak I, Simonato M, Corradi M, Carnielli VP, Baraldi E.
EIA and GC-MS analysis of 8-isoprostane in EBC of children with problematic
asthma. Eur Respir J. 2009 Nov 6. [Epub ahead of print] PubMed PMID: 19897556 doi: 10.1183/09031936.00074909

ABSTRACT

Background. Asthmatic airways are characterized by enhanced oxidative stress, which can be studied by measuring biomarkers, such as 8-isoprostane. Aims of the present study were: 1) to measure the concentrations of 8-isoprostane in exhaled breath condensate (EBC) and urine of children with problematic and well-controlled asthma; 2) to compare the concentrations of 8-isoprostane measured by gas chromatographic/negative ion chemical ionization mass spectrometry (GC/NICI-MS) and by an enzymatic immunoassay (EIA).

Methods. We recruited 20 asthmatic allergic children, 13 with well-controlled asthma and 7 with problematic asthma. They underwent exhaled nitric oxide measurements and spirometry, and both EBC and urine samples were collected. 8-isoprostane was measured in EBC by GC/NICI-MS and EIA.

Results. 8-isoprostane concentrations in EBC were significantly higher in children with problematic asthma than in children with well-controlled asthma ($p=0.01$). An acceptable reproducibility emerged between GC/NICI-MS and EIA (coefficient of reproducibility 11.5 pg/ml). 8-isoprostane levels measured in urine did not correlate with those measured in EBC.

Conclusion. We showed that 8-isoprostane in EBC was significantly increased in children with problematic asthma, suggesting a role for oxidative stress in this asthma phenotype. In addition we found an acceptable reproducibility of EIA compared to GC/NICI-MS, even if the latter method had higher accuracy.

INTRODUCTION

A number of recent studies have demonstrated an enhanced oxidative stress in asthma, as a consequence of an increased release of oxidant species and a decline in antioxidant defenses (1). It is difficult to measure reactive oxygen species (ROS) directly because they are highly reactive and short-lived, so oxidative stress is often studied by measuring products of the interaction between ROS and lipids, protein or deoxyribonucleic acid (1). Isoprostanes are prostaglandin-like compounds formed from the free radical catalyzed peroxidation of arachidonic acid, a mechanism independent of the cyclo-oxygenase (2,3). 8-isoprostane is therefore a stable product of lipid peroxidation and it is a reliable marker of oxidative stress (4). This marker can be measured in exhaled breath condensate (EBC), a biofluid collected non-invasively by cooling exhaled air during tidal breathing (5). EBC is a promising methodology inasmuch as the condensate composition is believed to reflect that of the airway lining fluid. However, the EBC technique has not been fully standardized yet and there are several methodological pitfalls such as the sensitivity of the available assays, and unresolved issues such as the definition of the exact anatomic origin of the biomarkers measured (5).

Increased levels of 8 isoprostane have been found in EBC of asthmatic subjects confirming the role of oxidative stress in the pathogenesis of asthma (6,7).

In many studies an immunoenzymatic technique (EIA) has been used to measure 8-isoprostane in EBC (7-10). Though the EIA approach is known to have some weaknesses in the analysis of EBC (inasmuch as the assay

works close to its detection limit), to our knowledge no published studies have compared EIA with a reference analytical method based on mass spectrometry in the study of EBC.

It is widely acknowledged that asthma can be easily controlled in most cases, although there is a small subgroup of children with recurrent severe exacerbations or chronic symptoms despite prescription of multiple drugs. The term “problematic asthma” has been recently been proposed to describe these children (11) and efforts should be made to better characterize this phenotype.

The aims of the present study were 1) to measure the concentrations of 8-isoprostane in exhaled breath condensate (EBC) of children with problematic and well-controlled asthma; 2) to compare the concentrations of 8-isoprostane measured by gas chromatographic/negative ion chemical ionization mass spectrometry (GC/NICI-MS) and by an enzymatic immunoassay (EIA). In addition, we compared the 8-isoprostane measured in EBC and in a urine sample, collected at the same time, in order to establish whether EBC, being a biofluid collected directly from the lung, can reflect pathological processes in the lung better than a systemic matrix such as urine.

METHODS

Patients

Twenty asthmatic children were enrolled, whose asthma was diagnosed by a pediatric respiratory physician based on their clinical history (cough, shortness of breath, recurrent wheezing, chest tightness) and an increase in FEV_1 after salbutamol (400 mg) >12%, according to international guidelines (12). Seven of these children had problematic asthma (11) while the other 13 children had well-controlled asthma (12). Problematic asthma was defined as the presence of chronic symptoms and/or frequent exacerbations and/or persistent airflow obstruction, despite treatment with high dose of inhaled steroids (ICS) (12) combined with long acting beta-2 agonists (n=7), montelukast (n=2) and theophylline (n=1). Cystic fibrosis, immunodeficiency, chronic rhinosinusitis had been excluded. Gastro-esophageal reflux had been demonstrated and treated in 3 of these children. Nine of the 13 children with well-controlled asthma were treated with low-medium doses of inhaled steroids and 7 were also taking long acting beta-2 agonists.

All asthmatic children were atopic, sensitized to at least one airborne allergen, as demonstrated by skin prick tests.

At recruitment, children underwent physical examination, FE_{NO} measurement and spirometry. Patients were instructed to abstain from short-acting bronchodilators for 8 hours and from long-acting bronchodilators for 18 hours before spirometry.

EBC and urine samples were collected and stored at –80°C, and subsequently analyzed by EIA (for EBC and urine) and GC/NICI-MS (for

EBC alone). In 8 children, two EBC samples were collected 1 hour apart to evaluate the analytical reliability.

All the study procedures were conducted in the afternoon.

EBC and urine analyses were performed at the Department of Pediatrics of Padova.

The Ethics Committee at our hospital reviewed and approved the protocol and all parents gave their informed consent.

Fractional exhaled nitric oxide (FE_{NO}) and pulmonary function measurement

FE_{NO} was measured using the NIOX system (Aerocrine, Stockholm, Sweden), following the ERS/ATS guidelines for measuring FE_{NO} in children (13). Children inhaled NO-free air to total lung capacity and exhaled through a dynamic flow restrictor with a target flow of 50 ml/sec for at least 6-7 seconds. No nose clip was used. The NIOX system was calibrated using a 200 ppb NO tank (Lindegas Hoek Loos Speciality gases, Amsterdam, Netherlands) according to the manufacturer's instructions.

Lung function was measured by means of a 10-liter bell spirometer (Biomedin, Padova, Italy) and the best of three maneuvers was expressed as a percentage (%) of predicted reference values, according to ERS/ATS guidelines (14).

EBC collection

EBC was collected and processed according to recent ATS/ERS recommendations (5), using the TURBO-DECCS (a transportable unit for research on biomarkers obtained from disposable exhaled condensate collection systems) (Medivac, Parma, Italy).

As reported elsewhere (15), the TURBO is a refrigerating system that relies on a thermoelectric module producing a Peltier effect. The cold side of the Peltier module is connected to an aluminium support shaped to house the test tube (16). The temperature is maintained constant during the collection (we used a collecting temperature of –4°C). The TURBO is supplied with the DECCS, a disposable respiratory system that consists of a mouthpiece equipped with a one-way valve and a reliable saliva trap, connected to a collecting vial (50 ml) by means of a tube (16). The children breathed tidally through the mouth for 15 minutes, while sitting comfortably and wearing a nose clip. They kept their mouth dry during EBC collection by periodically swallowing excess saliva. EBC samples were stored at –80°C in polypropylene tubes until assay.

