DM

A Powerful Strategy Based on Targeted Analysis for Detection of Asthma Biomarkers

The challenge for miniaturization

MASTER DISSERTATION

Pedro Henrique Fernandes da Silva Berenguer

MASTER IN APPLIED BIOCHEMISTRY

UNIVERSIDADE da MADEIRA

A Nossa Universidade

www.uma.pt

March | 2018

A Powerful Strategy Based on Targeted Analysis for Detection of Asthma Biomarkers

The challenge for miniaturization

MASTER DISSERTATION

Pedro Henrique Fernandes da Silva Berenguer

MASTER IN APPLIED BIOCHEMISTRY

ORIENTADOR
José de Sousa Câmara

CO-ORIENTADORA
Irene Gomes Câmara Camacho

A Powerful Strategy Based on Targeted Analysis for Detection of Asthma Biomarkers. The Challenge for Miniaturization.

Master dissertation presented to Madeira University in order to obtain the degree of Master in Applied Biochemistry, carried out under the scientific supervision of José de Sousa Câmara (CQM – Madeira Chemistry Research Centre) and co-supervision of Professor Irene Gomes Câmara Camacho (Center of Life Sciences).

Pedro Henrique Fernandes da Silva Berenguer

Campus Universitário da Penteada

Funchal – Portugal

March, 2018

For my parents, Constança and Cláudia.
Because they always believed in me.

"Live as if you were to die tomorrow.
Learn as if you were to live forever"

Mahatma Gandhi

Acknowledgments

The elaboration of my Master Dissertation depended on the support of several people and entities at different levels and fields. Without them, it would not be possible to perform it.

To my supervisor, Professor Doctor José de Sousa Câmara, and my co-supervisor, Professor Irene Gomes Câmara Camacho, I would like to express my deep gratefulness for the opportunity to perform this Master Dissertation and for the supervision at an excellence level, for their availability, understanding, transmission of knowledge and persistence during this dynamic process.

I would like to express my gratitude to Doctor Catarina Silva for all the support and patience, especially at the beginning of the experimental work with the analytical techniques, MEPS and UHPLC-PDA. Her knowledge was valuable in the execution of this work.

I want to thank all my colleagues at the Analytical Chemistry and Enology Laboratory (ACE_{CQM} Lab) for all their availability.

I also want to acknowledge Madeira Chemistry Research Center (CQM) for the physical and technological conditions created to develop this work. This work was also supported by Foundation for Science and Technology (FCT; project PEst-OE/QUI/UI0674/2013, CQM, Portuguese Government funds), and through Madeira 14-20 Program, project PROEQUIPRAM - Reforço do Investimento em Equipamentos e Infraestruturas Científicas na RAM (M1420-01-0145-FEDER-000008) and by ARDITI-Agência Regional para o Desenvolvimento da Investigação Tecnologia e Inovação, through the project M1420-01-0145-FEDER-000005 - Centro de Química da Madeira - CQM^{+} (Madeira 14-20).

The collaboration of the Health Service of Madeira Autonomous Region (SESARAM) under the guidance of Doctor Susana Oliveira and Doctor Rita Câmara was important since they helped me with the collection of patient samples and with ideas and technical concepts that were unknown to me. I also express my gratitude for the Hospital staff that helped me to reach patients and to collect samples including Dr. Filipa Sousa, Dr. Jorge Cabral, Dr. Cristina Freitas, Nurse Lina and Technician Katherine from the Department of Immunoallergology of Dr. Nélio Mendonça Hospital, and all staff at the Emergency of the Hospital Dr. Nélio Mendonça. To all patients who provided the samples, my gratitude

The collaboration of the Regional Direction of Education (DRE) and the elementary schools, EB1/PE Tanque – Monte and EB1/PE Visconde Cagongo, under the support of their Directors Dr. Raquel Queirós and Dr. Carla Teixeira, had a vital role since it was them who helped me with the collection of samples of the healthy control group. To all students for providing me the samples, my gratitude.

To all my colleagues and to my boss, Mr. Manuel Capelinha, at the restaurant Papa Manuel Lido for all the flexibility. Without it, this work would not be possible. I will never forget.

Last but not least, my deep, deep appreciation to my parents, family, my daughter Constança, my wife and friend, Cláudia and my friends especially João Pestana, Donato Rodrigues, Mrs. Herta and Mr. Karl Leis for all the support, strength and motivation to be persistent and for always being there.

Thank you,
Pedro Berenguer

Scientific work

During the development of this Master Dissertation, I was given the opportunity to develop the following scientific works. Furthermore, it is expected that this Master Dissertation results in the publication of two more scientific articles, one original work, relative to the experimental work, and one review, relative to the theoretical part of this Master Dissertation.

Posters

Pedro Berenguer, José S. Câmara, Irene C. Camacho. Exploring the ability of microextraction in packed syringe on extraction efficiency of lipid peroxidation biomarkers related with asthma. ExTech 2017. Santiago de Compostela, Spain. 2017.

Oral communications

Pedro Berenguer, Irene C. Camacho, Rita Câmara, Susana Oliveira, José S. Câmara. An improved MEPS/UHPLC-based methodology on the analysis of asthma biomarkers. 5th CQM Annual Meeting. Universidade da Madeira. Funchal, Portugal. 2018.

Pedro H. Berenguer, Catarina L. Silva, Irene C. Camacho, José S. Câmara. Looking for new contributions in asthma biomarkers – a chromatographic-based approach. 10º Encontro Nacional de Cromatografia. Bragança, Portugal. 2017

Pedro Berenguer, José S. Câmara, Irene C. Camacho. Exploring the ability of MEPS/UHPLCPDA methodology to determine the lipid peroxidation biomarkers related with asthma. 4th CQM Annual Meeting. Universidade da Madeira. Funchal, Portugal. 2017.

Pedro Berenguer, José S. Câmara, Irene C. Camacho. Non-invasive approach to establish the volatile metabolomic pattern on asthmatic children. 3rd CQM Annual Meeting. Universidade da Madeira. Funchal, Portugal. 2016.

Apart of this Master Dissertation, I also had the opportunity to contribute to other scientific works including:

Other publications – scientific articles

Priscilla Porto-Figueira, José A. Figueira, Pedro Berenguer, José S. Câmara. Exploring a volatomic-based strategy for a fingerprinting approach of Vaccinium padifolium L. berries at different ripening stages. Food Chemistry, 245 (2018) 141-149.

Irene Camacho, Agnieszka Grinn-Gofroń, Roberto Camacho, Pedro Berenguer, Magdalena Sadyś. Madeira – a tourist destination for asthma sufferers. International Journal of Biometeorology, 60, 11 (2016) 1739-1751.

Other publications – posters

Priscilla Porto-Figueira, Pedro Berenguer, Donato Rodrigues, José A. Figueira, Nelson Barros, Paulo Fernandes, José S. Câmara. Identification of phenolic compounds from fruits of *Vaccinium padifolium* by UHPLC-PDA and LC-ESI-MSMS. 18th EuroAnalysis 2015. The European Conference on Analytical Chemistry. Bordeaux, France. 2015.

Priscilla Porto-Figueira, José A. Figueira, Pedro Berenguer, José S. Câmara. Evaluation of the volatile profile of *Vaccinium padifolium* at different ripening stages using HS-SPME coupled with GC-MS. 18th EuroAnalysis 2015. The European Conference on Analytical Chemistry. Bordeaux, France. 2015.

Priscilla Porto-Figueira, Pedro Berenguer, Donato Rodrigues, José A. Figueira, Nelson Barros, Paulo Fernandes, José S. Câmara. Characterization of phenolic compounds from *Vaccinium padifolium* by UHPLC-PDA and LC-ESI-MS/MS analysis. HPLC 2015. Geneva, Switzerland. 2015.

Abstract

Asthma is a disease characterized by chronic airway inflammation and by its long term irreversible remodeling. The enzymatic peroxidation of the arachidonic acid leads to the formation of bioactive eicosanoids, key mediators of bronchial inflammation and response modulation in the airways contributing to the pathophysiology of this disease.

The present work aimed to develop an improved ultra-high pressure liquid chromatography (UHPLC)-based strategy to characterize biomarkers of lipid peroxidation: leukotrienes E_4 (LTE_4) and B_4 (LTB_4) and 11β-prostaglandin $F_{2\alpha}$ ($11\beta PGF_{2\alpha}$), present in urine of asthmatic patients and healthy individuals (control group). A semi-automatic eVol®-microextraction by packed sorbent (MEPS) format was used to isolate the target analytes. Several experimental parameters with influence on this procedure and on the chromatographic resolution, were evaluated and optimized. The method was fully validated with compatible values under optimal extraction (R-AX sorbent, 3 conditioning-equilibration cycles with 250 µL of ACN-H_2O at 0.1% FA, 10 extract-discard cycles of 250 µL of sample at a pH of 5.1, elution with 2 times 50 µL of MeOH and concentration of the eluate until half of its volume) and chromatographic conditions (14-min analysis at a flow rate of 300 µL min^{-1} in an UHPLC-PDA equipped with a BEH C18 column).

The developed method was successfully applied to the urine of asthmatic patients and healthy individuals. On average, the urine of asthmatic patients present significantly higher concentrations of $11\beta PGF_{2\alpha}$ (112.96 ng mL^{-1} *vs* 62.56 ng mL^{-1} in control group), LTE_4 (1.27 ng mL^{-1} *vs* 0.89 ng mL^{-1} in control group) and LTB_4 (1.39 ng mL^{-1} *vs* 0.76 ng mL^{-1} in control group). These results suggest the potential of the target eicosanoids on asthma diagnosis and on the follow-up of the therapeutic response, even though a larger and more extensive study will be necessary, using a bigger number of cases, to confirm the data obtained and to guarantee a greater robustness to the approach.

Keywords: Asthma; biomarkers, 11β-Prostaglandin $F_{2\alpha}$; Leukotriene E_4; Leukotriene B_4; Microextraction by packed sorbent; Ultra-high pressure liquid chromatography.

Resumo

A asma é uma doença caracterizada por inflamação crónica das vias aéreas e pela sua remodelação irreversível a longo prazo. A peroxidação enzimática do ácido araquidónico leva à formação de eicosanóides bioactivos, importantes mediadores da inflamação brônquica e da modulação da resposta nas vias aéreas contribuindo para a fisiopatologia desta doença.

O presente trabalho teve como objectivo desenvolver uma estratégia aperfeiçoada baseada em cromatografia líquida de ultra pressão (UHPLC) para caracterizar biomarcadores da peroxidação enzimática: leucotrienos E_4 (LTE_4) e B_4 (LTB_4) e 11β-prostaglandina F_{2a} ($11\beta PGF_{2\alpha}$), presentes na urina de pacientes asmáticos e de indivíduos saudáveis. Foi utilizada uma microextracção por sorvente empacotado (MEPS) em modo semiautomático (eVol®) para isolar os analitos alvo. Foram avaliados e optimizados vários parâmetros experimentais com influência neste procedimento e na resolução cromatográfica. O método foi totalmente validado, com valores compatíveis em condições óptimas de extracção (sorvente R-AX, 3 ciclos de condicionamento-equilíbrio com 250 µL de ACN-H_2O a 0,1% FA, com 10 ciclos de extracção-descarte de 250 µL de amostra a um pH de 5,1, eluição com 2×50 µL de MeOH e evaporação do eluato até metade do seu volume) e cromatográficas (análise de 14 minutos a uma taxa de fluxo de 300 µL min^{-1} num UHPLC-PDA equipado com uma coluna BEH C18).

O método desenvolvido foi aplicado com sucesso às amostras alvo. Em média, os pacientes asmáticos apresentaram concentrações significativamente mais elevadas de $11\beta PGF_{2\alpha}$ (112,96 ng mL^{-1} *vs* 62,56 ng mL^{-1} em indivíduos saudáveis), LTE_4 (1,27 ng mL^{-1} *vs* 0,89 ng mL^{-1} em indivíduos saudáveis) e LTB_4 (1,39 ng mL^{-1} *vs* 0,76 ng mL^{-1} em indivíduos saudáveis). Estes resultados sugerem o potencial destes eicosanóides no diagnóstico da asma e no seguimento da resposta à terapêutica. Contudo, será necessário um estudo mais alargado e extensivo, usando um maior número de casos, para confirmar os dados obtidos e garantir uma maior robustez desta estratégia.

Palavras-chave: Asma; 11β-Prostaglandina $F_{2\alpha}$; Leucotrieno E_4; Leucotrieno B_4; Microextracção por sorvente empacotado; Cromatografia líquida de ultra alta pressão.

Contents

Figure captions

List of tables

List of abbreviations

5-HPETE	5-Hydroperoxyeicosatetraenoic acid
8-isoP	8-Isoprostane
ACN	Acetonitrile
ANOVA	Analysis of variance
APC	Antigen-presenting cell
BAL	Bronchoalveolar lavage
BIN	Barrel insert and needle
BU	Blank urine
CAR	Carboxen
CAT	Catalase
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
CQM	Madeira Chemistry Research Center
Cys	Cysteinyl
DC	Dendritic cell
DESI	Desorption electrospray ionization mass spectrometry
df	degree of freedom
DI-SPME	Direct immersion-solid-phase microextraction
DLLME	Hollow fiber liquid-phase microextraction
DRE	Regional Direction of Education
DVB	Divinylbenzene
EBC	Exhaled breath condensate
eNO	Exhaled nitric oxide
EPOX	Epoxygenase
EtOH	Ethanol
EU	European Union
FA	Formic acid
FCT	Foundation for Science and Technology
FcεRI	High affinity IgE receptors
FcεRII	Low affinity IgE receptors
FD	Fluorescence detection

FEV_1	Forced expiratory volume in one second
FLAP	5-Lipoxygenase-activating protein
GA^2LEN	Global Allergy and Asthma European Network
GC	Gas chromatography/gas chromatograph
GC-MS	Gas chromatography-mass spectrometry
GINA	Global Asthma Initiative for Asthma
GPx	Glutathione peroxidase
GSH	Reduced glutathione
GST	Glutathione-S-transferase
HF-LPME	Liquid-liquid microextraction
HL	High level
HLB-DVB	Hydrophilic-lipophilic-balanced-divinylbenzene
HPETE	5-Hydroperoxyeicosatetraenoic acid
HS-SPME	Headspace-solid-phase microextraction
IgE	Immunoglobulin E
IL	Interleukin
IS	Internal standard
ISAAC	International Study of Asthma and Allergies in Childhood
IUPAC	International Union of Pure and Applied Chemistry
LC	Liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
LL	Low level
LLE	Liquid-liquid extraction
LO	Lipoxygenase
LOD	Limit of detection
LOQ	Limit of quantification
LPME	Liquid-phase microextraction
LT	Leukotriene
LTRA	Cys-LT receptor antagonist
LTX_Y	Leukotriene X_Y
LX	Lipoxin
LXX_Y	Lipoxin X_Y
MALDI	Matrix-assisted laser desorption ionization