Urine collection

Urine samples, collected right after the EBC collection, were immediately stored at –80°C in polypropylene tubes until assay.

Enzyme immunoassay of 8-isoprostane

One 50 ml aliquot of unextracted EBC was assayed in duplicate according to the manufacturer's protocol for the 8-Isoprostane (8-iso-$PGF_{2\alpha}$) specific enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI) and 8-

iso-$PGF_{2\alpha}$ concentrations in EBC were measured by plotting the values identified in the sample with the 8-iso-$PGF_{2\alpha}$ standard calibration curve (3.9-500 pg/ml).

8-iso-$PGF_{2\alpha}$ concentrations in urine were measured using the same EIA kit. Samples were prepared prior to the assay using a purification protocol recommended by the manufacturer (17). Briefly, the pH of the urine was adjusted to approximately 4.0 with 0.1 M HCl and a 0.5 ml aliquot of urine was extracted in duplicate on a SPE C-18 cartridge (Alltech), previously rinsed with 5 ml of methanol followed by 5 ml of UltraPure water. The columns were then washed with 5 ml of UltraPure water, 5 ml of hexane and allowed to dry. The 8-iso-$PGF_{2\alpha}$ was eluted with 5 ml ethyl acetate containing 1% methanol. The eluate underwent silica gel chromatography carried out with a solution of chloroform / methanol / acetic acid / water (80:18:1:0.8 v/v). 8-iso-$PGF_{2\alpha}$ was eluted with 4 ml ethanol, dried at 37°C under a nitrogen stream and reconstituted with EIA buffer. The antiserum used in the EIA has 100% cross-reactivity with 8- isoprostane and 20.6%, 4.00%, 1.84%, 1.70% respectively with $PGF_{3\alpha}$, 2,3-dinor-$PGF_{2\alpha}$, $PGE_{2\alpha}$, 2,3-dinor-$PGF_{1\alpha}$ PGE_1, as declared by the manufacturer. The lowest detection limit of the assay was 3.9 pg/ml. 8-iso-$PGF_{2\alpha}$ concentrations were expressed in pg/ml for EBC and in ng/mmol creatinine for urine samples. Creatinine urine concentrations were measured with the SPOTCHEM II Creatinine Reagent Strip in the SPOTCHEM Analyzer (A Menarini Diagnostics).

Gas chromatography / negative ion chemical ionization mass spectrometry (GC/NICI-MS) of 8-isoprostane

8-iso-$PGF_{2\alpha}$ concentrations in EBC were measured using a gas chromatographic/negative ion chemical ionization mass spectrometric (GC/NICI-MS) approach with a stable isotope dilution method modified from Milne et al. (18) using a quadrupole mass spectrometer (Voyager, Thermoquest, Rodano, Milano, Italy). Sample preparation was carried out prior to assaying, first by extraction and then by derivatization. After acidifying 0.5 ml aliquots of EBC, in duplicate, to pH 3 with HCl 0.1 M, 2 ng of the deuterated internal standard 8-iso-$PGF_{2\alpha}$-d_4 (Cayman Chemical, Ann Arbor, MI) was added. After adding the internal standard, the mixture was vortexed and applied on an HLB (Oasis Waters) cartridge previously prepared by rinsing with 2 ml of methanol followed by 2 ml of UltraPure water (pH 3.0) and left to dry. The columns were washed with 3 ml of UltraPure water (pH 3.0) followed by 3 ml of hexane. 8-iso-$PGF_{2\alpha}$ was eluted with 4 ml of ethyl acetate and dried at 40°C under a nitrogen stream. The extract was then converted into pentafluorobenzyl (PFB) ester by treating it with a mixture of 40 ml of 10% pentafluorobenzyl bromide in acetonitrile and 20 ml of 10% *N,N*-di-isopropyl ethylamine (DIPEA) in acetonitrile at room temperature for 30 minutes. The reagents were dried under a nitrogen stream, then the 8-iso-$PGF_{2\alpha}$ was converted into trimethylsilyl ether derivative by adding 20 ml of N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide (BSTFA) and 10 ml of pyridine; the mixture was incubated at 40°C for 20 minutes, dried under a nitrogen stream and the derivatized 8-iso-$PGF_{2\alpha}$ was redissolved in 20 ml of

undecane for analysis by GC/NICI-MS. 8-iso-$PGF_{2\alpha}$ was separated on a 30 m*0.25mm*0.25 µm ULTRA2 fused silica capillary column (J&W Scientific Agilent Technologies Italia S.p.A., Cernusco sul Naviglio, Milano, Italy). The oven temperature is programmed for 1 minute at 190°C, 20°C per minute from 190°C to 300°C, and maintained at 300°C for 12 minutes. Methane was used as carrier gas for the GC/NICI-MS. The ion monitored for 8-iso-$PGF_{2\alpha}$ was *m/z* 569, while for the internal standard it was *m/z* 573.

Statistical analysis

Normally distributed data were recorded as mean ± SEM. Non-normally distributed data (urinary 8-isoprostane and FE_{NO} values) were reported as medians and interquartile ranges (IQR) and were log transformed (achieving a normal distribution) to perform the statistical analysis.

The between method reproducibility of the 8-isoprostane measurements using EIA and GC/NICI-MS was assessed by Bland Altman analysis (19). The coefficient of reproducibility was calculated as 1.96*SD of the differences between the measurements performed with the two methods, and it was used to define the limits of agreement.

The reliability of EIA and GC/NICI-MS was evaluated by calculating the intraclass correlation coefficient in a subgroup of children who performed two consecutive EBC collections 1 hour apart.

The comparison between children with problematic asthma and children with well-controlled asthma was performed by t-test for 8-isoprostane EBC concentrations and log-transformed urinary 8-isoprostane concentrations and FE_{NO} values.

Correlations were evaluated by applying Pearson's test. Results were considered significant at a value of $p< 0.05$.

Power calculation revealed that a sample size of 7 enables a difference in EBC 8-isoprostane levels of 30 pg/ml to be detected with a power of 83% at a 2 sided a level of 0.05.

RESULTS

8-isoprostane in children with problematic and well-controlled asthma

We found that children with problematic asthma had significantly higher 8-isoprostane levels than children with well-controlled asthma. This was true for the measurements obtained by both GC/NICI-MS (p=0.008) (figure 1) and EIA (p=0.01) (table).

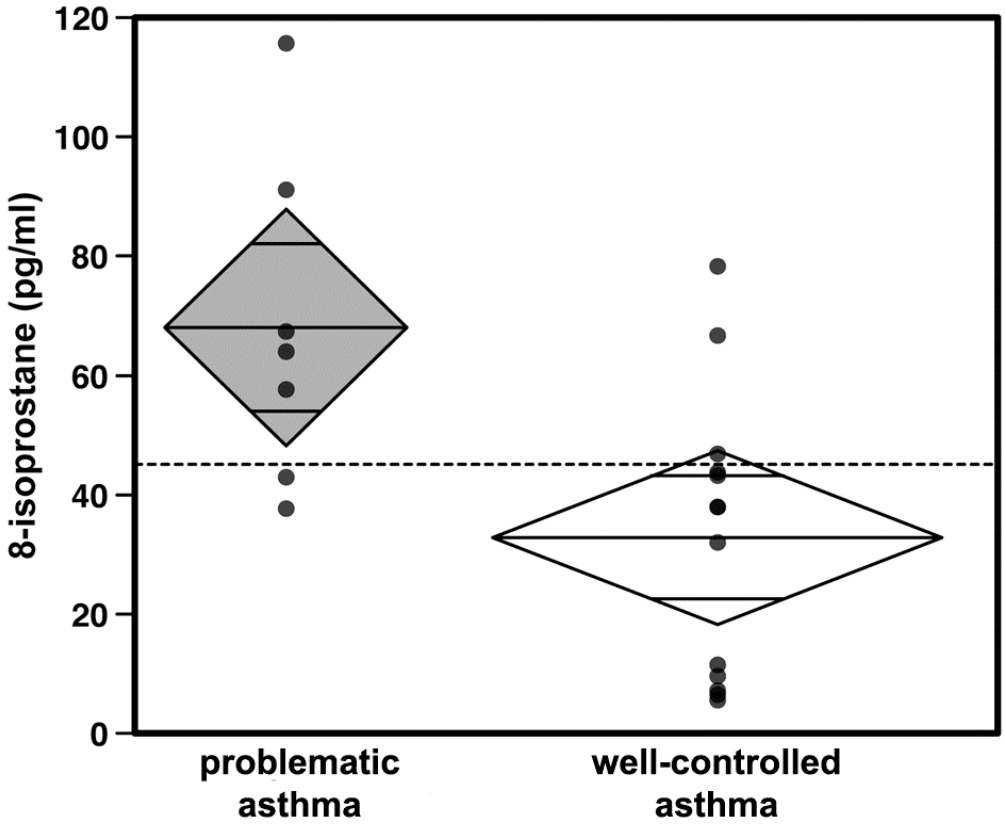

Figure 1. The diamond plot represents EBC 8-isoprostane levels in children with problematic asthma and with well-controlled asthma measured by GC/NICI-MS. The line through the center of each diamond represents the group mean. The top and bottom vertices are the upper and lower 95% confidence limits. The horizontal dashed line is the overall mean. The black dots are the individual values. The difference between the two groups is statistically significant (p=0.008).

On the contrary, the comparison of urine 8-isoprostane levels did not show any difference between problematic and well-controlled asthma (p= 0.4) (table).

FE_{NO} was no different in problematic and well-controlled asthmatic children (p=0.32) (table).

When either the whole group of children or the well-controlled group was considered, no difference was found between those treated with ICS and those steroid naive (p=0.63 and p=0.59, respectively).

	Problematic asthma	Well-controlled asthma	p-values
Number (males)	7 (4)	13 (7)	
Age (years)	9.6 (range 6-13)	11.1 (range 6-15)	
Height (cm)	140.6 ± 6.3*	146.8 ± 3.1*	
Weights (Kg)	35.6 ± 3.9*	45.8 ± 3.6*	
8-isoprostane in EBC by GC/NICI-MS (pg/ml)	68.0 ± 10.3*	32.8 ± 6.6*	0.008
8-isoprostane in EBC by EIA (pg/ml)	74.0 ± 12.5*	35.3 ± 7.4*	0.01
8-isoprostane in urine (EIA) (ng/mmol creatinine)	35.4 (30.6-60.6)**	50.6 (35.1-74.9)**	0.44
FVC (%pred)	89 ± 6*	96 ± 3*	0.26
FEV_1 (%pred)	76 ± 5*	89 ± 3*	0.048
FEF_{25-75} (%pred)	59 ± 7*	82 ± 7*	0.05
FEV_1/FVC (%)	79 ± 4*	84 ± 2*	0.20
FE_{NO} (ppb)	16 (9.5-47.5)**	27 (17.3-48.0)**	0.36

Table. Anthropometric characteristics, 8-isoprostane concentrations, spirometric parameters and exhaled nitric oxide values in children with problematic and well-controlled asthma. GC/NICI-MS: gas chromatographic/negative ion chemical ionization mass spectrometry; EIA: enzymatic immunoassay; EBC: exhaled breath condensate; *data expressed as mean and SEM; **data expressed median and IQR

Reproducibility of EBC 8-isoprostane measurements

The Bland Altman plot showed an acceptable reproducibility of the 8-isoprostane measurements obtained by GC/NICI-MS and EIA (figure 2). The coefficient of reproducibility was 11.5 pg/ml and the 95% limits of agreement were –15.4 pg/ml and +7.7 pg/ml, with all the values falling within this range.

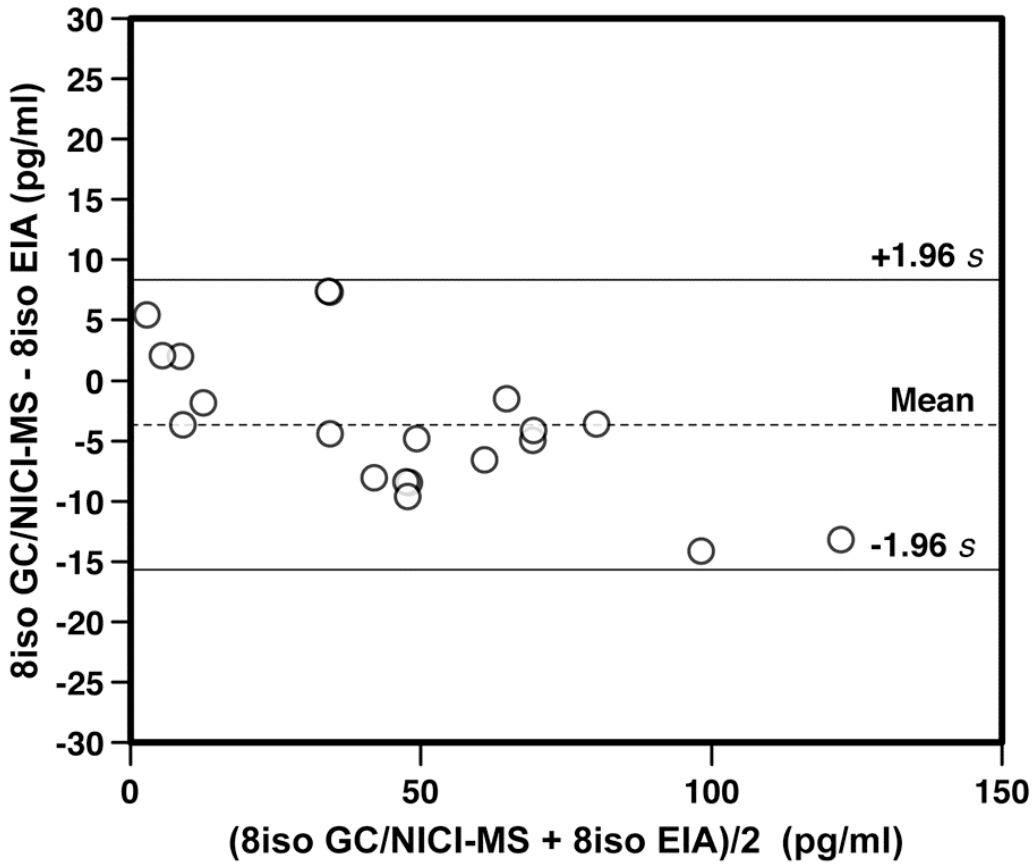

Figure 2. Bland and Altman plot of EBC 8-isoprostane measurements obtained with GC/NICI-MS and EIA (coefficient of reproducibility 11.5 pg/ml). In the y-axis are reported the differences and in the x-axis the means of the measurements performed with the two methods. The coefficient of reproducibility is 1.96*SD (standard deviation of the differences).