MAPK	Mitogen-activated protein kinase
MEPS	Microextraction by packed sorbent
MET	Microextraction technique
MeOH	Methanol
ML	Medium level
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MVSA	Multivariate statistical analysis
NADP	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NF	Nuclear factor
NIMS	Nanostructure-imaging mass spectrometry
NK	Natural killer cell
NKT	Natural killer T cell
NMR	Nuclear magnetic resonance
NOS	Nitric oxide synthases
Nrf-2	Nuclear erythroid 2 p45-related factor 2
NSAID	Nonsteroidal anti-inflammatory drug
NTD	Needle trap device
PA	Polyacrylate
PCA	Principal component analysis
PDA	Photodiode array
PDMS	Polydimethylsiloxane
PEF	Peak expiratory flow
PEG	Polyethylene glycol
PEP	Polar enhanced polymer
PET	Positron emission tomography
PG	Prostaglandin
PGC	Porous graphitic carbon
PGX_Y	Prostaglandin X_Y
pK_a	Acid dissociation constant

PLS	Partial least squares
PLS-DA	Partial least squares-discriminant analysis
PPAR-α	Peroxisome proliferator-activated receptor alpha
PSA	Primary-secondary amine
PS-DVB	Polystyrene-divinylbenzene
PUFA	Polyunsaturated fatty acid
qMS	Quadrupole mass spectrometry
QSM	Quaternary solvent manager
QuEChERS	Quick, easy, cheap, effective, rugged and safe technique
r	Coefficient of correlation (Pearson's r)
r^2	Coefficient of determination
R-AX	Retain anion exchange
R-CX	Retain cation exchange
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RSD	Relative standard deviation
SAX	Strong anion exchange
SCX	Strong cation exchange
SDME	Single drop microextraction
SIL	Silica
SLDA	Stepwise linear discriminant analysis
SOD	Superoxide dismutase
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
SU	Synthetic urine
TGF	Transforming growth factor
Th2	Type 2 T helper lymphocyte
ToFMS	Time of flight mass spectrometry
TX	Thromboxane
TXX_Y	Thromboxane X_Y
UHPLC	Ultra-high performance liquid chromatography
UPLC	Ultra-performance liquid chromatography
USRU	Unspiked real urine

UV	Ultraviolet
UVSA	Univariate statistical analysis
UV-Vis	Ultraviolet-visible
VAX	Verify anion exchange
VOM	Volatile organic metabolite

Author's note

Asthma is a disease that affects millions of children all over the world. It affects not only patients but also their parents and the social system where they belong. Its diagnose is somehow difficult and require the expertise of a health technician. Usually it is performed based on symptomatic history, long-term follow-up, extensive differential diagnosis and by the response of the child to bronchodilator or anti-inflammatory medication. Therefore, the main motivation of this work was to contribute to the progress of asthma diagnose and precision medicine with the characterization of three biomarkers of this disease (LTE_4 and LTB_4 and $11\beta PGF_{2\alpha}$) by means of two powerful analytical strategies, a sample preparation technique – microextraction by packed sorbent, and a separation and detection technique – ultra-high pressure liquid chromatography hyphenated to a photodiode array detector.

This Master Dissertation was developed in two complementary parts, one theoretical and another experimental. Initially, the theoretical part of this Master Dissertation was meant to be done in only one chapter but, due to the relevance of both the pathological field and analytical chemistry, this information was deepened in terms of disease mechanisms and metabolomics, the first and second chapters respectively. Throughout the first chapter, the reader is guided through the "history" of asthma, a worldwide social and economic burden and the main biochemical mechanisms and pathways that characterizes this common disease. The second chapter describes the role of metabolomics, the potential of non-invasive sample procedures and the potential of the most recent analytical techniques to be used in biomarker characterization. The experimental part consisted of the third chapter of this Dissertation. It describes the "fieldwork", the selection of the individual and sample collection, and the laboratory work where samples were treated, and the analytical method was developed in terms of optimization and validation and finally, in order to explore its analytical and discriminatory potential, the developed method was applied to real samples of asthmatic children and healthy controls. At the end, it can be found the fourth and last chapter of this Master Dissertation concerning the main conclusions and my future perspectives.

Chapter I – Epidemiology, etiology, pathophysiology and pathogenesis of asthma

1.1 The epidemiology of childhood asthma – a social and economic burden

1.1.1 Prevalence and incidence

All over the world, about 334 million people of all ages and ethnicities might suffer of asthma. It affects not only the asthmatic population but also their families, health system and government [1, 2]. A study carried out by the International Study of Asthma and Allergies in Childhood (ISAAC), which involved a total of 306 centers in 105 countries in all Phases and about 1.2 million children in the third Phase, concluded that the symptoms of asthma are more common than initially thought. Asthma is the most common childhood chronic airway disease in the high-income countries, more common in these countries than in the low- and middle-income counterparts although some of these also had high prevalence of asthma symptoms (current wheeze – wheeze in the past 12 months). ISAAC concluded that the prevalence of asthma symptoms among adolescents (13-14 years old) varied from values above 20% in English language countries (e.g. 32.6% in Wellington, New Zealand) and Latin America and values below 5% in countries belonging to the Indian Sub-Continent, Asia-Pacific (e.g. 0.8% in Tibet, China), Eastern Mediterranean and Northern and Eastern Europe (Figure 1A).

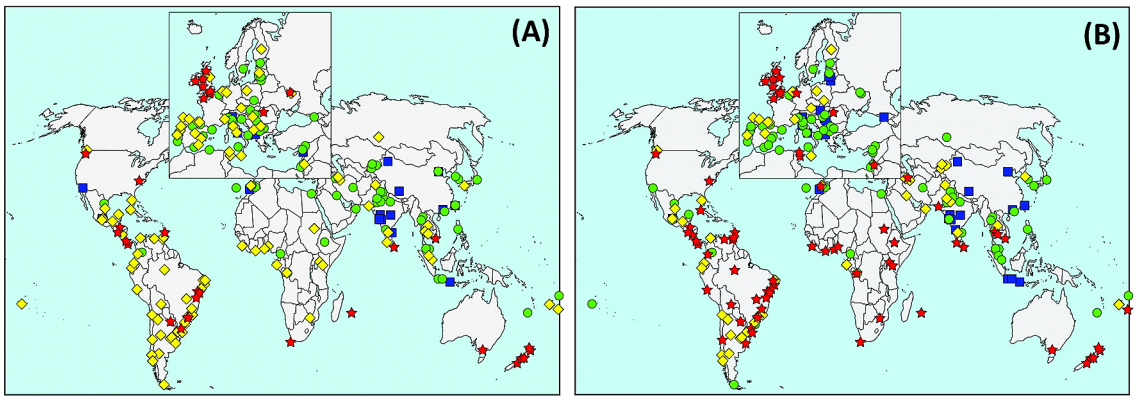


Figure 1 – Prevalence of (A) asthma symptoms and (B) severe asthma symptoms among 13-14-year-old children. Symbols indicate prevalence values of < 5% (blue squares), 5 to < 10% (green circles), 10 to < 20% (yellow diamonds) and > 20% (red stars). Adapted from [3].

Similar results were found among children (6-7 years old). It was observed a prevalence above 20% in almost all English language and Latin America countries (e.g. 37.6% in Costa Rica) and the lowest prevalence (< 5%) was in the Indian Sub-Continent (e.g. 2.4% in Jodhpur, India), Asia-Pacific and Northern and Eastern Europe. According to ISAAC, low- and middle-income countries, which represents 85% of world population, have the highest prevalence of severe asthma including African, Indian Sub-Continent

and Eastern Mediterranean countries for adolescents and Africa, Latin America and East of the Mediterranean (Figure 1B) for children with about 50% of them with current wheeze [1, 3] possibly due to the lack of resources [4]. Globally, 4.9% of children had symptoms of severe asthma (e.g. regular exacerbations and daily asthma symptoms), while this number increase to 6.9% among adolescents [3]. This study also obtained an increase in the global prevalence of asthma between the 1990s (ISAAC Phase I) and the 2000s (ISAAC Phase III), from 11.1% to 11.6 among children and from 13.2% to 13.7% among adolescents. In general, asthma is becoming more frequent and the "gap" between the high-income and the middle- and low-income countries is becoming less apparent [1]. In part, it is believed that the asthma incidence is increasing in the last decades due to urbanization and western lifestyle being expectable that in 2025, this increase will be more pronounce since urbanization will reach 45% to 59% and the asthmatic population will account for more than 400 million people worldwide [5].

Globally, 400 thousand people died from asthma in 2015. Even though this number is concerning, compared to 1990, it reflects a decrease of 26.7% in mortality due to better treatments and to the development of medicine. Asthma prevalence has increased about 12.6% during these 25 years being the most prevalent chronic respiratory disease worldwide [6].

The social burden of asthma in Portugal

The Global Allergy and Asthma European Network (GA^2LEN) defined asthma as "the patient ever had asthma throughout his life" and at least one of the following symptoms in the last 12 months: "wheeze or whistling", "waking with chest tightness", "waking with shortness of breath" and "waking with an attack of coughing" [7]. According to this definition, in 2012, Sa-Sousa et al. [8] estimated that about 7.8% of people suffered from asthma in Portugal and 10.5% suffered from asthma at some point in their life. The same authors improved the specificity of this definition and included the "asthma attack" and "waking with breathlessness" and withdrew the "waking with a feeling of tightness in chest" and "waking with cough". According to this new definition of "current asthma", they estimated the prevalence of asthma in Portugal at 6.8% comparable to European Union (EU): Gothenburg, Sweden – 7.1%; Krakow and Lodz, Poland – 7.1% and 6.0%; Amsterdam, Netherlands – 6.4%; Belgium – 7.6% and Brandenburg, Germany – 6.3% [7]. Most of the individuals (72.8%) who suffered from

asthma at some time in their life (10.5%) had their first exacerbation before age 18. From the individuals with "current asthma", 5.7% also had chronic bronchitis and 52.3% had rhinitis. Among individuals under 18 years of age, the prevalence of "current asthma" was around 7.2% and it is only surpassed by individuals over 65 (8.0%). In older individuals, between 18-65 years old, the prevalence was of about 6.3%. According to ISAAC, the incidence of asthma symptoms (wheeze) in the last 12 months in Madeira, Portugal (Table 1) was of about 14.5% in 1995, decreasing significantly to 11% in 2002 among children aged 6-7 years. Among adolescents with 13-14 years old, the values did not change significantly in the same period of 7 years remaining between the 9 to 10.2% [9]. Although in 1995, Funchal was the region of the country with the highest rate of asthma among 6-7 years children, it was the only region covered by the study where the same rate decreased significantly between 1997 and 2002, with a highest rate of asthma among 6-7 years old in the Centre – Lisbon (14.1%), North – Oporto (13.1%) and South – Portimão (13.2%) [9].

Table 1 – Results of the surveys proposed by ISAAC on the different Regional Centers in Portugal. Percentages indicate the percentage of the population that suffered symptoms of asthma (wheezing) in the last 12 months ("current asthma") between Phases I (1995) and III (2002) of the study. Lifetime asthma and wheeze refers to the populations surveyed who have had these symptoms at least once in their lifetime [9]. Abbreviations: N – number of individuals of the study population; % – percentage of individuals of the study population, asterisk refers to significant differences between the % of 1995 and 2002.

Region	**Age**	**% (N), 1995**	**% (N), 2002**	***p* value**
Lisbon	6-7	12.8 (2115)	14.1 (2484)	0.177
	13-14	10.9 (3030)	14.6 (3029)	<0.001*
Funchal	6-7	14.5 (1732)	11 (1821)	0.001*
	13-14	10.2 (3313)	9.0 (3163)	0.103
Portimão	6-7	10.7 (1189)	13.2 (1071)	0.069
	13-14	8.0 (1058)	9.7 (1109)	0.164
Oporto	13-14	7.2 (3100)	13.1 (3337)	<0.001*
Coimbra	13-14	8.1 (926)	10.7 (1177)	0.043*
Total	6-7	12.9	12.9	0.983
	13-14	18.2	21.8	<0.001*
Lifetime asthma	6-7	11	9.4	0.008*
Lifetime wheeze	6-7	28.2	28.1	0.936

1.1.2 Economic impact

Part of the injury caused by asthma is related to the economic costs, one of the highest among chronic diseases due to the high healthcare needed to treat this condition. In high-income countries, they vary from some tens of millions to thousands of millions US dollars [1]. Direct costs are related to treatment, prevention, and management and

accounted for the greatest part in most of the studies (53-100%) reviewed by Bahadori et al. [10]. It is estimated that 47-86% of these costs are related to hospitalization and 38-89% are related to medication. Indirect costs accounted for 52-75% of the overall costs and include, for example, school days missed by the asthmatic children, work days missed by their parents, loss of productivity and waiting time. Asthma costs varies with the quality of the health system, location and level of education and are strongly related to symptoms, age, and severity. The asthmatic children have a much higher indirect cost proportion than healthy children and adult asthmatics [10]. Generally, when an asthmatic child is with symptoms, it is unable to continue their most common activities such as school. If it becomes worse, the child needs immediate medical treatment, sometimes requires more intensive care and hospitalization. In the US, pediatric patients are nearly half of asthma hospitalizations (44%) being this disease the third highest cause of hospitalization among children in this country [11]. Usually parents need to miss work to take care of their children. Thus, asthma and especially under-treated asthma, causes great economic health care costs (preventive and reliever medicine, devices used in treatments such as spacers and aerosols, ambulatory care, emergency, hospital care, patient education, management and planning) and productivity losses [1]. Despite this, It is believed that an increase in medical expenses leads to a reduce in the total health care and in the indirect costs due to the improvement of the control level of the asthmatic patients [10].

1.2 An etiologic, pathophysiologic and pathogenetic approach

Asthma is defined by some authors as a syndrome [12]. Although this definition is contested by other authors [13], a clear consensus says that asthma is a heterogeneous disease with both genetic and environmental components. Genetic factors are defined by the genes related to the development of asthma [14, 15]. Atopy is a genetic predisposition for the development of an immunoglobulin E (IgE)-mediated response to common aeroallergens [16]. In addition, asthma is not equally distributed between sexes in some way due to hormonal factors [17]. Environmental components are related to a multitude of factors including aeroallergen exposure [18], viral or bacterial infections [19], active or passive smoking [20] as well as in utero tobacco smoke exposure [20] that associated to parental stress leads to the development or complication of asthma, good nutrition as a way to treat or prevent asthma [21], exercise that leads to the manifestation of symptoms

in most patients [22], climatic conditions like extreme temperatures and high humidity that are associated to the behavior of asthma and development of exacerbations [23].

Asthma is described as chronic inflammation and long-term irreversible remodeling of the airways. Furthermore, it is also defined by the clinical history of respiratory symptoms such as: dyspnea, wheeze, breathless, chest tightness, cough and variable expiratory airflow limitation that vary over time and in intensity [24-27]. When associated to one or more triggers (with an additive or synergetic effect on the development of mild symptoms or exacerbations [27]), this symptoms can culminate in an inappropriate inflammatory response: contraction of the airways and consequent narrowing and obstruction of the lumen due to airway wall edema and the excess of mucus (Figure 2). The excess of airway mucus is a common characteristic of asthma and is associated to an increase of mucin secretion (glycoproteins that are the main constituent of mucus) by hypertrophied submucosal glands related to the large cartilaginous airways, where its excessive secretion can be cleared by cough; and goblet cells hyperplasia related to the small non-cartilaginous airways, which clearance cannot be made through cough but rather by a healthy mucociliar system instead. The compromised small airways by goblet cells hyperplasia leads to mucus plugging and consequently to airway obstruction having a more significant effect on the pathophysiology of asthma than the hypertrophied submucosal glands [28].

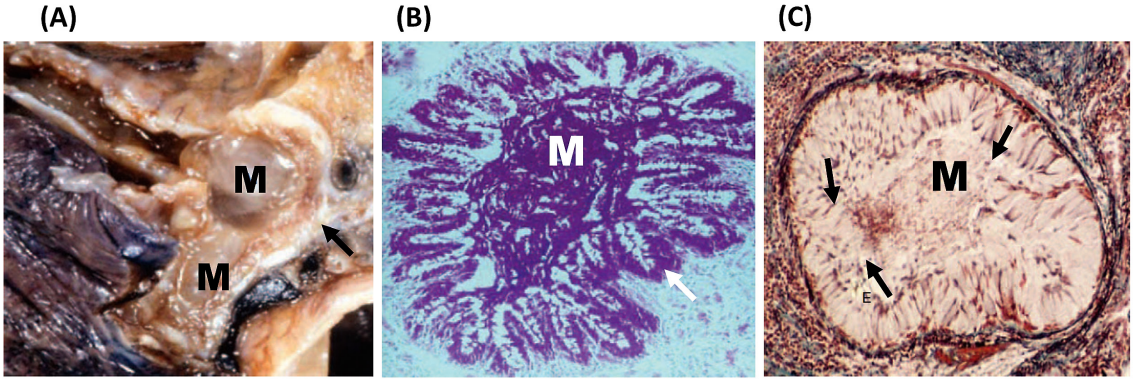


Figure 2 – Obstruction of the airways by mucus in asthmatic patients. (A) Longitudinal section of a bronchus (arrow) completely obstructed by mucus (M) in a fatal case of severe asthma. (B) Cross-section of a bronchus that suffered bronchoconstriction. Epithelium (arrow) with folds formed by contraction of the smooth muscle and occlusion by mucus (M) of the remaining luminal space in a fatal case of severe asthma. (C) Cross section of a bronchiole showing incomplete release or "continuity" of mucin segregated by the goblet cells of the airway epithelium (arrows) in a case of asthma. Adapted from [29].

1.2.1 Clinical diagnosis and classification of asthma

Currently, there are no single diagnostic methods or specific biomarkers for the detection of asthma, especially in children. This disease is suspected whenever the subject

has recurrent episodes of wheezing and cough. Usually, it is made by long-term follow-up, extensive differential diagnosis and by the response of the subject to bronchodilator or anti-inflammatory treatment.

As described by the Global Initiative for Asthma (GINA) [24], a current standard asthma diagnosis is typically performed based in two criteria: pattern of respiratory symptoms (e.g. wheezing, dyspnea, chest tightness and cough) and variable airflow. Asthma is often characterized by persistent symptoms or recurrent exacerbations triggered by numerous agents such as allergens, irritants, exercise, laughter, changes of weather and viral infections. It is also common for the asthmatic patients to have a personal history of variable respiratory symptoms and atopy (e.g. rhinitis, conjunctivitis, and eczema). The clinical diagnose begins with a physical examination in order to confirm symptoms of asthma or other atopic conditions that support its diagnosis.

The lung function evaluation is an important step of an asthma diagnosis and therapeutic response. For this purpose, spirometry is often used, a technique recommended for patients older than 5-7 years, in which among the obtained data, it is highlighted the forced expiratory volume in 1 second (FEV_1) and the peak expiratory flow (PEF). The tabulated FEV_1 values for an asthma diagnosis in children is of at least 80% of the predicted and this value should be reversible in more than 10-12% or 200 mL after bronchodilation (i.e. use of a bronchodilator short- or long-acting β_2-agonist) [24, 26, 30].

In other situations, techniques including bronchial provocation (e.g. methacholine or histamine challenge), exercise challenge, allergy tests (e.g. skin prick tests or measurement of specific IgE in serum), chest x-ray, fractional concentration of exhaled nitric oxide (eNO) quantification, exhaled breath condensate (EBC) analysis, eosinophil counting in serum or induced sputum and quantification of histamine release by basophils may be indicative of an allergic inflammation [24, 26]. In order to validate the interpretation of the results obtained by less invasive techniques, more evasive techniques including tissue analysis such as endobronchial biopsy or bronchoalveolar lavage (BAL) are used. These techniques cannot be used routinely in children once they require strong sedation [31].

In an initial clinical approach, asthma is classified according to its severity based on symptoms, frequency, air flow limitation and lung function variation. Thus, asthma is classified as persistent or intermittent. Persistent asthma can also be sub-classified as mild, moderate or severe (Table 2). Based on this classification, the most appropriate

treatment for the patient can be determined. The classification of asthma in a given patient may change over a period of months or years. Thus, in a first approach, asthma can be classified as severe persistent and then, at a later stage, be classified as moderate persistent if there is a good response to treatment [32]. In further assessments, asthma is dynamically classified based on its level of control: well controlled, partly controlled and uncontrolled with high relevance in clinal practice towards which asthma management is evaluated [30].

Table 2 – Classification of asthma by gravity based on clinical characteristics prior to treatment [32].

Classification	**Clinical symptoms**
Intermittent	Presents symptoms less than once a week; Short term exacerbations; Nocturnal symptoms no more than 2 times a month – Prediction of FEV_1 or PEF ≥ 80% – Variability of PEF or FEV_1 < 20%
Mild persistent	Present symptoms more than 1 time per week but less than 1 time per day Exacerbations can affect activity and sleep Nocturnal symptoms more than 2 times per month – Prediction of FEV_1 or PEF ≥ 80% – Variability of PEF or FEV_1 < 20-30%
Moderate persistent	Present daily symptoms Exacerbations can affect activity and sleep Nocturnal symptoms more than 1 time per week Daily use of short-acting β_2-agonists – Prediction of FEV_1 or PEF = 60-80% – Variability of PEF or FEV_1 > 30%
Severe persistent	Present daily symptoms Regular exacerbations Regular nocturnal symptoms Limitation of physical activity – Prediction of FEV_1 or PEF ≤ 60% – Variability of PEF or FEV_1 > 30%

1.2.2 Atopic asthma – from a phenotype to an endotype classification

In 1947, Francis Rackemann classified asthma in two clinically distinct main phenotypes: atopic or extrinsic asthma and non-atopic or intrinsic asthma [33]. The atopic phenotype is highly related to allergies, characterized by airway hyperresponsiveness, infiltration of the mucosa with eosinophils and type 2 T helper lymphocytes (Th2), circulating specific IgE and positive skin prick tests to common allergens (e.g. pollens, mold, mites and animal epithelia). It is more common, contributing with about 73% of the asthmatic cases and arises earlier, before 30 years old. Its diagnosis is relatively simple

and generally based on clinical and familiar history. It may complicate due to infections and it is also associated to asthmatic bronchitis, cough and rhinitis that can lead to a severe form of asthma. Non-atopic asthma is hard to diagnose once its patients do not have a clinical familiar history of allergies, having its origin in factors that are not related to allergies such as bacterial infections. “Polypoid sinusitis”, dyspnea, negative skin prick tests and total IgE concentration in serum between the normal values without significant evidence of specificity to common aeroallergen, are common characteristics among these individuals. They show sensitization to aspirin more frequently, and psychosomatic exhaustion factors are also considered. This phenotype affects about one fourth (i.e. 27%) of the asthmatic population that tend to be older than the atopic asthmatics (older than 30-40 years old) with a predominance of female individuals [18, 33-35].

According to Bacharier et al. [26], phenotypes of asthma are defined by age and triggers. Symptoms persistence (i.e. wheezing) in infant children (0-2 years old) is an important indicator of severity. Among preschool children (3-5 years old), symptoms persistence during the last 12 months is a differentiating indicator of asthma phenotype. Depending on the symptoms, asthma can be classified as virus- (more common at this age), exercise- or allergen-induced. Among school-age children (6-12 years old), even though symptoms are comparable to those of preschool children, the allergen-induced asthma becomes more common and apparent. Seasonality may become evident and severity can become an issue due to the lack of response to therapy and lung function. In adolescence (13-16 years old), atopic asthma has more new cases than remissions and non-atopic asthma can have its onset. Some issues like smoking, follow-up of therapy, and change of the pediatric to another physician have their beginning at this age. A study by de Blic et al. [36] suggested that severe asthma could also be considered as a unique standalone phenotype. It is age-dependent, related to persistence and to a poor response to therapy and can be measured by the frequency of symptoms and lung function. In infant children, persistence should be considered as severe asthma, in older children, severe exacerbations are those with respiratory distress with need of oxygen and consequent hospitalization [26].

Despite the classification of asthma in different phenotypes being extensively used, it has shown to be insufficient regarding its heterogeneity at a molecular level and its failure in correlating inflammation, damage functional impairment and symptoms [37]. In order to make progress in terms of new treatments, preventions and cures, a recent

approach has sub-classified each phenotype into groups based on their different pathogenetic mechanisms at a molecular and cellular level, and based on their response to treatment – endotypes [37, 38]. The allergic asthma phenotype is defined by different endotypes including Th2-driven inflammation (allergen exposure), eosinophilic inflammation (eosinophils infiltration in the mucosa), responsive to steroids, responsive to allergen immunotherapy, responsive to anti IgE and responsive to specific cytokines such as interleukin (IL)-4, IL-13 and IL-5 [39]. The sub-classification of the phenotypes into endotypes gives the opportunity to identify biomarkers of disease and consequently guide to a better and more precise diagnosis that leads to a personalized treatment and management [38].

1.2.3 Pathogenesis of inflammation in allergic asthma

Airway inflammation is an inherent feature of allergic asthma. A better understanding of the specific steps of this process, may improve the advance of new therapies which can be applied to patients and consequently improve the control of asthma and its symptoms.

The genetic predisposition to trigger a local IgE-mediated response (i.e. atopy) and the environmental influence, such as timing and allergen dose to which the subject is exposed, contribute to the appearance of an inappropriate inflammatory response to allergens that, in a normal situation, should be harmless [27, 40].

Allergic inflammation in asthma comprises a well-orchestrated interaction between the immune system and the respiratory epithelium. An ideal immune function depends on the dynamic balance between both innate and adaptive systems and their immunoregulatory components [40]. The innate immune system forms a peripherical network of immunological surveillance in order to initiate an immediate beneficial response against foreign agents [41]. The adaptive immune system generates lymphocytes that circulate between the lymphatic and the blood circulatory systems capable of mounting an antigen-specific immune response able to acquire long lasting memory against the same antigen [42]. Even though the definition of both systems seems clear, their distinction has become increasingly difficult because of their dynamic interaction [40].

Allergic asthma is widely dependent on IgE sensitization. Airway inflammation is a response of the immune system to injury which, in a normal situation, should be

advantageous. In an anomalous situation, such an allergic asthmatic reaction, the inflammatory response leads to a chronic inflammation in the presence of non-pathogenic agents. In these cases, exacerbations can occur even in the absence of continuous allergen exposure. The combined action between the multi-cellular inflammatory infiltrate and the parenchymal lung tissue cells is performed by bioactive mediators such as eicosanoids, antibodies, cytokines and growth factors [40].

The inflammatory cascade associated to asthma, is a process that consists of 4 main phases: (*i*) sensitization or antigen presentation phase, (*ii*) re-exposure to the allergen, (*iii*) airway remodeling, and (*iv*) resolution. In this chapter, it is briefly described the main phases of the inflammatory process.

(i) Sensitization

The development of allergic asthma involves sensitization to a specific allergen which can arise years before the onset of the characteristic symptoms of asthma (Figure 3). This phase occurs when the allergen (i.e. antigen) stimulates the production of Th2. These Th2, do not react directly to allergens. They have antigen-presenting cells (APCs) as intermediates, such as dendritic cells (DCs), considered the most powerful APCs, that constantly scan the airways through pattern recognition receptors associated to damage or pathogenic agents, promoting their detection and elimination. The activation of Th2 from naïve $CD4^{+}$T cells by APCs leads to the synthesis of inflammatory cytokines (e.g. IL-4 and IL-13) that stimulate the interaction between Th2 and B-lymphocytes to proliferate and synthesize specific IgEs for the antigen. The specific IgEs circulate in the blood stream and bind essentially to high affinity IgE receptors (FcεRI) – more common, present on mast cells surface of the airways epithelium and lower layers of the mucosa, and on the peripheral blood basophils, less abundant granulocytes, which are found in the blood making them highly reactive – or to low affinity IgE receptors (FcεRII) – present on a wide variety of leukocytes, making these cells accessible to the allergen in a future re-exposure [40, 43-45].

The genetic predisposition for atopy, and the continuous exposure to factors with a harmful effect to a good lung growth and proper development of the immune system, immature at an early age, such as alcohol, active or passive smoking (particularly during pregnancy), air pollution and exposure to volatile chemicals influence the sensitization process. The constant exposure to antigens and the systematic stimulation of asthma

symptoms, intensify their persistence throughout life as well as decrease the probability of remission [43, 46].

(ii) Re-exposure to the allergen

A re-exposure to the allergen, leads to a complexity of intercellular signaling events that promote the degranulation and discharging of mediators (e.g. proteases), cytokine transcription, histamine and synthesis of eicosanoids derived from phospholipids such as serotonin, leukotrienes (LTs) and prostaglandins (PGs) by mast cells and basophils. These mediators cause the early-phase (i.e. acute-, immediate-phase) reaction (Figure 3) characterized by airway hyperresponsiveness, mucus hypersecretion and vasodilation (bronchospasms and edema) before inflammatory cells recruitment. At this time, occurs activation of macrophages and production of reactive oxygen species (ROS) [40, 44, 45] as will be discussed in more detail later.

After hours or days, about half of the asthmatic patients experience the late-phase (i.e. chronic phase) reaction (Figure 3) characterized by the strong infiltration of Th2 in the mucosa, mediated by eosinophilic inflammation [47] and the involvement of macrophages, epithelial, endothelial and smooth muscle cells that promote the chronic inflammatory symptoms of asthma by producing their mediators such as chemokines, cytokines and LTs [44, 45].

Mast cells are an initial source of IL-4 and IL-5 (recruitment, differentiation, proliferation and maturation of eosinophils present in the vascular system). Absent in non-asthmatic patients such as eosinophilic bronchitis patients, they are recruited and maintained by the smooth muscle of the airways of asthmatic individuals responsible for the characteristic airway constriction [40]. Eosinophils are found mainly in the lung tissue and BAL of asthmatic patients. Their precursors, $CD34^+$ cells, are recruited from the bone marrow to the lungs by the release of PGs, cysteinyl-LTs (cys-LTs), cytokines and chemokines. Its selective recruitment to the airways is driven by chemoattractant eotaxins synthesized by the epithelial cells in response to IL-5 and IL-13 from Th2 and degranulate to release cationic proteins, harmful to the lung tissue. Some studies also demonstrated that eosinophils have the ability to activate the pathogenic response of Th2 [48]. Chronic inflammation becomes persistent due to the repetitive cycle of tissue damage and inflammatory cells recruitment, even in the absence of allergens [40, 45, 49]. Basophils act in the same way as eosinophils. They have FcεRI, APC function and release

eicosanoids and histamine by degranulation contributing to the pathogenesis of asthma [40].