Reliability

A subgroup of children provided two EBC samples 1 hour apart. The intraclass correlation coefficient for these pairs of samples was 0.72 for GC/NICI-MS (n= 8) and 0.53 for EIA (n=5).

Correlations of EBC 8-isoprostane with lung function and FE_{NO} measures

The measures of 8-isoprostane performed by means of EIA show a negative correlation with FEF_{25-75} and with FEV_1/FVC close to statistical significance (p=0.05, r=-0.4 and p=0.06, r=-0.4 respectively). When the EBC measurements obtained by GC/NICI-MS were considered, the correlations with FEF_{25-75} and FEV_1/FVC were both statistically significant (p=0.03, r=-0.5).

No correlation emerged between the 8-isoprostane levels measured by EIA and FVC (p=0.67) or FEV_1 (p=0.12), or between the 8-isoprostane measured by GC/NICI-MS and FVC (p=0.66) or FEV_1 (p=0.08).

No correlation was found between EBC 8-isoprostane levels and FE_{NO}.

The measurements obtained in urine samples showed no correlation with the spirometric parameters or FE_{NO} levels.

Correlation between measurements in urine and EBC

No correlation emerged between the 8-isoprostane concentrations in urine and in EBC, measured either by GC/NICI-MS (p=0.27) or by EIA (p=0.23).

DISCUSSION

The present study showed that EBC 8-isoprostane, a marker of oxidative stress, was significantly higher in children with problematic asthma than in children whose asthma was well controlled (figure 1). 8-isoprostane was measured with both a reference analytical method and an enzymatic immunoassay: we found an acceptable reproducibility (figure 2) although GC/NICI mass spectrometry afforded a greater accuracy.

We also found a significant negative correlation between 8-isoprostane and both FEF_{25-75} and FEV_1/FVC, which are sensitive indicators of airway obstruction and show a declining gradient in children with increasingly severe asthma (20). 8-isoprostane is a stable product of free-radical catalyzed arachidonic acid peroxidation independent of the cyclo-oxygenase, and it is a recognized marker of oxidative stress (4). Oxidative stress can cause airway narrowing, both by directly damaging the airway epithelium due to lipid peroxidation and because of the bronchoconstriction induced by the release of arachidonic acid (21-22). There is also evidence of a receptor-mediated contraction of airway smooth muscle caused by isoprostane (4). Our data confirm a close link between increased oxidative stress and airflow limitation in asthmatic children. This finding is in keeping with previous data reported by our own (7) and other groups (6,10,23), supporting the existence of a correlation between oxidative stress and asthma severity. In a recent study, Fitzpatrick et al (24) demonstrated an imbalance between oxidants and antioxidants, with reduced GSH (reduced glutathione) and increased GSSG (oxidized glutathione) levels in bronchoalveolar lavage of children

with severe refractory asthma, supporting a key role for oxidative stress in the pathogenesis of severe asthma.

In our study, FE_{NO} levels did not differ between problematic and well-controlled asthma cases, nor did they correlate with 8-isoprostane EBC levels. FE_{NO} is considered a biomarker of eosinophilic airway inflammation (25) and its levels can predict response to inhaled corticosteroids (26). Our findings confirm that FE_{NO} and 8-isoprostane reflect different aspects of the pathogenic mechanisms behind asthma. Taken together, our data suggest that children with problematic asthma may have an inflammatory phenotype in which oxidative stress plays a central role. The resulting oxidative damage may lead to a persistent airway obstruction and poor response to steroid therapy. In keeping with our results, previous studies have already reported that EBC 8-isoprostane levels are poorly affected by ICS therapy (6-8). A potential role for antioxidant treatments in asthma management has recently been proposed (1) and measuring 8-isoprostane might be used in the future to identify asthmatic children likely to benefit more from the use of such new therapeutic approaches.

From a methodological standpoint, in the present study we compared the measurements of 8-isoprostane (8-iso-$PGF_{2\alpha}$) in the EBC of asthmatic children using two different methods, i.e. GC/NICI-MS (a reference analytical method) and an EIA assay, that is cheaper and easier to perform but less sensitive and specific (5).

When the Bland Altman plot was used to compare the measurements obtained with the two methods, we found a coefficient of reproducibility of 11.5 pg/ml, with all values falling within the limits of agreement (figure 2),

showing that the reproducibility between the two methods is acceptable. The weaknesses of the EIA technique should be borne in mind nonetheless. In fact, when we calculated the intraclass correlation coefficient (ICC), we found that the reliability of EIA was not as good as that of GC/NICI-MS (ICC 0.53 and 0.72, respectively). In addition, the visual inspection of the Bland Altman plot (figure2) shows that the differences between the measurements obtained with the two methods were negatively skewed, suggesting that EIA tends to overestimate the 8-isoprostane concentrations in EBC. This may be due to the cross-reactivity of the EIA antibody with structurally related 8-iso-$PGF_{2\alpha}$ isomers as reported in the method section and also by other authors (27-28). Neither of these isomers coelutes with the 8-iso-$PGF_{2\alpha}$ used to measure 8-isoprostane by GC/NICI-MS, as amply explained by Milne et al. (18).

The advantages of GC/NICI-MS over commercially-available immunoassay kits include the high sensitivity and specificity of the mass spectrometric approach (29), which yields quantitative results in the low picogram range; its drawbacks are that it is labor-intensive and requires considerable outlays for equipment by comparison with the low cost and relative ease of use of immunoassay kits. Furthermore the sensitivity/specificity of the immunoassay kits vary considerably from one manufacturer to another. More recently, new methods for 8-IsoPGF2a analysis, based on liquid chromatographic mass spectrometry (LC/MS), have been developed, offering the advantage of a simpler sample preparation than in GC/MS because no derivatization of the molecule is required (30). Despite major advances in the sensitivity of LC/MS

instrumentation, one concern with these assays relates to the detection limits in biological fluids, which are often higher than those using GC/MS (30-31). For these reasons we can infer that the GC/ MS analysis can still be considered the reference analytical method.

In this study, a urine sample was collected right after collecting the EBC and subsequently analyzed for the presence of 8-isoprostane. To our knowledge, no previous studies on asthmatic subjects have measured the same biomarker in both urine (a systemic matrix) and EBC, which is a biological fluid reflecting airway lining fluid composition (5). Though EBC collection is easy and entirely non-invasive, it is time-consuming and younger children may have trouble cooperating for long enough to complete the procedure, whereas urine samples are very quick and easy to collect. These observations provided the rationale for simultaneously analyzing urine and EBC samples with a view to ascertaining whether urine can reflect the inflammatory processes in the asthmatic lung as accurately as EBC does. Our results demonstrate that urinary 8-isoprostane levels do not correlate with those measured in EBC, nor do they show any correlation with lung function parameters or with the asthma phenotype. Urine samples are likely to be affected by the metabolism of the whole body and our data suggest that EBC is by far superior for studying lung inflammation and oxidative stress.