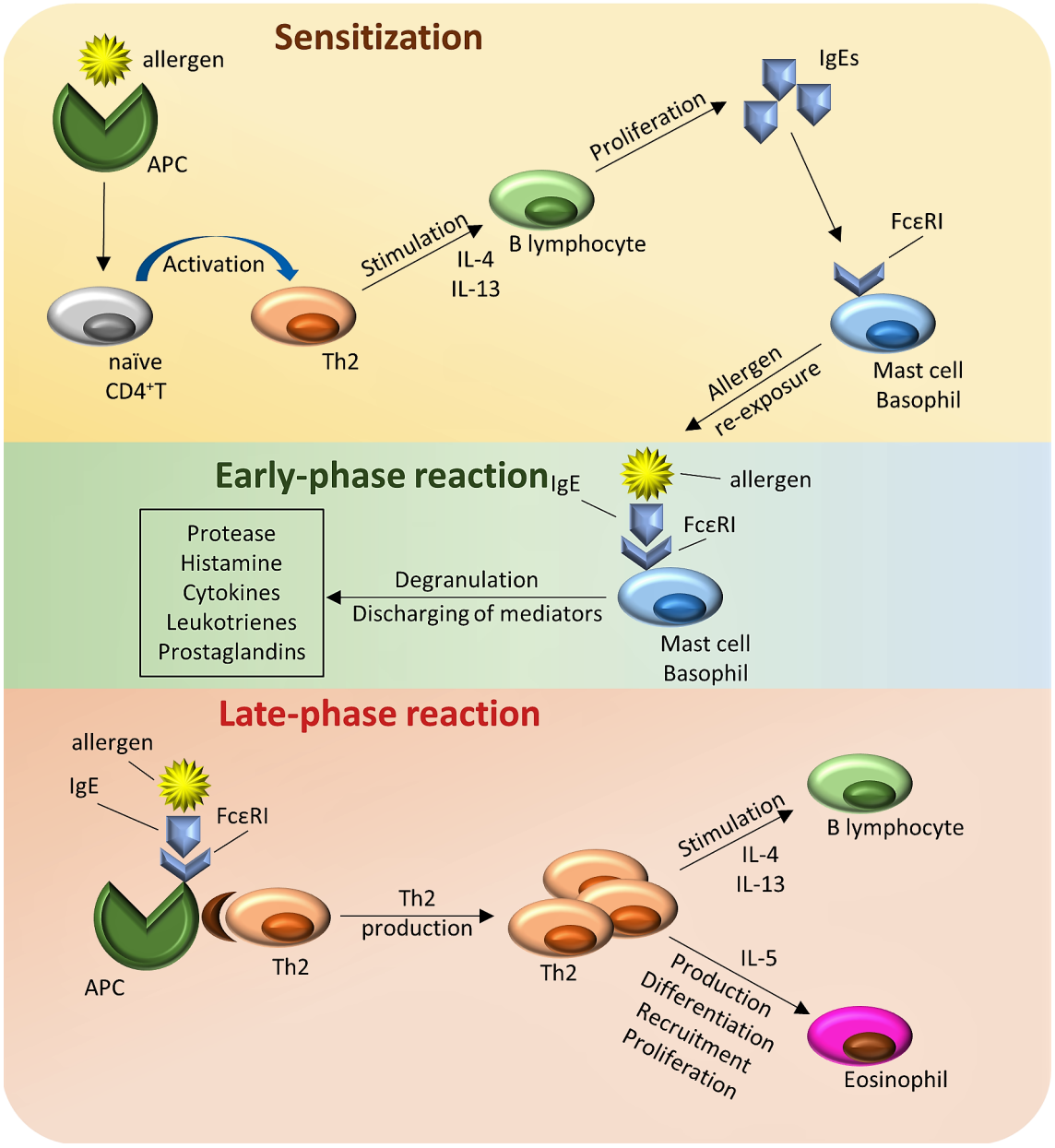


Figure 3 – Sensitization, early-phase and late-phase reaction in the inflammatory response. Abbreviations: APC – antigen presenting cell; IL – interleukin; IgE – immunoglobulin E; FcεRI – high affinity IgE receptor. Adapted from [47].

Even though eosinophilic inflammation is the most common type of asthma and even considered a distinct endotype, neutrophils have also been associated with cases of severe asthma, exacerbations and non-atopic asthma, being considered a distinct endotype represented in a significant group of asthmatics, who respond poorly to corticosteroids [40].

The Th2, cell biomarkers of disease severity, migrate into the lungs and allow the airways to recognize and respond to the presence of allergens. Their effect is amplified by the production of inflammatory cytokines such as IL-4, -5, -9, -13 and -25 promoting the IgE production, inflammation, airway hyperresponsiveness and remodeling [40].

The presence of lymphocytes with characteristics from both innate and adaptive immune systems are classified as non-classical lymphocytes. These cells include natural killer cells (NKs), NK T cells (NKTs) and γδT cells. NKs are naturally present in the lungs. However, although their role is still questionable, they were observed in an increased concentration in asthmatic patients. The activation of NKTs by glycolipids promotes the synthesis of Th1 and Th2 cytokines (e.g. IFN-γ and IL-4). In the same way as NKs, γδT cells were found in a greater concentration in asthmatic patients essentially during periods of exacerbations being considered as both regulators of airway homeostasis or pro-inflammatory mediators [40, 49].

(iii) Airway remodeling

The characteristic chronic inflammation of the asthmatic patients is typically accompanied by changes in tissues at the level of extra cellular matrix, protein deposition, basement membrane, increased smooth muscle mass, nerves, vessels and epithelial barrier function followed by hyperplasia – airway remodeling. These changes contribute to the thickening of the airway walls and to the decrease of the airway diameter resulting in obstruction, hyperresponsiveness and poor patient response to therapies such as corticosteroids, β_2-agonists and anti-LTs [40, 43].

The transforming growth factor (TGF)-β is upregulated in the asthmatic airways and is considered the main regulator of remodeling. It promotes the synthesis of extracellular matrix and the transformation of myofibroblasts from airway smooth muscle and fibroblasts, which in a normal individual would have a repair function. However, in asthmatic patients, myofibroblasts appear to be correlated with thickening of the basement membrane. In addition, TGF-β propagates the airway remodeling through the induction of pro-inflammatory mediators with effect on smooth muscle contractibility. Other mediators identified in asthmatic airways contribute to remodeling, such as platelet-derived growth factor that promotes proliferation of fibroblasts and smooth muscle; and the vascular endothelial growth factor that promotes angiogenesis and irregular

production of extracellular matrix metalloproteinases responsible for matrix degradation during remodeling [40, 44, 45].

(iv) Resolution

The resolution phase of the inflammatory process in asthma concerns homeostasis. It includes the removal of apoptotic cells and release of IL-10 with anti-inflammatory function by restoring tissues and growth factors that lead to the return of tissue integrity [40, 44, 50].

The chemokine receptor D6 as well as certain infiltrating leukocytes have a resolution function by cleansing inflammatory chemokines that recruit leukocytes. During this step, other anti-inflammatory agents are also produced among which lipid mediators, such as resolvins and protectins, produced from eicosapentaenoic acid and docosahexaenoic acid in order to control the infiltration of granulocytes. In this way, they prevent the release of pro-inflammatory chemokines and activation of inflammatory cells, promoting the clearance of inflammatory cells [40].

1.3 The role of enzymatic and non-enzymatic lipid peroxidation in asthma

During lipid peroxidation, a polar group hydroperoxide (-OOH) is added to the hydrophobic tails of the unsaturated fatty acids. Hydroperoxide lipids are generally characterized as harmful since they lead to changes in lipid-lipid and lipid-protein interactions, to the formation of free radicals and to interruptions in the functions of cell membranes, compromising the integrity of organelles and cell as a whole. However, if lipid peroxidation occurs in a controlled manner in specific cell sites, it may have beneficial effects on the organism such as destructive processes of cell metabolism, cell differentiation and cell maturation, which require changes in cell and subcellular membranes. Hence, it is expected that lipid peroxidation can be activated or deactivated when necessary and its level of control is not possible by non-enzymatic reactions thus, different enzymes are involved in this process [51] such as the lipoxygenases (LOs) inherent to the LTs and lipoxins (LXs) pathway [51, 52] and the cyclooxigenases (COXs) inherent to the pathway of PGs and thromboxane A_2 (TXA_2) [53].

1.3.1 The arachidonic acid cascade – an enzymatic peroxidation approach

As discussed before, mast cells play an important role in the inflammatory response by the release of pro-inflammatory mediators such as LTs and PGs with origin on the arachidonic acid present in the cell membrane.

The arachidonic acid cascade (Figure 4) begins with its hydrolysis under the action of phospholipase A2. Its enzymatic oxidation is mediated by COXs, LOs and epoxygenases (EPOXs) leading to the formation of important bioactive metabolites belonging to the group of eicosanoids – oxygenated polyunsaturated fatty acids (PUFAs) containing 18 to 22 carbons [54]. Even though metabolites derived from the EPOX pathway, epoxyeicosatrienoic acids, are of great importance to the body as blood pressure regulators in addition to their beneficial effects on the vascular, cardiac, nervous and renal system [55], it will be essentially discussed over this master dissertation, metabolites derived from the COXs and LOs pathways such as (*i*) LTs (eicosanoids containing 3 conjugated double bonds, trienes), (*ii*) PGs (eicosanoids containing a 5 carbon ring) and (*iii*) LXs (distinguished by having a system of 4 conjugated double bonds, tetraenes) since they are powerful inflammatory mediators and are directly related to atrophy and weakening of airway muscles in chronic respiratory diseases [52, 56-58].

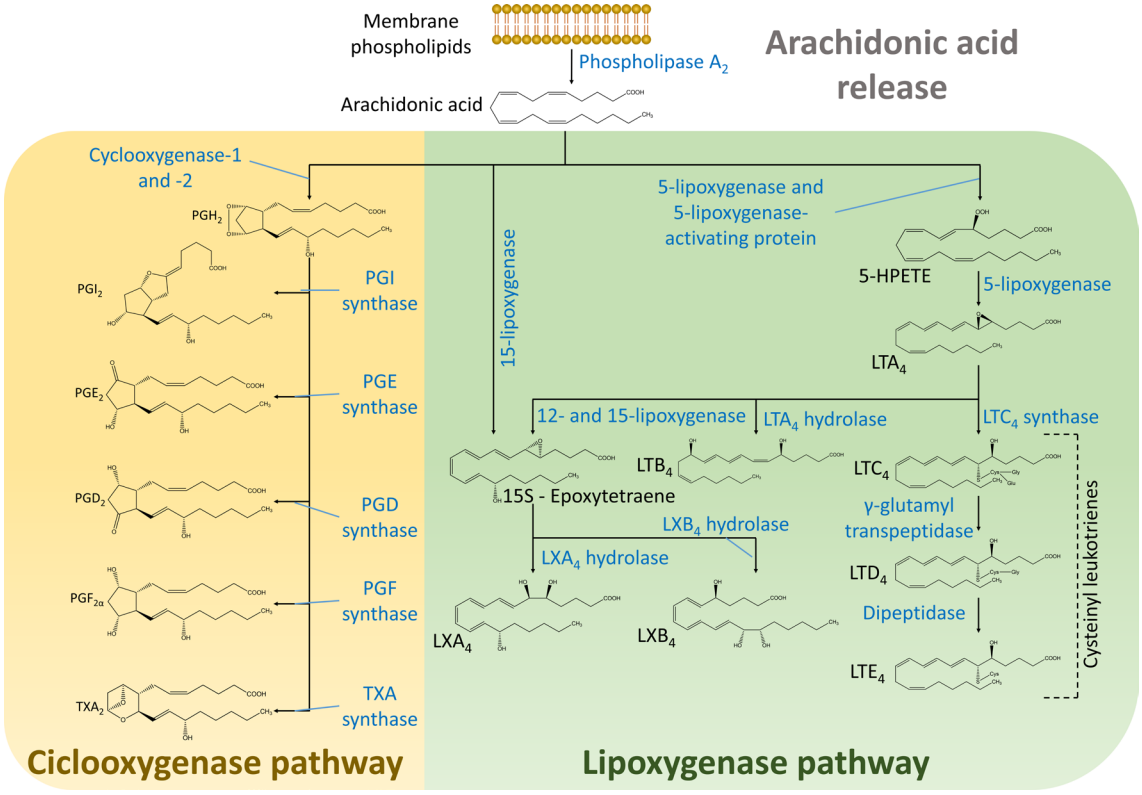


Figure 4 – The arachidonic acid cascade. An enzymatic pathway that leads to the formation of bioactive eicosanoids. Abbreviations: 5-HPETE, arachidonic acid 5-hydroperoxide; LX_Y, lipoxin X_Y, LTX_Y, leukotriene X_Y, PGX_Y, prostaglandin X_Y, TX_Y, thromboxane X_Y.

In Table 3 is shown studies perform with different analytical strategies to characterize the eicosanoids levels present in different biosamples of asthma and COPD sufferers.

(i) Leukotrienes

After hydrolysis, the arachidonic acid is converted by the enzyme 5-LO and the 5-LO-activating protein (FLAP) into 5-hydroperoxyeicosatetraenoic acid (5-HPETE) by the insertion of a hydroperoxide group (-OOH) into the carbon 5 of the arachidonic acid. A subsequent dehydration reaction, catalyzed by the 5-LO, results in the formation of LTA_4 [54, 59]. This LT is a precursor of the LTB_4, a dihydroxy leukotriene, when hydrolyzed by the action of the enzyme LTA_4 hydrolase. The LTA_4 can be also converted to cys-LTs (LTC_4, LTD_4 and LTE_4), when conjugated to intracellular reduced glutathione (GSH) by the enzymatic action of LTC_4 synthase [52, 57]. The FcεRI-mediated release of LTC_4 by mast cells, depends essentially on the levels of intracellular GSH. The intracellular GSH levels increase with the levels of oxidative stress leading to the intensification of inflammation [54, 59, 60]. LTs formation is controlled by the differential expression of enzymes in cells. For example, neutrophils express LTA_4 hydrolase and produce LTB_4 whereas mast cells and eosinophils express LTC_4 syntax and produce LTC_4 although transcellular biosynthesis occurs in 60 to 70% of cases. This process occurs, for instance, when a cell A (e.g. neutrophil or mast cell) produces LTA_4 which is then used by a cell B to produce LTC_4 (e.g. platelets) or LTB_4 (e.g. erythrocytes) [54].

The LTB_4 (Figure 5) is a powerful proinflammatory chemoattractant whose main target are neutrophils promoting their activation, adhesion to endothelium and chemotaxis – selective recruitment to the lungs. The signaling of these eicosanoids is mediated by both LTB_4 receptor-1 of high affinity and specificity, and LTB_4 receptor-2 of low affinity and specificity. These receptors also differ in their distribution in tissues and belong to the family of G protein-coupled receptors. The LTB_4 also activates degranulation and release of other mediators such as IgEs and cytokines, enzymes and superoxides in neutrophils, and it is also involved in inflammatory pain by processes dependent on these neutrophils. It also has a ligand function for the peroxisome proliferator-activated receptor alpha (PPAR-α), which plays a key role in the oxidative degradation of fatty acids and their derivatives including LTB_4 itself [52, 59].

Table 3 – Relevant studies that investigated levels of eicosanoids behavior among asthmatic populations, COPD sufferers and healthy individuals. Abbreviations: LX_Y – lipoxin X_Y; LTX_Y – leukotriene X_Y; PGX_Y – prostaglandin X_Y; TX_Y – thromboxane X_Y; EBC – exhaled breath condensate; COPD – chronic obstructive pulmonary disease; AIA – aspirin-induced asthma; ATA – aspirin-tolerant asthma; MEPS – microextraction by packed sorbent; LL – liquid-liquid extraction; EIA – enzyme immunoassay; SPE – solid-phase extraction; UHPLC – ultra-high performance liquids chromatography; UHPLC-PDA – UHPLC-photodiode array; UHPLC-MS/MS – UHPLC-tandem mass spectrometry; GC – gas chromatography; GC-NICI-MS – GC-negative ion chemical ionization-mass spectroscopy; GC-MS – GC-mass spectrometry; ANOVA – analysis of variance.

Analyte	**Biosample**	**Population**	**Groups**	**Extraction platform**	**Analytical platform**	**LOD/LOQ**	**Statistical analysis**	**Comments**	**Ref.**
LTB_4	Urine	Adults and children	Case vs control	MEPS	UHPLC-PDA	0.37/1.22 ng/mL		Increased in asthmatics	[61]
LTB_4	Sputum	- (COPD)	Case vs control	LL	UHPLC-MS/MS	-/0.2 ng/mL		Increased in COPD patients	[62]
LTB_4	EBC	Children	Case vs control	EIA	-	4/- pg/mL	Spearman's correlation, Mann-Whitney and Wilcoxon signed-rank test	Increased in asthmatics	[63]
Cys-LTs and LTB_4	Sputum	-	Case vs control	SPE	UHPLC-MS/MS	9.8/19.5 pg/mL		Increased in asthmatics. Undetectable LTC4	[78]
11β$PGF2_\alpha$ and LTE_4	Urine and plasma	Adults	AIA vs ATA vs control	SPE	GC-NICI-MS	-	Pearson's correlation and ANOVA	Increased in asthmatics	[65]
11β$PGF2_\alpha$	Urine	Adults	AIA vs atopic vs control	EIA	-	15.6/- pg/mL	T test	Increased in atopic asthmatics and AIA after induction	[79]
11β$PGF2_\alpha$ and LTE_4	Urine	Children	Case vs control and obese vs non-obese	EIA	-	-	Wilcoxon signed-rank and Kruskal-Wallis test	Increased only in asthmatics. More pronounced in obese asthmatics	[80]
Cys-LTs, TXB_2, PGE_2, PGD_2 and $PGF_{2\alpha}$	Sputum	Adults	Case vs control	EIA (Cys-LTs) and LL (PGs and TX)	GC-NICI-MS		Pearson's correlation and Kruskal-Wallis test	Increased Cys-LTs in asthmatics. More pronounced in persistent and severe asthma.	[81]
Cys-LTs and LTB_4	EBC	Adults and children	Case vs control	LL	GC-MS	1/- pg/mL	Spearman's correlation, Kruskal-Wallis and Mann-Whitney test	Increased in asthmatics	[82]
LTB_4 and LXA_4	EBC	Children	Case vs control	SPE and EIA		3.9/- pg/mL	χ^2, T test, ANOVA, ROC and AUC	Increased in asthmatics	[83]

As shown in Table 3, the LTB_4 levels tend to be higher in different biological matrices such as urine, EBC and sputum of individuals with asthma [61-63].

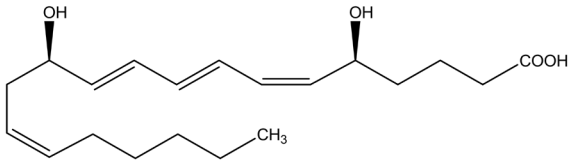


Figure 5 – Chemical structure of leukotriene B_4.

The Cys-LTs contain a cysteine amino acid in their structure and are considered the most powerful bronchoconstriction mediators known to date, about 100 to 1000 times more powerful than histamine, and play an important role in inflammatory disorders, particularly in asthma. They are eosinophil chemoattractants, possess a potent vasoconstricting and vasodilating capacity and increase vascular permeability allowing the migration of macromolecules from the plasma to airway edema, characteristic of asthma. In addition, they stimulate mucus secretion and inhibit mucociliar clearance. As for LTB_4, the effects of these LTs are mediated by G protein-coupled receptors (Cys-LT receptor-1 and -2) with differential tissue distribution [52, 59]. Cys-LTs are potential biomarkers of asthma [64, 65] and are released following allergen exposure, exercise or ingestion of COX-1 enzyme inhibitors known as nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin [66, 67]. The use of cys-LT receptor antagonists (LTRAs) such as montelukast, zafirlukast and pranlukast, as well as inhibitors of its synthesis such as zileuton, have been used to ease clinical symptoms of asthma [68] as well as in several studies in order to determine their role in the pathophysiology of this disease [67, 69]. Since LTC_4 and LTD_4 are biologically converted to LTE_4 (Figure 6), little or none of these precursors is detected in urine. Therefore, LTE_4 is considered a urinary biomarker of total cys-LTs [70-72] as well as a potential biomarker of asthma severity [73] (Table 3). It has been demonstrated an increase in urinary LTE_4 levels following inhalation of either LTC_4 and LTE_4 [74]. Furthermore, baseline LTE_4 values are essentially related to age [75] and genetic predisposition [76].

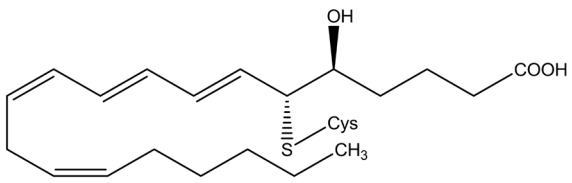


Figure 6 – Chemical structure of leukotriene E_4.

(i) Prostaglandins

PGs are formed by metabolization of the arachidonic acid catalyzed by COX-1 and COX-2 in PGH_2, an unstable PG, rapidly converted by PG terminal synthases into bioactive PGs - PGE_2, PGI_2, $PGF_{2\alpha}$, PGD_2 and thromboxane A2 (TXA_2). PGE_2 and PGD_2 can be recruited by inflammatory cells such as neutrophils, macrophages and mast cells [57, 58]. During an inflammatory response, the level and profile of PG production changes from very low values in non-inflamed tissues to very high values due to differential expression of enzymes in inflamed tissues [77].PGD_2 is a major eicosanoid released by the activation of mast cells and basophils [84, 85] and plays an important role in both inflammation and homeostasis of the organism. For example, in the brain, it is involved in the regulation of sleep and other activities of the central nervous system as the perception of pain. It has long been associated to inflammation and atopy despite, in some cases, exerting anti-inflammatory functions. PGD_2 can subsequently be metabolized into $11\beta PGF_{2\alpha}$ and PGs of the J series (e.g. PGJ_2). It has been detected in the BAL fluid of asthmatic patients, concentrations about 150 times higher after an allergic reaction. In tests performed with this PG, it has been observed bronchoconstriction and eosinophilic infiltration. Its pro-inflammatory effects are mediated by DP1 and DP2/CRTH2 receptors. DP1 receptors are expressed in the bronchial epithelium and are mediators of chemokines and cytokines production that recruit lymphocytes and eosinophils which in turn lead to inflammation and airway hyperreactivity. In addition, the activation of DP1 receptors also leads to the reduction of eosinophils in allergic inflammation. DP2/CRTH2 receptors contribute to the pathogenic response by controlling cell migration (i.e. chemotaxis). PGD_2 recruits Th2 and eosinophils directly through the DP2/CRTH2 receptors present in inflammatory cells such as Th2, eosinophils and basophils. The anti-inflammatory action of PGD_2 is associated with the presence of DP1 receptors in DCs which prevents them from migrating from the lungs to the lymph nodes and may play a role in the immune adaptive response to antigens by reducing the proliferation of cytokines by antigen-specific T cells [77]. The PGD_2 is not excreted in the urine. The first and most abundant metabolite of PGD_2 is $11\beta PGF_{2\alpha}$ (Figure 7), which also has biological activity such as bronchoconstriction and contraction of the coronary arteries and it is enzymatically synthesized by the action of the reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent 11-ketoreductase [79, 85].

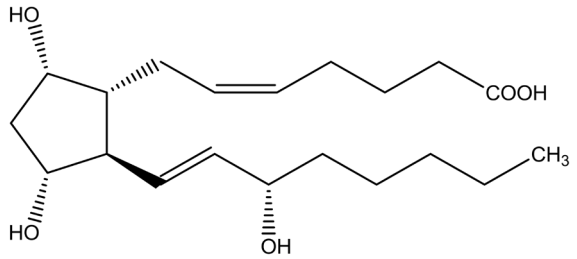


Figure 7 – Chemical structure of 11β-prostaglandin $F_{2\alpha}$.

(ii) Lipoxins

Although LXs, such as LXA_4 and LXB_4, do not belong to the LT family, they are eicosanoids whose origin is mediated by LOs being the product of the action of the 5-LO enzyme together with 15-LO and/or 12-LO. Their main functions are cell regulation, inflammatory inhibition in the lungs, increase phagocytosis of macrophages, resolution of pulmonary edema, inhibition of cell proliferation, COX-2 activity and angiogenesis [52, 57]. Therefore, their expression is decreased in the airways and systemic circulation of patients with severe asthma [86].

1.3.2 Oxidative stress and inflammation

Oxidative damage is defined by the injury that occurs in the body when reactive species outgrow the antioxidant defenses of the body [87].

ROS and reactive nitrogen species (RNS), either endogenous or exogenous, play an important role in airway inflammation and are a determining factor in the severity of asthma. An excess of reactive species in the organism leads to deep structural cell damage through oxidation of the main biomolecules including lipids, proteins and DNA [88].

According to the hierarchical model of oxidative stress (Figure 8) [89], when the organism is exposed to low levels of oxidative stress, it is able to recover cellular homeostasis through the production of different enzymatic antioxidants such as catalase (CAT), superoxide dismutase (SOD) 3, hemoxygenase-1, glutathione-S-transferase (GST), quinone oxidoreductase, glutathione peroxidase (GPx) and UDP glucuronosyltransferase induced through the nuclear erythroid 2 p45-related factor 2 (Nrf-2) pathway responsible for activating the transcription of more than 200 genes. Due to its ability to respond to low levels of oxidative stress, Nrf-2 may be a determinant predisposition factor to develop asthma as a response to environmental stimuli such as air pollution [90]. When exposed to intermediate levels of oxidative stress, which overwhelmed the antioxidant capacity of the organism, other potentially pro-

inflammatory pathways are developed such as mitogen-activated protein kinase (MAPK) and nuclear factor (NF)-κB pathways – leading to the expression of cytokines, chemokines, and adhesion molecules relevant to airway inflammation and consequently to asthma *per se*. An increase to high levels of oxidative stress can lead to a mitochondrial cytotoxic response resulting in cellular apoptosis or necrosis [89, 91].

Asthma is also characterized by the unbalance between oxidant and antioxidant agents. Antioxidants protect cells and tissues from the continuous production of ROS and RNS from normal organism metabolism [92]. An unbalance in the state of redox homeostasis of the airways is a determining factor in the initiation of asthma and its severity [91]. Consequently, asthma is characterized by a loss of antioxidant activity, as demonstrated by Sackesen et al. [93] where they found that the SOD and GPx as well as non-enzymatic compounds such as glutathione, ascorbic acid, α-tocopherol, lycopene and β-carotene were found to be significantly lower in asthmatic children. The loss of enzymatic antioxidant activity (e.g. SOD, CAT and GPx) reflects the oxidative stress in the airways of asthmatic patients [94]. The genetic component is also reflected in the expression of antioxidant enzymes such as GSTs expressed in the lungs. Different polymorphisms with high frequency in the population lead to a loss in the expression of these enzymes, resulting in a pathophysiological alteration that is reflected in inflammation of the airways and symptoms, increase of IgE values and a histamine response [91].

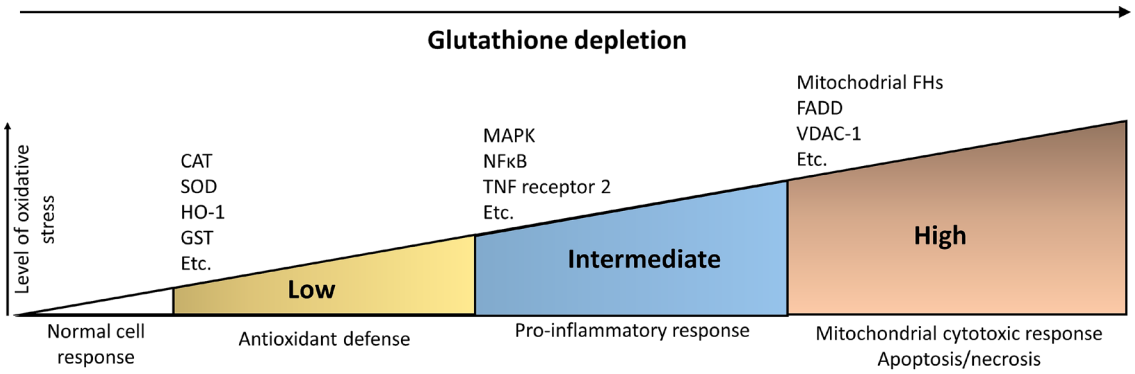


Figure 8 – The hierarchical model of oxidative stress. At a low level of oxidative stress, several antioxidant enzymes are induced via Nrf-2 in order to recover the redox homeostasis. At an intermediate level, there is induction of proinflammatory responses by the activation of MAPK and NFκB. At a high level, apoptosis or cell necrosis occurs. Abbreviations: CAT – catalase; SOD – superoxide dismutase; HO-1 – hemoxygenase-1; MAPK – mitogen-activated protein kinase; NFκB – nuclear factor-κB; TNF – tumor necrosis factor; FH – fumarate hydratase; FADD – Fas-associated death domain protein; VDAC-1 – voltage-dependent anion-selective channel-1 protein. Adapted from [89].

1.3.2.1 The role of endogenous and exogenous reactive species in asthma

Inflammatory cells including mast cells, eosinophils, macrophages and neutrophils, and the epithelial and smooth muscle cells of the airway produce endogenous ROS (Figure 9) after their activation by a variety of stimuli [44, 91, 95].