The present study has a number of limits. Children with problematic asthma are only a minority of the asthmatic children and the small sample size prevents us from comparing the two sub-phenotypes of problematic asthma described by Bush et al. (i.e. difficult-to-treat asthma and severe

therapy resistant asthma) (32). As previously suggested (11) there is a need for multicenter studies enabling the recruitment of a sufficient number of patients to allow for these aspects to be investigated.

We also recognize the methodological limits of the EBC technique (5). As regards 8-isoprostane, it has been demonstrated that different condenser coatings lead to different percentage of recovery in EBC (9). That is why the results of studies applying different condensers cannot be readily compared. Moreover, from an analytical point of view, interferences due to matrix effect are possible when 8-isoprostane is measured by EIA, and they are favored by the very low concentration of detected substances (33).

In conclusion, our study demonstrated that EBC 8-isoprostane levels are higher in children with problematic asthma, suggesting a role for airway oxidative stress in this asthma phenotype. In addition, we found an acceptable reproducibility of an immunoenzymatic assay compared to GC/NICI-MS, even if the latter method had higher accuracy.

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METABOLOMIC ANALYSIS OF BREATH CONDENSATE IN THE CHARACTERIZATION OF ASTHMA PHENOTYPES IN CHILDREN

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The paper has been submitted as abstract to the ERS Annual Meeting (Barcelona 18-22 September,2010)

ABSTRACT

Background. Asthma is a heterogeneous disease and there is a need for a better characterization of the different asthma phenotypes from a biochemical-inflammatory standpoint. The metabolomic analysis of a biofluid, by means of a non-selective approach, leads to the identification of patterns of metabolites that enables the discrimination of healthy from ill subjects and the characterization of different subgroup of patients.

Methods. We recruited 42 asthmatic children (age 8-17) with either mild asthma (treated or not with inhaled steroids) or severe asthma (poor control of the disease in spite of regular therapy with multiple drug). A group of 15 healthy subjects was recruited as control. Children performed spirometry, exhaled nitric oxide measurement, exhaled breath condensate collection. Condensate samples were analyzed by means of the metabolomic approach based on mass spectrometry.

Results. The metabolomic analysis demonstrated a clear discrimination between healthy children, children with mild asthma (either treated with inhaled steroids or steroid naive) and children with severe asthma, suggesting that a different EBC metabolic profile characterize these 3 groups. In the characterization of the mild asthma group a metabolite, which belongs to the prostanoid family, emerged as important. For of the severe asthma group, although no single variables emerged, a clear characterization was possible on the base of the overall metabolic fingerprint

Conclusions. The metabolomic approach enables distinguishing children with different degree of asthma severity. Further studies, testing in new groups of asthmatic subjects the built model, could confirm the role of the EBC metabolic profile in the early characterization of asthma phenotype in children.

INTRODUCTION

The heterogeneous nature of asthma is widely recognized. In the past, several classifications of asthma have been proposed mainly based upon description of symptom triggers, lung function measurements or clinical characteristics (1). Nowadays we know that asthma is a chronic inflammatory disease of the airways, but again there is evidence that different inflammatory patterns (e.g. eosinophilic, neutrophilic, paucigranulocytic) can sustain asthma symptoms (2,3). Some researchers are working on the characterization of the different asthma sub-phenotypes at a molecular level (4). The identification of specific biochemical pathways underlying the disease may in fact provide useful information that, taken together with clinical data, could lead to a more accurate characterization of these sub-phenotypes (5). Even more important is the consequent possibility of developing targeted therapeutic strategy, based upon the knowledge of the specific molecular mechanisms involved.

The metabolomic analysis is a non selective approach which simultaneously considers a great number of bio-compounds in a sample, and, through the application of bioinformatic tools, evaluates whether it is possible to identify characteristic profiles able to discriminate between different groups of subjects (6).

Recently the metabolomic approach has been applied to the study of exhaled breath condensate (EBC) (7,8), a biofluid collected non-invasively by cooling the air expired during tidal breathing (9). The EBC technique, although not fully standardized yet, is easy to perform and leads to the

collection of a matrix – the condensate- which is believed to mirror the composition of airway lining fluid, providing information on the pathological processes involving the lung (9).

Aim of the present study was to analyze, by means of the metabolomic approach, the EBC samples collected in asthmatic children with different levels of disease severity, to evaluate whether it is possible to discriminate these clinical subgroups on the base of the EBC metabolic profiles. These profiles could substantially contribute to the early characterization of specific asthma sub-phenotypes.

METHODS

Study subjects and study design

We recruited 42 atopic asthmatic children, aged 8-17, among patients attending the Pulmonology/Allergy outpatient's clinic at the Pediatrics Department in Padova. The children had suffered no acute upper or lower airway infection in the last 3 weeks. The diagnosis of asthma was based on clinical history and medical examination, pulmonary function parameters and response to β_2-agonist agents, according to international guidelines (10).

The following groups of children were recruited:

1) 14 children with well-controlled asthma, using beta2 short acting agonists as needed and with no need for controller medications.
2) 17 children with well-controlled asthma in regular treatment with controller medications. All these children were treated with low-medium dose ICS (200-400 mcd/day of budesonide or equivalent); 13 of them where also treated with LABA, 2 with montelukast and 1 with theophylline
3) 11 children with poorly controlled asthma although regularly treated with multiple controller medications (severe asthma). All these children were treated with high dose of ICS (400-1000 mcg/day of budesonide or equivalent) combined with LABA; in addition 3 were treated with montelukast and 1 was treated with theophylline. In this group asthma was considered poorly controlled because of chronic symptoms and/or frequent severe exacerbations requiring a course of oral steroids (all the included children had 3 or more

exacerbations in the previous year). In children belonging to this group, the main co-morbidities (e.g. RGE and rhinosinusitis) had been excluded or treated and adherence to therapy was regularly checked.

As control group we recruited 15 healthy children, aged 9-17, with no history of atopy or respiratory diseases.

At recruitment all the children underwent physical examination and performed FE_{NO} measurement and spirometry. EBC was collected, stored at –80° C and subsequently analyzed by mass spectrometry (MS).

The Ethics Committee of our hospital reviewed and approved the protocol and all parents gave their informed consent.

EBC collection

EBC was collected and processed according to recent ATS/ERS recommendations (9). EBC was collected using the TURBO-DECCS (transportable unit for research on biomarkers obtained from disposable exhaled condensate collection systems) (Medivac, Parma, Italy).

TURBO is a refrigerating system that relies on a thermo electrical module giving rise to a Peltier effect. The cold side of the Peltier module is connected to an aluminium support shaped to house the test tube. TURBO is supplied with DECCS, a disposable respiratory system that consists of a mouthpiece equipped with a one-way valve and a reliable saliva trap, connected to a collecting vial (50 ml) by means of a tube. The children breathed tidally through the mouth for 20 minutes, while sitting comfortably and wearing a nose clip. They kept their mouth dry during

EBC collection by periodically swallowing excess saliva. EBC samples were immediately stored at –80°C in polypropylene tubes until assay.

Orbitrap LC–MS analysis of metabolites in EBC

Analyses were performed with a Ultimate 3000 Dionex HPLC system (Dionex, Softron GmbH, Germany) coupled to an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with an Advion Triversa NanoMate source (Advion BioSciences, inc. Ithaca, NY, USA).

20 μL of EBC were injected on a trap Atlantis T3 (Waters) column 3μm particle size, 10mm x 2.1mm inner diameter, and diverted, after a time period of 1.25 min and from 1.25 min up to 7 min, on an analytical Atlantis T3 (Waters) column 3μm particle size, 150mm x 1mm inner diameter at 40°C. The trap column Solvent A was H_2O with 0.1% formic acid and solvent B was acetonitrile with 0.1% formic acid. Solvent B varied as follows: 0 min 2%, 1.25 min 100%, 8 min 2%; the flow rate was 40 μL/min.

The analytical column solvent A was H_2O with 0.1% formic acid, solvent B was acetonitrile with 0.1% formic acid, solvent C was methanol with 0.1% formic acid; the flow rate was kept at 40 μL/min. Solvent B and C varied as follows: 0 min B 0% C 5%, 1.5 min B 0% C 5%; 10-11 min B 48% C 50%; 11.1-12 min B 98% C 0%; 12.1 min B 0% C5%. The column was then equilibrated for 5 min at the initial conditions prior to the analysis of the next sample.

The LC flow was split (1/100) on the Advion source and directed to the mass spectrometer. MS analysis was performed in positive FTMS mode at a resolution of 30,000 (at m/z 400) with a 50 – 1000 scan range using the

following source parameters: tube lens was 150 V, capillary voltage was 45 V, capillary temp 200°C, and ion spray voltage was 1.7 kV.

All chemicals were purchased from Sigma (St. Louis, MO, USA). Acetonitrile, formic acid, and ammonium used for HPLC solvents were of LC–MS degree.

All samples were injcted three times in three random sequence to avoid any effect on the classification relate to the analytical condition.

A standard solution was used as internal control for the data extraction parameters by using the software MarkerLynx (Waters)

Fractional exhaled nitric oxide (FE_{NO}) measurement

FE_{NO} was measured with the NIOX system (Aerocrine, Stockholm, Sweden) using a single-breath on-line method according to the ERS/ATS guidelines for measuring FE_{NO} in children (11). Children inhaled NO-free air to total lung capacity and exhaled through a dynamic flow restrictor with a target flow of 50 ml/sec for at least 6-7 seconds. No nose clip was used. The NIOX system was calibrated using a 200 ppb NO tank (Lindegas Hoek Loos Speciality gases, Amsterdam, Netherlands) according to the manufacturer's instructions.

Lung function test

Lung function parameters were measured with a 10-liter bell spirometer (Biomedin, Padova, Italy) and the best of three maneuvers was expressed as a percentage (%) of the predicted reference values according to Polgar and Promadhat (12).

Statistical analysis

Normally distributed data (spirometric parameters) were recorded as mean±SEM, while non-normally distributed data (FE_{NO} values) were reported as medians and interquartile. Spirometric parameters and log-transformed FENO values were analyzed by means of ANOVA, followed by Holm-Sidak test for between-group comparisons.

Multivariate statistical analysis was applyed to analyse metabolomic data. Firtsly principal component analysis (PCA) was used as unsupervised method to study the metabolomic differences between each asthmatic group and the healthy control group.

To further study the differences between groups the supervised methods PLS-DA and O2PLS-DA were used. The Bidirectional-Orthogonal Projections to Latent Structures (O2PLS) is a multivariate projection method that extracts linear relationships from two data blocks X and Y by removing the so-called structured noise (13,14) When structured noise is present in a dataset X (or Y), traditional projection techniques as PLS regression can produce systematic variation of X (or Y) which is not correlated to Y (or X). O2PLS removes this structured noise from both X and Y without imposing particular direction in the prediction model. As consequence, O2PLS decomposes the systematic variation in the X-block (or Y-block) into two model parts: one, the so called predictive or parallel part, which models the joint X-Y correlated variation and another, called as orthogonal part, that is not related to Y (or X). O2PLS can be used to perform Discriminant Analysis (DA) by introducing suitable dummy variables. The main benefit using O2PLS-DA technique is the reduced

model complexity (15). In the case of N classes, the dimension of the predictive space is N-1 and, then, the model can be explained by using only N-1 components. The number of latent components of the model can be determined by cross-validation techniques. Multivariate data analysis was performed by using SIMCA P+ (Umetrics, Umea, Sweden).

RESULTS

Metabolomic data

As part of the multivariate statistical analysis the dataset has been reduced to 596 metabolomic variables by eliminating all the variables for which the value was different from 0 in less than 6 samples.

The application of the O2PLS-DA demonstrated that it is not possible to create a good model for the discrimination of the 4 groups studied (i.e. the healthy subjects and the 3 groups of asthmatic subjects).

On the contrary a robust model can be built (2 parellel and 2 orthogonal latent components, R2=0.75 and Q2=0.47) by means of the OPLS-DA considering the following 3 groups: 1) healthy children; 2) children with mild asthma (including both ICS treated and steroid naive); 3) children with severe asthma (poor control in spite of high-dose therapy)