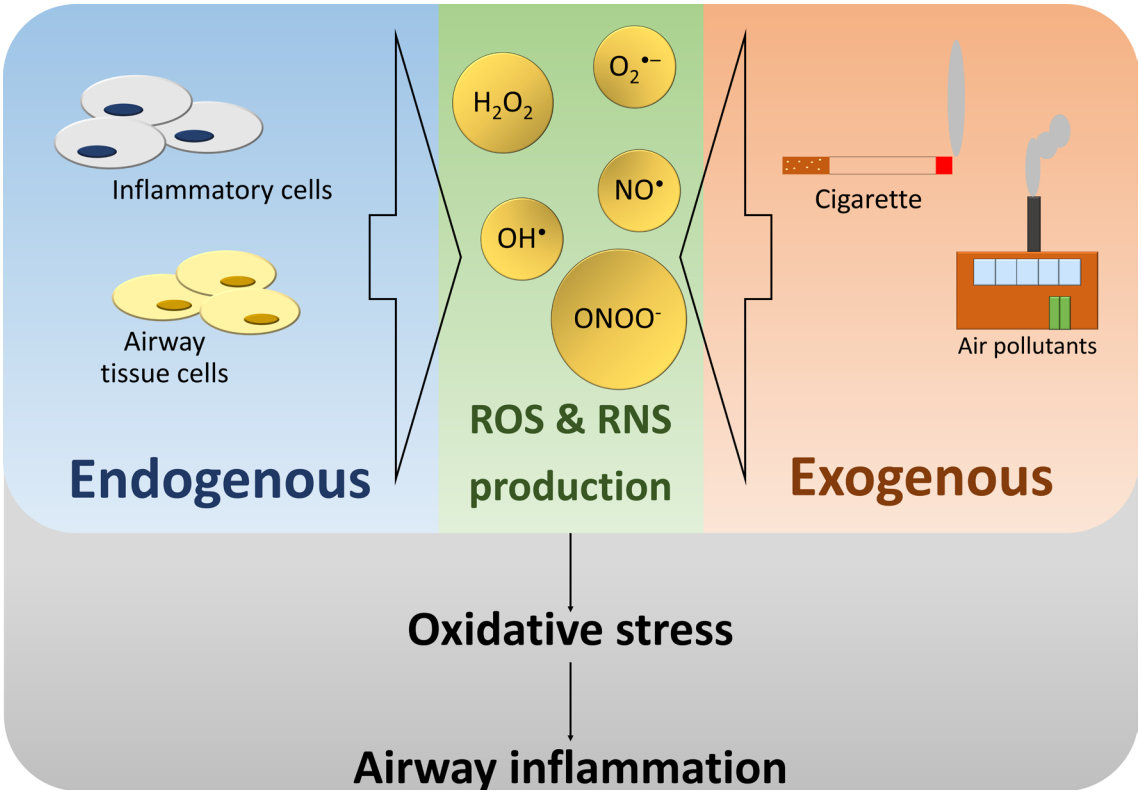


Figure 9 – The role of endogenous and exogenous reactive species in airway inflammation. Oxidative stress is caused by the excess of both endogenous (produced by inflammatory cells and airway tissue cells) and exogenous reactive species (e.g. environmental and occupational pollution) that lead to airway inflammation. RNS, reactive nitrogen species; ROS, reactive oxygen species.

The superoxide anion ($O_2^{\bullet-}$) is produced by the nicotinamide adenine dinucleotide phosphate (NADP) oxidase-dependent complex, by the cytosolic xanthine oxidase system and by the mitochondrial electron transport chain. This species is then enzymatically or spontaneously (reacting with itself) converted to hydrogen peroxide (H_2O_2). Reactions with $O_2^{\bullet-}$ and H_2O_2 can be catalyzed into hydroxyl radical ($OH^{\bullet}$), a much more reactive species than $O_2^{\bullet-}$ and H_2O_2, by transition ions such as the iron present in the body. In fact, much of the damage caused by $O_2^{\bullet-}$ and H_2O_2 is due to the formation of $OH^{\bullet}$. For example, Fe^{3+} is reduced to Fe^{2+} (Haber-Weiss reaction; Reaction 1) and Fe^{2+} is then oxidized into Fe^{3+} leading to the formation of $OH^{\bullet}$ and OH^- from H_2O_2 (Fenton reaction; Reaction 2) [96]. $OH^{\bullet}$ is also synthesized through the formation of hypohalides (e.g. hypochlorous acid – HOCl and hypobromous acid – HOBr) from the reaction between H_2O_2 and halides (compounds that result from the reaction between a halogen and another species)

catalyzed by peroxidases present in inflammatory cells such as eosinophils, neutrophils and monocytes. Subsequently, the hypohalides react with $O_2^{\bullet-}$ forming $OH^{\bullet}$ [94, 97, 98].

The main RNS produced in the organism is $NO^{\bullet}$ produced by NO synthases (NOS). These enzymes catalyze the conversion of L-arginine into L-citrulline with the generation of $NO^{\bullet}$ [99]. In a normal situation, $NO^{\bullet}$ is constitutively produced in small amounts by isoenzymes NOS1 (neuronal) and NOS3 (endothelial), which are assumed to play a regulatory role in neurotransmission and bloodstream respectively, or in large amounts when induced by cytokines or bacterial products on inflammation or infection by the isoenzyme NOS2 (inducible) [100, 101]. There is evidence that asthmatic patients show increased levels of $NO^{\bullet}$ in the exhaled breath essentially due to an NOS2 overexpression in the endothelial cells of the lungs and respiratory tract [102], although NOS1 and NOS3 seem to be involved [103, 104]. Even though the increased expression of NOS2 can be reduced by corticosteroids [105], no $NO^{\bullet}$ removal system is currently known [106].

$$O_2^{\bullet-} + Fe^{3+} \rightarrow Fe^{2+} + O_2 \qquad \text{Reaction 1}$$

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + OH^{\bullet} \qquad \text{Reaction 2}$$

$O_2^{\bullet-}$ can also react with $NO^{\bullet}$ forming peroxynitrite ($ONOO^-$), which reacts with proteins such as tyrosine, through its high nitration power [101, 107]. In atopic asthmatics, high levels of nitrotyrosine, a product of tyrosine nitration, were detected [108]. It has also been detected an increased concentration of peroxynitrite in the EBC of chronic obstructive pulmonary disease (COPD) patients [109], which in turn reduce de detectable values of $NO^{\bullet}$ in the airways [100].

The reaction between radical species and lipids can culminate in the formation of isoprostanes, saturated hydrocarbons (e.g. ethane and pentane) and aldehydes (e.g. malondialdehyde) [88, 110]. Higher values of isoprostanes (i.e. 8-isoprostane, 8-isoP) were detected in either the exhaled breath [73, 111], urine [65] or plasma [87] of asthmatic patients being an *in vivo* way of accessing oxidative stress-related diseases such as asthma, diabetes and cardiovascular diseases [56]. Other products, including 3-bromotyrosine, 3-chlorotyrosine and malondialdehyde, a marker of lipid peroxidation of radical action, are also indicators of the severity of asthma [112]. In 2006, Ercan et al.

[113] obtained increased levels of malondialdehyde and lower levels of glutathione in a study performed with asthmatic patients. In the same way, it was detected more than a 10-fold increase in 3-bromotyrosine levels and a 2 to 3-fold increase in 3-chlorotyrosine in the BAL of asthmatic patients compared to heathy subjects after allergen challenge.

The exposure to ultrafine particles (< 100 nm) present in air pollutants can directly reach the mitochondria, damaging them and making it difficult for the lungs to deal with the oxidative stress [114]. In addition to air pollutants, cigarette smoke and its more than 4000 compounds distributed throughout the gaseous, tar and aqueous phase contribute widely to the oxidative stress. High concentrations of $O_2^{\bullet -}$ and $NO^{\bullet}$ are present in the gaseous phase of smoke. The tar phase has organic radical species that react with the molecular oxygen (O_2) present in the air and forms $O_2^{\bullet -}$, H_2O_2 and OH^-, besides being a powerful chelating agent of iron and generating H_2O_2 continuously. The aqueous phase of cigarette smoke also contributes since its compounds may undergo redox reactions in the lung epithelium [91, 94, 115].

1.3.2.2 Non-enzymatic lipid peroxidation in asthma

One of the consequences of the oxidative stress is the non-enzymatic lipid peroxidation and it involves the oxidation of fatty acids that occurs *in vivo* by reactive species-mediated reactions [56], being a complex process essentially divided into three phases: initiation, propagation and termination (Figure 10) [116].

Initiation occurs when a hydrogen is abstracted, or a radical is added to a PUFA resulting in oxidative damage. PUFAs are more fragile than saturated fatty acids since they have double bonds adjacent to a methylene group (CH_2). Once the C-H bond of this methylene group is weak, the hydrogen is susceptible of being abstracted. The resulting molecule undergoes rearrangement forming a conjugated diene which combines with oxygen forming a peroxyl radical, with the ability to abstract one hydrogen from another PUFA. This reaction can be catalyzed by metal complexes forming alkoxy radicals ($RO^{\bullet}$) and $OH^{\bullet}$, which also participate in the lipid peroxidation chain reaction by abstracting a hydrogen from the PUFAs. In this way, it is only necessary a starting point for many PUFAs being converted to peroxyl radicals ($ROO^{\bullet}$) initiating the propagation of this chain reaction. The resulting radical fatty acids are stabilized by rearrangement into conjugated dienes which preserve the most stable products such as hydroperoxides (ROOH), alcohols, aldehydes and alkanes. The chain propagates until two radical species conjugate

to terminate the chain or until they find an antioxidant that breaks the propagation [56, 116].

The resulting oxidized lipids have bioactivity as vasoconstrictors, vasodilators, bronchoconstrictors and may induce the characteristic inflammation of asthma. In addition, they have the ability to change the membrane fluidity and consequently its associated enzymes and receptors [56].

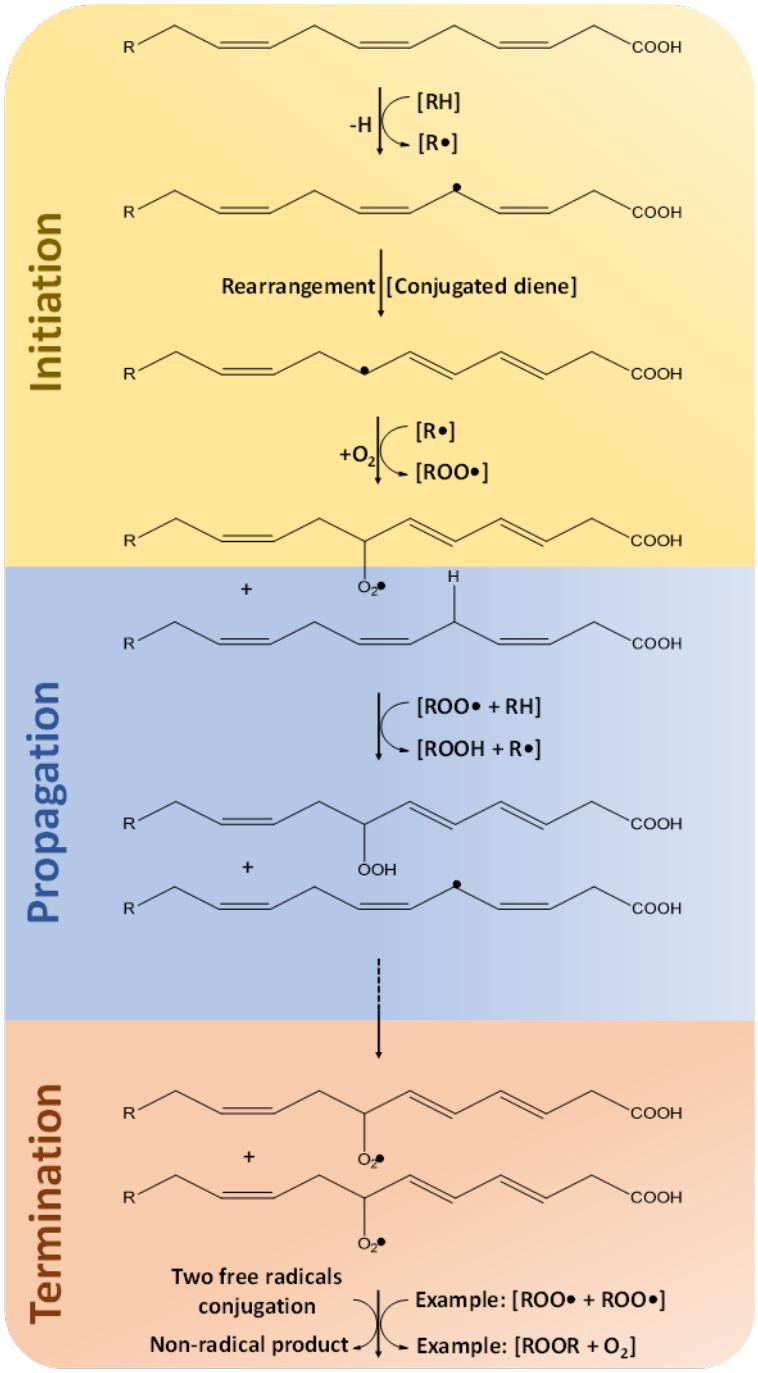


Figure 10 – Basic scheme of non-enzymatic lipid peroxidation of a fatty acid.

1.3.3 The link between enzymatic and non-enzymatic lipid oxidation of the arachidonic acid

As previously discussed, cys-LTs are biomarkers of asthma [64, 65] and products of the enzymatic lipid peroxidation of arachidonic acid [52, 57]. The 8-IsoP is a biomarker of non-enzymatic lipid peroxidation mediated by reactive species [117] strongly related to asthma [65, 73, 87, 111].

Few studies have linked cys-LTs levels to 8-IsoP levels. In a study performed by Samitas et al. (2009) [73], a positive correlation ($r = 0.61$; $p < 0.0001$) could be observed between 8-IsoP levels and cys-LTs in the EBC of asthmatic patients. This correlation persisted only in severe asthmatics ($r = 0.69$; $p = 0.044$), being an indicator that, in addition to reflecting different aspects of inflammation, they are somehow related. Baraldi et al. (2003) [118] observed a significant positive correlation between the levels of cys-LTs and 8-IsoP ($r = 0.62$; $p = 0.01$) in the EBC of children with asthma exacerbations although this correlation did not remain significant after treatment with steroids ($r = -0.14$; $p = 0.63$). Zanconato et al. (2004) [64] obtained a significant positive correlation between cys-LTs and 8-IsoP levels ($r = 0.47$; $p < 0.01$) in the EBC of asthmatic children, with different levels of control and severity, who participated in their study. A possible explanation is the synthesis of LTC_4 regulated by the activity of LTC_4 synthase and by the amount of intracellular GSH as a substrate. The FceRI-mediated release of LTC_4 by mast cells, depends essentially on the levels of intracellular GSH, which in turn increase with the levels of oxidative stress leading to the aggravation of inflammation [60].