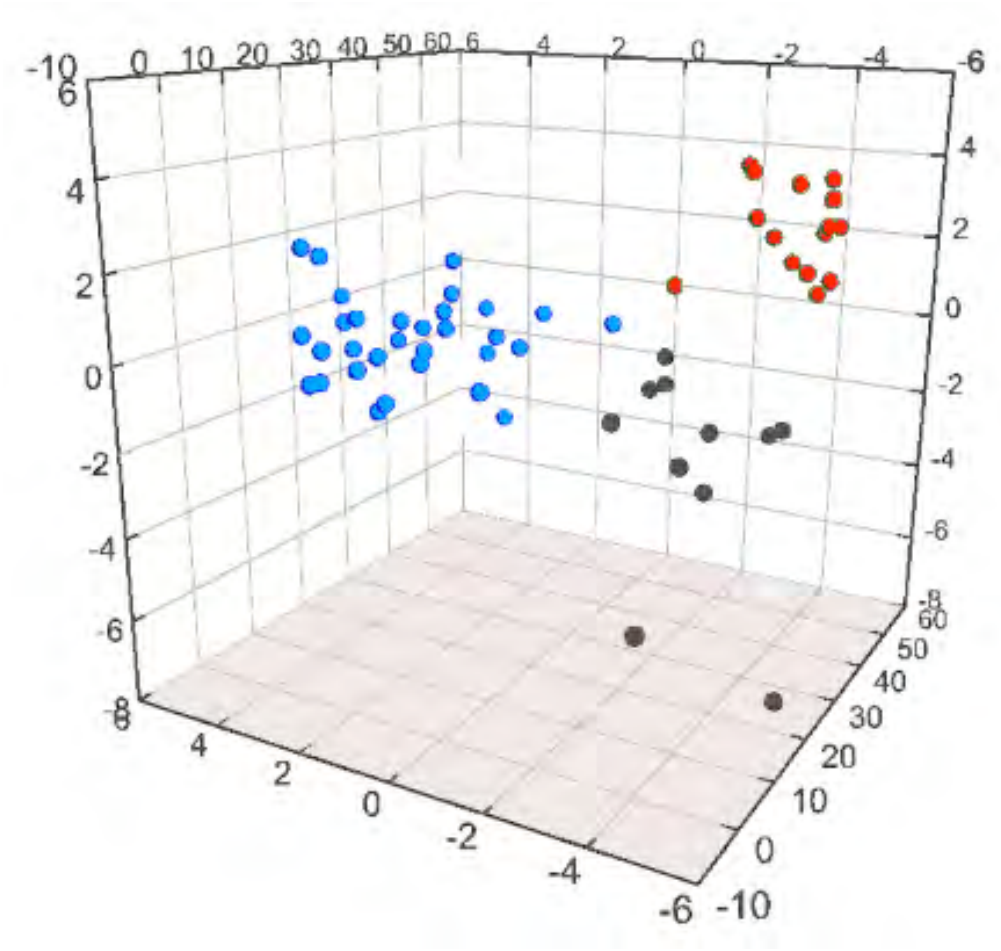

Figure 1. Blue dots represent mild asthma, black dots severe asthma and red dots healthy controls

Moreover, by O2PLS-DA two class models were built and a clear separation was found between healthy children and severe asthmatics (1 parallel and 2 orthogonal latent components R^2=0.88 Q^2=0.62) as well as between healthy children and mild asthmatics (1 parallel and 2 orthogonal latent components R^2=0.85 Q^2=0.51).

Permutation testing (200 times) showed that the models are not over-fitted.

By means of the s-plot we have identified the variables that explain most of the variance and that are therefore more important for the discrimination between groups. The variable 293 (with retention time of 10.23 min and with 371.24 m/z) clearly emerged as a potential biomarker for the characterization of the mild asthma group.

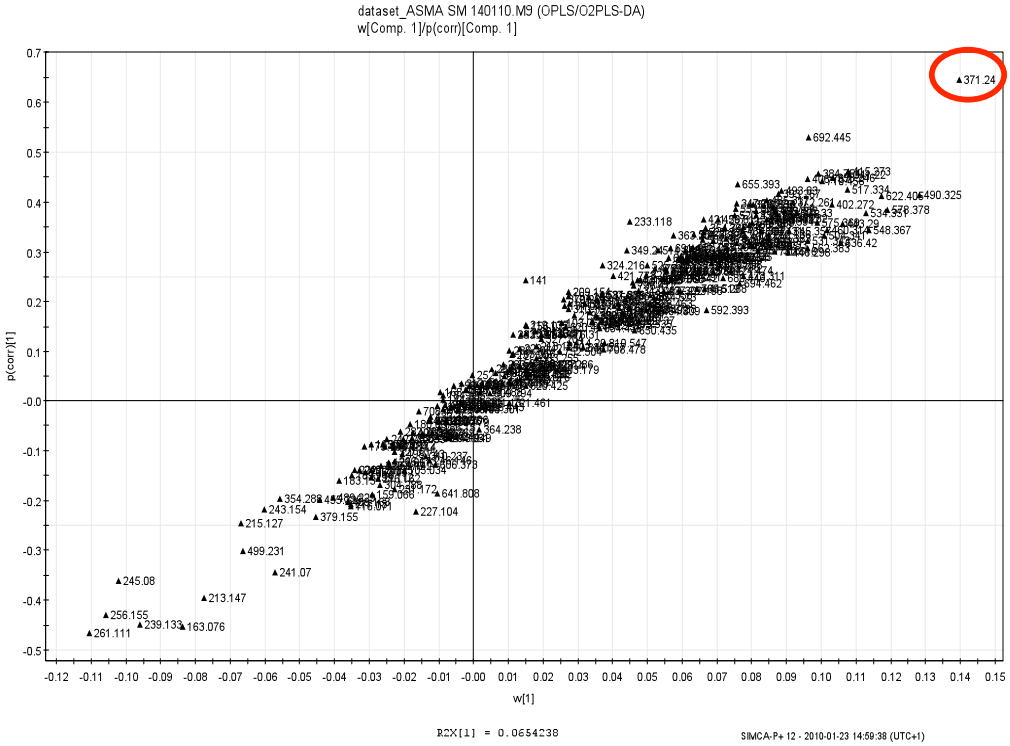

Figure 2. The S-plot visualizes the variable influence in a model and is particularly useful for discriminant analysis models, e.g. OPLS/O2PLS-DA. It is a scatter plot that combines the covariance and correlation loading profiles. This corresponds to combining the contribution or magnitude (covariance) with the effect and reliability (correlation) for the model variables with respect to model component scores. In this plot, the w loading weight profile (w) of the first predictive (class-separating) OPLS/O2PLS-DA component is plotted on the x-axis and represent X-variable contribution (covariance). The p correlation loading vector of the first predictive (class-discriminating) component is plotted on the y-axis and represents the correlation (reliability) of each X-variable with the first predictive score component t1 and spans between theoretical minimum of -1 and a maximum of +1

By searching The Human Metabolome Database (HMDB) (16) we have found few possible cadidates for this variable: 20-Hydroxy-PGF2a, Thromboxane B2 and 6-Keto-prostaglandin F1a.

Lung function and exhaled NO data

The values for lung function parameters and exhaled NO for each of the 4 recruited groups are reported in the table.

	Nr (male)	**Age (range)**	**FVC* (%pred)**	**FEV_1* (%pred)**	**FEV_1/FVC* (%)**	**FEF_{25-75}* %pred**	**FE_{NO}° (ppb)**
Healthy	15(7)	12.6 (9-17)	101 (1.9)	98 (1.9)	90 (1.5)	106 (4.9)	11.3 [9.7-16.1]
Mild asthma without regular therapy	14(9)	12.5 (8-17)	108 (3.2)	103 (3.3)	87 (1.6)	104 (6.3)	41.2 [26.4-61.9]
Mild asthma with regular treatment	17(12)	10.4 (8-15)	98 (1.6)	93 (1.9)	86 (1.8)	89 (5.8)	20.2 [10.6-27.7]
Severe asthma	11(5)	10.4 (8-16)	86 (4.2)	73 (3.3)	78 (1.9)	55 (4.2)	41.5 [19.5-90.0]

*data are expressed as mean ± SEM; ° data are expressed as median and IQR

Comparing the 3 groups discriminated by the metabolomic analysis (healthy, mild asthma and severe asthma) we found significantly lower values in children with severe asthma for all the spirometric parameters ($p<0.001$ for FVC, FEV_1, FEF_{25-75} and $p<0.01$ for FEV_1/FVC). In detail, FVC: healthy 101% $\pm$ 1.9 (mean$\pm$SEM), mild asthma 102% pred $\pm$ 1.9, severe asthma 86% $\pm$ 4.2; FEV_1: healthy 98% $\pm$ 1.9, mild asthma 98% pred $\pm$ 2.0, severe asthma 73% $\pm$ 3.3; FEV_1/FVC: healthy 90% $\pm$ 1.5, mild asthma 87% pred $\pm$ 1.2, severe asthma 78% $\pm$ 1.9; FEF_{25-75}: healthy 106% $\pm$ 4.9, mild asthma 96% pred $\pm$ 4.4, severe asthma 55% $\pm$ 4.2.

Exhaled NO was higher ($p<0.01$)in asthmatic than in healthy subjects (11.3 ppb [9.8-16.1]) but there was no significant difference between mild (26.3 ppb [17.6-46.2]) and severe asthmatics (41.5 ppb [19.5-90]).

Discussion

By means of the metabolomic analysis we demonstrated that it is possible to clearly separate children with different levels of asthma severity, on the base of their EBC metabolic profile.

As recently reported (17), severity and control are two tightly bound aspects of asthma: control is defined as the absence of symptoms, reliever use, night waking, as well as the absence of activity limitations and exacerbations; severity, on the other hand, is defined as the intensity of treatment required to achieve and maintain asthma control (17).

In pediatric asthma most of the cases are mild since a good control of the disease is reached with low-to-moderate dose of ICS. Nonetheless a subset of asthmatic children has a severe disease characterized by persistent symptoms and/or frequent exacerbations in spite of being treated with high-dose ICS combined with other drugs (17, 18). Several authors have underlined the need for a better comprehension of this heterogeneity of asthma through the characterization of the different phenotypes not only from a clinical standpoint but also from a biochemical-inflammatory one (18,19).

In the present study we recruited 3 groups of asthmatic children. The first group included children with well controlled asthma requiring only occasional use of short acting beta2 agonists, the second group included children with a good control of their asthma obtained with low dose ICS combined or not with long acting beta 2 agonists (LABA). The children included in these two groups were classified as having “mild asthma” since their asthma was easily controlled (17). The third group included children

with persistent/recurrent symptoms and/or frequent exacerbations although treated with high dose ICS combined with other drugs. Because of their clinical characteristics children included in this last group were classified as having “severe asthma” (17).

The metabolomic analysis enabled a clear separation between the following 3 groups: 1) healthy children, 2) children with mild asthma, 3) children with severe asthma, suggesting that these groups are characterized by a different EBC metabolic profile.

It is worth noting that, within the mild asthma group, the metabolomic analysis does not discriminate between children in whom the control is obtained with regular ICS therapy and those who remain in good control without any regular treatment. This observation means that, in spite of requiring or not regular treatment, children with mild asthma have a similar EBC metabolic profile, suggesting common metabolic-inflammatory processes. Previous studies, from ours and other groups, in which a single biomarker was measured in EBC (e.g. 8-isoprostane or cysteinil leukotrienes), already found no significant differences between treated and non-treated children with mild asthma (20-22). Our finding strengthens this previous observation because of the nature of the metabolomic approach. In fact, the metabolomic analysis considers a great number of metabolites altogether, drawing a general fingerprint of a sample, and our study suggests that the overall picture of the metabolites, as studied with the method that we applied, is similar in all the children whose asthma is easily controlled either with low dose ICS or without any maintenance therapy.

When interpreting the data, one of our points was to evaluate whether the discrimination among the 3 groups (healthy, mild asthma and severe asthma) could be an artifact due to the presence or absence of drug metabolites in the EBC of children treated and non-treated respectively. The lack of discrimination within the mild asthma group between children ICS treated and those steroid naïve clearly plays against the hypothesis that the separation found, was related to drug metabolites. As a further confirmation we compared the metabolomic profiles of all the non-treated children (i.e. healthy children plus mild asthmatics steroid naïve) with those of all the regularly treated children (i.e. mild asthmatics in treatment plus severe asthmatics) finding no difference between these two groups. Taken together these observations strongly suggest that the discrimination is possible because of phenotype differences between the groups, and it is not artifactual.

After demonstrating that the discrimination between the 3 described groups is possible we moved on trying to identify one or more variables important for such discrimination.

We could recognize one variable that plays an important role in the characterization of the mild asthma group.

By searching the Human Metabolome Database (Version 2.5) (16) we found few possible molecules that could correspond to the identified variable. These molecules belong to the class of prostanoids and they are 20-Hydroxy-PGF2a, Thromboxane B2 and 6-Keto-prostaglandin F1a.

This finding confirms several previous studies that demonstrated that asthmatic subjects have increased levels of prostanoids. Among the

candidate metabolites, Thromboxane B(2), a stable metabolite of thromboxane A(2), is a potent bronchoconstrictor (23) and increased levels of this mediator have been demonstrated in EBC of asthmatic subjects (24,25).

Although the design of the present study does not allow to establish the cellular source of the identified metabolite, the observation that it is characteristic of mild asthma but not of severe asthma, let us speculate that a different inflammatory profile underlie these two conditions.

Alike in previous metabolomic studies (26), the identification through the untargeted metabolomic analysis of a metabolite already recognized as important for the disease pathophysiology, validate the capability of the untargeted metabolomic approach of identifying relevant biomolecules (6).

Our metabolomic analysis did not enable the identification of single molecules important for the characterization of the severe asthma group. Nonetheless this group could be completely discriminated by the other two groups, meaning that an overall metabolite fingerprint exists that specifically characterize severe asthmatic children.

Compared to the other measurements performed in this study (spirometry and FE_{NO} measure), the metabolomic analysis is much more informative. In fact, spirometry parameters are lower in severe than in mild asthmatic children, but they provide no information at all on the biochemical-inflammatory underlying processes. Exhaled NO, on the other hand, is an isolated marker of eosinophilic inflammation greatly affected by steroid therapy (27), which in our study cannot distinguish between mild and severe asthma.

A limit of the present study is related to the EBC technique itself. Although many studies have been published applying this method, different laboratories still use different devices for EBC collection and the technique is not completely standardized yet so that its use is by now limited to the research filed (9).

Another limit is the relatively low number of subjects recruited in each group. Nonetheless a suitable permutation test was applied as control for the statistical analysis in order to guarantee that the results are not due to casualty or that overfitting does not affect the model.

In conclusion our metabolomic analysis of exhaled breath condensate enabled the identification of 2 main metabolic profile in asthma: one characteristic of children with mild disease, in whom control is either reached with low-dose ICS therapy or maintained with no regular therapy, and the other characteristic of severe asthmatic children. Thromboxanes/prostaglandins are important mediator of the inflammatory profile that underlies mild asthma. In severe asthma, although no single mediators could be identified, an overall metabolic fingerprint exists that characterizes these subjects. Further study testing in new groups of asthmatic subjects the built model could confirm the role of the EBC metabolic profile in the early characterization of asthma phenotype in children.

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RINGRAZIAMENTI

Voglio ringraziare in primo luogo il Dr Giuseppe Giordano e il Dr Matteo Stocchero per il preziosissimo aiuto nell'analisi e interpretazione dei dati di metabolomica.

Ringrazio inoltre le Dottoresse Barbara Andreola, Luisa Freo e la Professoressa Liviana Da Dalt per l'aiuto nella raccolta e interpretazione dei dati nello studio sulle polmoniti acquisite in comunità. Ringrazio il Dr Massimo Corradi, la Dr.ssa Paola Cogo e le sue collaboratrici, dottoresse Ilena Isak e Manuela Simonato per i dosaggi di 8-isoprostano e LTB4 nel condensato.

Infine vorrei ringraziare il Professore Eugenio Baraldi per i suoi insegnamenti e la Dottoressa Stefania Zanconato per la collaborazione quotidiana nell'attività clinica.