Chapter II – Metabolomics in asthma

2.1 From the concept to the experiment

Metabolomics is an analytical approach defined as an "omic" science dedicated to the study of metabolites, which in turn are defined as small dimension bioactive compounds (substrates and products, < 1 kDa). They represent the end product of cell biochemistry [119, 120] (e.g. ATP, glucose, cholesterol and lipid signaling molecules) which involved proteins (proteome) translated from mRNA (messenger RNA; transcriptome) transcribed from genes (genome) [121]. These metabolites do not comprise static end products of a metabolic cascade but rather a dynamic interplay [122]. The complete set of metabolites produced in a cell or tissue at any one time is defined as metabolome [123]. Currently, the human metabolome consists of around 114100 known metabolites (obtained from "The Human Metabolome Database" version 4.0 – http://www.hmdb.ca/ on February 2018).

The study of metabolites has several approaches which can be defined as untargeted or targeted analysis [124]. Untargeted metabolomics includes the metabolite fingerprinting, which consists of the identification of metabolite patterns, not necessarily involving an extensive identification and quantification of a large set of metabolites since multivariate statistical analysis (MVSA) is usually used in order to reduce the dimension of data with discriminatory capabilities. The untargeted metabolomics leads to new findings and the identification of unexpected metabolites and consequently new hypotheses can arise based on findings [122, 124, 125]. On the other hand, targeted metabolomics is the identification, quantification and validation of previously discovered biomarkers or potential biomarkers of disease or products of a specific pathway or drug degradation at any one time. These are hypothesis-based analyzes and include the targeted analysis of a single or few metabolites and the metabolomic profile, which is the analysis of a large set of metabolites of a cell type, tissue or organism metabolism [121, 122] that could be candidate biomarkers discovered in untargeted analysis [126]. Recently, imaging techniques are being applied in metabolite profiling. They consist of the spatial localization of selected metabolites present in a cell or tissue sample allowing the direct observation of the pathological process [120] and include magnetic resonance imaging (MRI), positron emission tomography (PET) [127], and mass spectrometry-based techniques including, matrix-assisted laser desorption ionization (MALDI), desorption electrospray ionization mass spectrometry (DESI), nanostructure-imaging mass

spectrometry (NIMS), among others [120, 128], without the need of metabolite extraction and separation processes [128].

Different metabolomics-based concepts have branched out based on the subject of study such as pharmacometabolics – the study of the behavior of drugs in the organism based on its metabolomic profile [129]; on the sample such as breathomics – the study of the metabolites present in breath [130], for example the studies by Caldeira et al. [131, 132] with asthmatic children populations; or on the physical state of the metabolites such as volatomics – the study of volatile organic metabolites (VOMs) emitted from a cell type, tissue or organism present in a variety of matrices or body fluids including urine, exhaled breath, blood, feces and sweat [133].

2.1.1 The experimental design in metabolomics – the search for biomarkers

The experimental design in metabolomics epidemiologic studies (Figure 11) begins with the selection of the metabolomics approach (targeted or untargeted) and the selection of population [134]. If it is intended to include subjects from different groups (e.g. control and asthmatic patients), these individuals should be representative of that group in terms of number and similarity at the baseline in terms of genetics (i.e. ethnicity, gender) age, body mass index, lifestyle (e.g. diet and physical activity), health (disease status and medication), since these variables can influence the results [135]. Samples should be treated uniformly in terms of sampling, transport, process and storage in order to avoid new variables between these factors and minimize changes in the metabolomic profile. Fast transport, immediate freezing and the avoid of multiple freeze and thaw cycles are vital since some metabolites may deteriorate if enzymatic activity is not suspended [134, 136].

In order to avoid operator-dependent errors and to obtain the best results possible, sample pre-treatment and treatment are fundamental steps in metabolomics, which are dependent on the matrix type, properties of the desired metabolites, volume and the analytical method that will be used [135]. Despite being less discussed in metabolomics-themed reviews, sample preparation techniques also play a vital role concerning time, cost, ease of use, automation/semi-automation possibility and analytical performance.

Extracted metabolites are usually separated and characterized through diverse metabolomic strategies essentially based on liquid (LC) and gas chromatography (GC) hyphenated to diverse spectroscopic techniques [125].

Minimum effort, time, and resources are essential in analytical metabolomics studies. Regarding this, method optimization [137] in terms of metabolites extraction efficiency and separation, as well as method validation in terms of analytical performance including selectivity, linearity, instrumental sensitivity, precision, accuracy, matrix effect and extraction efficiency, are essential parameters that need to be taken into account during a method development [138, 139].

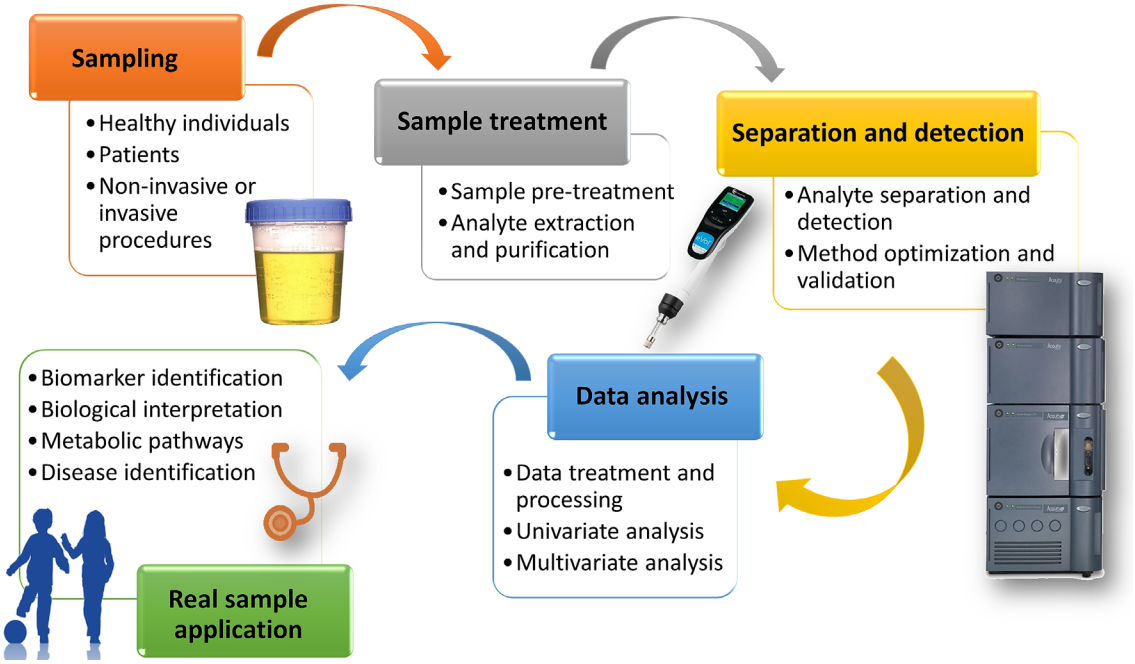


Figure 11 – General strategy in a metabolomics epidemiologic study.

Sample characterization, metabolite profiling, fingerprinting as well as correlation studies are based on chemometrics (untargeted analysis) and quantitative metabolomics (targeted analysis) such as univariate (UVSA) and MVSA in order to identify candidate biomarkers of disease [134, 140]. These techniques can be used in screening, diagnosis and monitoring of disease progression, leading also to the reduction of disease heterogeneity. Thus, being biomarkers precise numbers, they are intended to be reliable and valid in terms of specificity, sensitivity and predictive power [141]. Regarding this, there is a need for biomarkers with an active role in asthma phenotype/endotype identification, diagnosis and treatment, leading to the development of the personalized medicine [142, 143].

2.1.2 Biological samples

The metabolomics studies in asthma comprise several biological samples (biosamples) including urine, exhaled breath, blood, saliva, induced sputum, nasal lavage

fluid, BAL fluid and stool. Even though blood (serum and plasma) mostly contains endogenous analytes and provides a good overview of the metabolic status of the patient, and induced sputum, nasal lavage and BAL fluids are highly relevant to access the airway physiology and integrity, they are particularly invasive to obtain and generally require trained personnel to make the collection, being relatively inaccessible to obtain [134, 144]. Therefore, in this section, it is given special attention to biosamples that can be collected through totally non-invasive approaches such as (*i*) urine, (*ii*) saliva and (*iii*) exhaled breath due to its ease of collection from infants comparatively to the more invasive mentioned approaches, and its potential to be used in asthma studies.

(i) Urine

Urine is the main way the body eliminates waste and excess products of metabolism. It is a transparent, sterile, yellowish-colored fluid produced by the kidneys. These organs extract excess products and soluble wastes from the bloodstream resulting in a product with high concentrations of urea, inorganic salts, creatinine, ammonia, organic acids, toxins and pigmented species such as urobilin inherent to hemoglobin degradation, which gives its characteristic color [145].

Regarding clinical diagnosis, urine has been used since the ancient Egypt until the present times routinely through simple dipstick tests [145] (e.g. human chorionic gonadotropin in pregnancy) or through more complex analysis in laboratorial environment.

Urine sampling is a non-evasive procedure which, in opposition to other biological matrices, is available in large volumes, requires no previous preparation of the patient, and it is easily collected among pediatric ages, for example. In metabolomics studies, urine has the advantage of being relatively stable, less complex than other matrices, having low concentration of proteins, reflecting the pathophysiological changes in the organism, age, sex, among others [134]. To date, 4297 metabolites are known in the human urine (obtained from "The Urine Metabolome Database" – http://www.urinemetabolome.ca/ on March 2018). Urine also has the advantage of its metabolites for volatomics analysis being preconcentrated by kidneys before excretion and have reduced matrix effects compared to other biofluids such as blood, resulting in better analytical sensitivity even though several substances such as drugs may interfere with VOM analysis [133]. Due to these properties, it is believed that urine can be routinely

used to identify biomarkers of oxidative stress and inflammation. A volatomics approach has first been applied by Linus Pauling et al. in 1971 with the determination of about 280 volatile substances in a human urine sample [146]. More recently been applied in the urine of an asthmatic population by Loureiro et al. [147].

The use of urine metabolomics approaches has increased in the past years (Figure 12). It has been used in the study of asthma [147-150] and also in the study of other chronic respiratory diseases such as COPD [149], neurodegenerative diseases such as Alzheimer's [151] and Parkinson's disease [152], different types of cancer such as prostate [153] and kidney cancer [154], cardiovascular diseases [155], as well as in the determination of urinary excretion of antipsychotics [156] and antidepressants metabolites [157], among others.

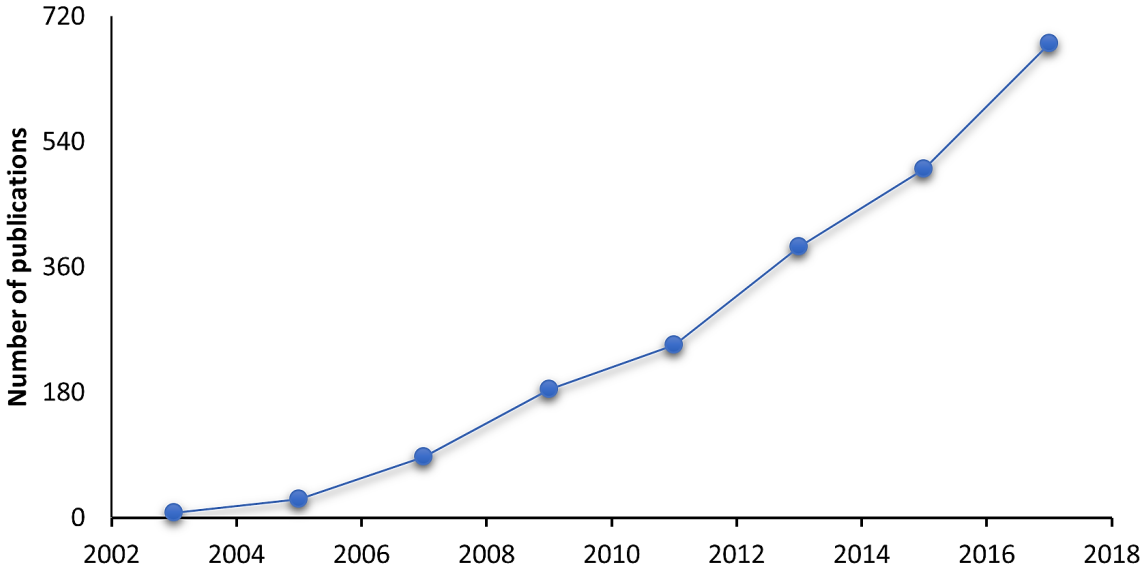


Figure 12 – Number of publications that include urine in metabolomics studies. Data obtained from Clarivate Analytics' Web of Science, https://clarivate.com/products/web-of-science/, Core Collection Database using the following topic search keywords: "urine metabolomics".

(ii) Saliva

Saliva is a biological fluid produced by the major (sublingual, submandibular and parotid) and several minor salivary glands in the oral cavity. This fluid is mostly composed by H_2O, inorganic and organic constituents and other substances from the airways, food, blood, among others. Water accounts for 99% of saliva components. The inorganic constituents (e.g. Na^+, Cl^- and Ca^{2+}) give to saliva a buffer capacity, tooth integrity functions and organic components activity regulation. The organic constituents such as urea, lipids and proteins have enzymatic activity (e.g. amylase, lysozymes and peroxidases) relevant to oral health, lubrification and digestive functions [158, 159].

This biofluid is readily accessible through easy and non-invasive techniques and the exchange of substances between saliva and systemic circulation, through a thin layer of epithelial cells, makes it a potential excellent alternative to serum analysis as a source of information. Collection of saliva has the advantage of a reduce risk of infection compared to serum, the advantage of avoiding privacy and absence issues being able to be collected momentarily although in a reduced volume compared to urine. Its composition is more stable and not so complex as serum and thus, reflects with more accuracy the metabolic condition at any time. In addition it contains metabolites that can be used as biomarkers of diseases [159]. To date and according to "The Saliva Metabolome Database" – http://www.salivametabolome.ca/ on March 2018), 1236 metabolites are known in the human saliva.

Due to its ability to reflect the internal state of the organism [160, 161], saliva has become a biosample of interest in forensic analysis [162], occupational pollutants monitorization [163] and in metabolomics as a potential diagnostic tool for several diseases including, among others, oncological diseases such as breast cancer [164] and oral squamous cell carcinoma, periodontal diseases and Sjögren's syndrome, as reviewed by Liu and Duan in 2012 [159].

(iii) Exhaled breath

Exhaled breath is mainly composed by a mixture of several inorganic gases (e.g. H_2O, NO, CO_2, CO and O_2) VOMs (e.g. saturated and unsaturated hydrocarbons and acetone) [110] and non-volatile species (e.g. cytokines, isoprostanes and peroxynitrite), from which some are biomarkers of asthma, lung function and airway remodeling [165].

Along with urine and saliva, exhaled breath represents one of the most accessible biosamples because it is naturally produced in large quantities through the gas exchange in the lungs (about 10000 L per day). The potential of breath as a diagnostic tool begun with Antoine Lavoisier in 1790 with the study of gas exchange [166]. The modern era of breath analysis started with Linus Pauling et al. in 1971 with the determination of about 250 VOMs in a sample of human breath [146]. Since then, research in the breath analysis has been made both in its volatile fraction and in its non-volatile aqueous fraction in the form of aerosol particles that can be measured through condensation (i.e. EBC).

Concentrations of VOMs present in the exhaled breath are a challenge. These values are dependent on the concentrations in ambient air, the physiological state of the

individual (e.g. age, body mass index and menstrual cycle length), food intake and occupational pollutants, as reviewed by Lourenço and Turner [167]. Therefore, the origin of the substances detected in breath analysis can be divided into two categories: environmental exogenous (environment, food, …) and endogenous (metabolism) metabolites. Even though this classification is relatively difficult, endogenous metabolites are interesting due to their potential as biomarkers of disease.

Several breathomics approaches have been applied in the study of diseases including, pulmonary diseases such as asthma and COPD [83, 168, 169], oncological diseases such as lung, breast, colorectal and prostate cancer [170, 171], and heart failure [172].

2.1.3 Sample preparation techniques in metabolomics – the challenge for miniaturization

The traditional sample preparation techniques are liquid-liquid (LLE) and solid phase extraction (SPE). Frequently, these techniques have the disadvantages of not being easily hyphenated with an analytical platform, are time consuming, unfeasible sampling procedure, needs considerable amounts of harmful organic solvents (e.g. methanol, MeOH; acetonitrile, ACN; acetone; and ethanol, EtOH), with strong environmental implications, especially in the case of LLE, and requires substantial amounts of sample in the range of mL or g [173].

Considering this, microextraction techniques (METs) emerged in the early 90's with SPME [174] followed by liquid-phase microextraction (LPME) [175]. Up to date, a noteworthy number of different METs are continuously being developed in the field of LLE, such as single-drop microextraction (SDME), hollow fiber liquid-phase microextraction (HF-LPME) and dispersive liquid-liquid microextraction (DLLME); and in the field of SPE including needle-based SPME sampling techniques such as (*i*) MEPS and (*ii*) fiber SPME [176, 177], in addition to other (*iii*) microextraction strategies (QuEChERS, mSPEed and NTDs) with potential application to the asthma field.

(i) MEPS

MEPS is a miniaturized SPE technique consisting of a removable MEPS cartridge and a sampler device designed for the characterization of metabolites with different properties. The sampler device has the possibility to be manual through a Hamilton gas-

tight syringe; semi-automatic by means of a digitally controlled and programmable semi-automatic device (eVol® XR; Figure 13A) that controls the aspiration/dispensing volume and flow rate coupled to a gas-tight XCHANGE® analytical syringe (5-1000 µL; Figure 13B); and fully-automatic by means of the previous XCHANGE® analytical syringe on-line with LC or GC autosamplers. The possibility of a semi/fully automation require minimal user interference allowing better precision, accuracy and consequently better reproducibility.

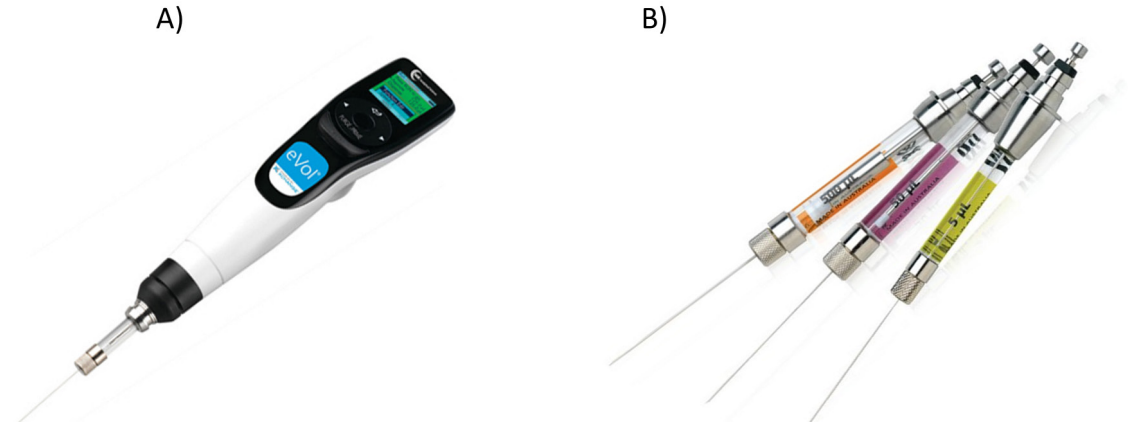


Figure 13 – An eVol® XR device for semi-automatic MEPS (A) and respective gas-tight XCHANGE® analytical syringes (B).

The MEPS cartridge, a metallic cylinder known as "barrel insert and needle" (BIN; Figure 14), contains 1-4 mg of a solid packed sorbent bed through which small volumes (usually 10-250 µL) of sample and solvent passes retaining and eluting the metabolites. Depending on the nature of the metabolites (hydrophobic or hydrophilic) and the matrix (aqueous or organic), different sorbent materials are available including silica, carbon and polymeric polystyrene-divinylbenzene (PS-DVB) based sorbents [178]. Unlike SPE, which only can be used once, MEPS cartridges can be re-used several times with human biosamples, such as urine and plasma, without loss of performance reducing in this way the costs of extraction [179].

Samples used for MEPS purpose, usually require pre-preparation in terms of centrifugation, filtration, dilution and the adjustment of pH and temperature in order to extend the life of the sorbent and its re-usability and enhance the extraction efficiency [178]. MEPS procedure consist of 4 main steps: conditioning/regeneration, sample loading, washing and elution, which can be optimized in order to obtain better extraction performance in terms of efficiency and matrix effect. The conditioning/regeneration of the sorbent is performed with an organic solvent followed by its equilibration with H_2O (pure or acidified) before loading the sample and ends with the elution followed by the

regeneration of the sorbent that match with conditioning step if further extractions are made. Sample loading cycles consists of a certain number of drawing up into the syringe and discharge. Washing the fiber with H_2O (pure or acidified) allows the removal of undesired matrix interferences that can be weakly retained on the sorbent while the elution step consists on the use of the best solvent and the lowest volume possible that is able to elute the target analytes retained on the surface of the sorbent [178-180].

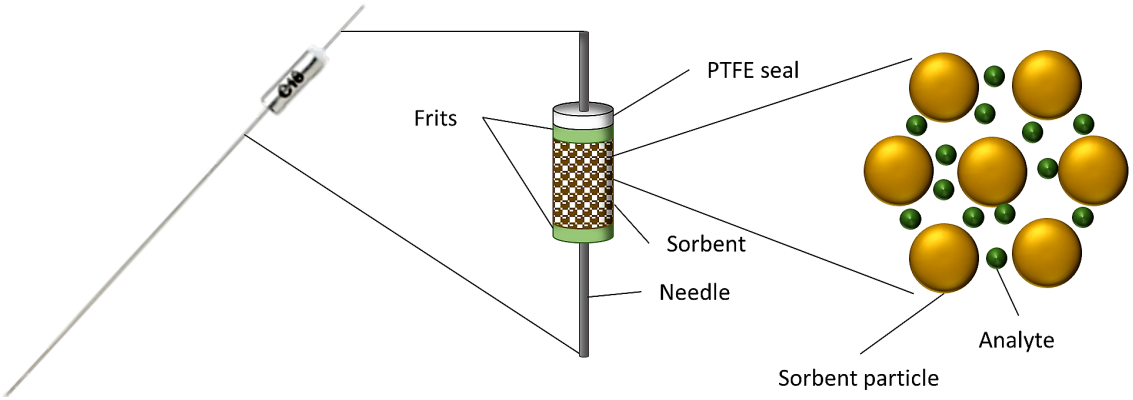


Figure 14 – MEPS BIN (barrel insert and needle) and schematic magnification of a MEPS BIN. Magnification illustrate an analyte adsorbed on the sorbent particles. Adapted from [178].

The use of MEPS procedure has increased (Figure 15) since it was firstly purposed by Abdel-Rehim in 2004 for determination of local anesthetics in human plasma [181]. Even though MEPS procedure was only applied once in the study of asthma by Perestrelo et al. [61] on the characterization of urinary LTB_4 (Table 4), it has been applied in the study of other diseases such as cardiovascular diseases [155] breast and lung cancer [182, 183] as well as other fields such as pharmacology, in the study drugs behavior including psychoactive metabolites in oral fluid [184] or urine [156, 157], toxicology [185], food chemistry [186], among others.

(ii) SPME

In 1990, Arthur and Pawliszyn [174] developed the first successful MET called SPME. This technique had several advantages over the mentioned classical sample preparation techniques including the absence or minimum requirement of solvents, the need for small amounts of sample, short sample preparation time, possibility for laboratory automation and on-site sampling.

Table 4 – Microextraction techniques used in the study of asthma biomarkers. Abbreviations: MEPS – microextraction by packed sorbent; HS-SPME – headspace-solid-phase microextraction; DVB/CAR/PDSM – divinylbenzene/carboxen/polydimethylsiloxane; VOM – volatile organic metabolite; ANOVA – analysis of variance; PLS – partial least squares; PLS-DA – partial least squares-discriminant analysis; UHPLC-PDA – UHPLC-photodiode array; GC×GC-ToFMS – two-dimensional gas chromatography–time of flight mass spectrometry; GC-qMS – gas chromatography-quadrupole mass spectrometry; NMR – nuclear magnetic resonance spectroscopy.

Extraction platform	Stationary phase	Analytical platform	Biosample	Population	Groups	Analyte	Statistical analysis	Comments	Ref.
MEPS	Porous graphitic carbon	UHPLC-PDA	Urine	Adults and children	Case vs control	Targeted LTB_4	-	Increased in asthmatics. LOD = 0.37 ng/mL LOQ = 1.22 ng/mL	[61]
HS-SPME	DVB/CAR/PDMS	GC–qMS	Exhaled breath	Children	Case vs control	Untargeted. 44 identified VOMs present only in breath	ANOVA and PLS-DA	28 VOMs with discriminatory power. Asthmatics characterized by VOMs linked to oxidative stress such as alkanes and aldehydes	[131]
HS-SPME	DVB/CAR/PDMS	GC×GC-ToFMS	Exhaled breath	Children	Case vs control	Untargeted. Full set of 134 VOMs identified	PLS-DA and Monte Carlo cross validation	23 VOMs with discriminatory power. Asthmatics characterized by VOMs linked to oxidative stress belonging to alkanes	[132]
HS-SPME	DVB/CAR/PDMS	GC×GC-ToFMS	Exhaled breath	Adolescent swimmers	Case vs control	Untargeted. Hundreds of VOMs identified. Targeted 5 aliphatic alkanes and 3 aldehydes linked to oxidative stress	PCA	Swimming associated to a decrease in oxidative stress VOM levels. More pronounced in controls	[190]
HS-SPME	DVB/CAR/PDMS	GC×GC-ToFMS	Urine	Adults	Case	Untargeted. Several dozen VOMs identified. Targeted 34 aliphatic alkanes and aldehydes	PLS	Metabolites associated to eosinophilic inflammation, lung function and disease severity	[147]
HS-SPME	DVB/CAR/PDMS	GC×GC-ToFMS and ^{1}H-NMR	Urine	Adults	Case	Targeted aldehydes and alkanes. Untargeted ^{1}H-NMR	PCA	Oxidative stress (aldehydes, alkanes, carnitine, acetylcarnitine) Tricarboxylic acid cycle metabolites (e.g. citrate, alanine) linked to exacerbations	[148]

When it was first introduced, this technique involved a fused silica fiber coated with polyimide inside a Hamilton gas-tight syringe. This syringe was intended to protect the fiber and also allow the introduction of the fiber into a GC injector. Currently, commercial SPME consists of a syringe-like device containing a fiber assembly and a fiber assembly holder, which can be manual or automated (Figure 16A). The fiber assembly includes a fiber coated with 1 to 2 cm of different polymeric stationary phases to extract and concentrate the analytes from the sample protected into a needle. The manual fiber assembly holder, consists of a plunger with a spring, a stainless-steel tube and an adjustable depth gauge [173].

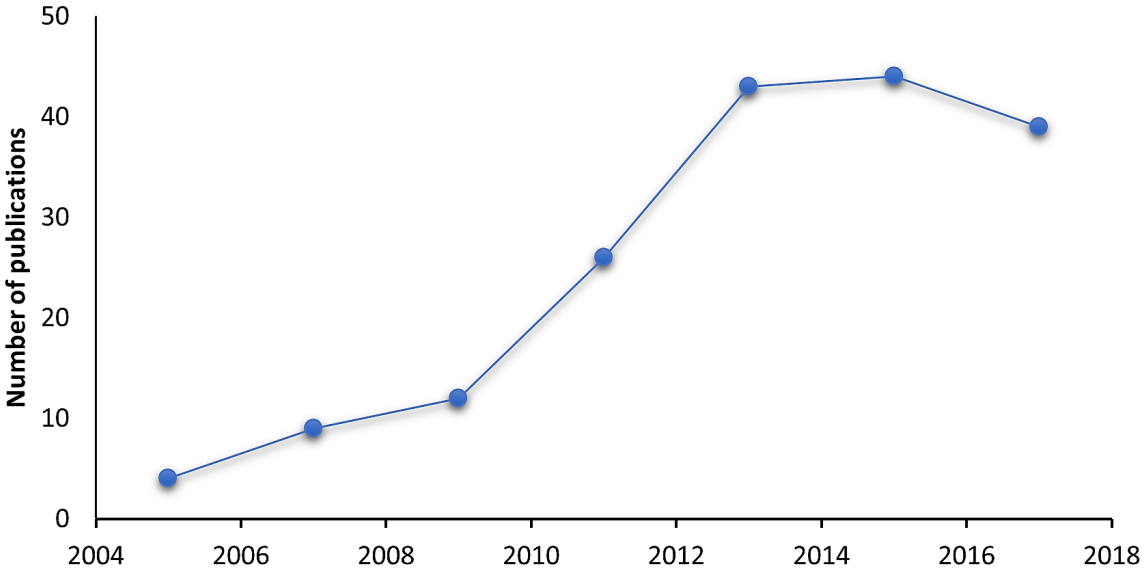


Figure 15 – Number of publications that include MEPS as a tool in metabolomics studies. Data obtained from Clarivate Analytics' Web of Science, https://clarivate.com/products/web-of-science/, Core Collection Database using the following title search keywords: "microextraction in/by packed sorbent/syringe".

Depending on the nature of metabolites, matrix and the purpose of the analysis, it is necessary to optimize different *device* and *sample pre-treatment parameters* that influence the analytical performance of the method [173, 180].

Device parameters

The device parameters with influence in the extraction performance includes the extraction mode, nature of the fiber (coating), agitation method and extraction time [173, 180, 187, 188].

The extraction of metabolites from a gaseous sample, liquid or solid, can be performed in two ways, via headspace-SPME (HS-SPME) mode, in which the fiber is exposed to the gas phase above the sample (Figure 16B), or by direct immersion (DI)-

SPME in which the fiber is directly immersed into the liquid sample. The co-extraction of non-volatile interferences (e.g. proteins) can complicate the extractive process in complex bio-samples such as urine, requiring, in some cases, the introduction of barriers in the form of porous membranes between the sample and the fiber (membrane-protected SPME) or in the form of a gaseous space between the sample and the fiber (headspace; HS-SPME) allowing a greater selectivity as well as a good sensitivity for the extraction of volatile compounds [173, 180, 188].

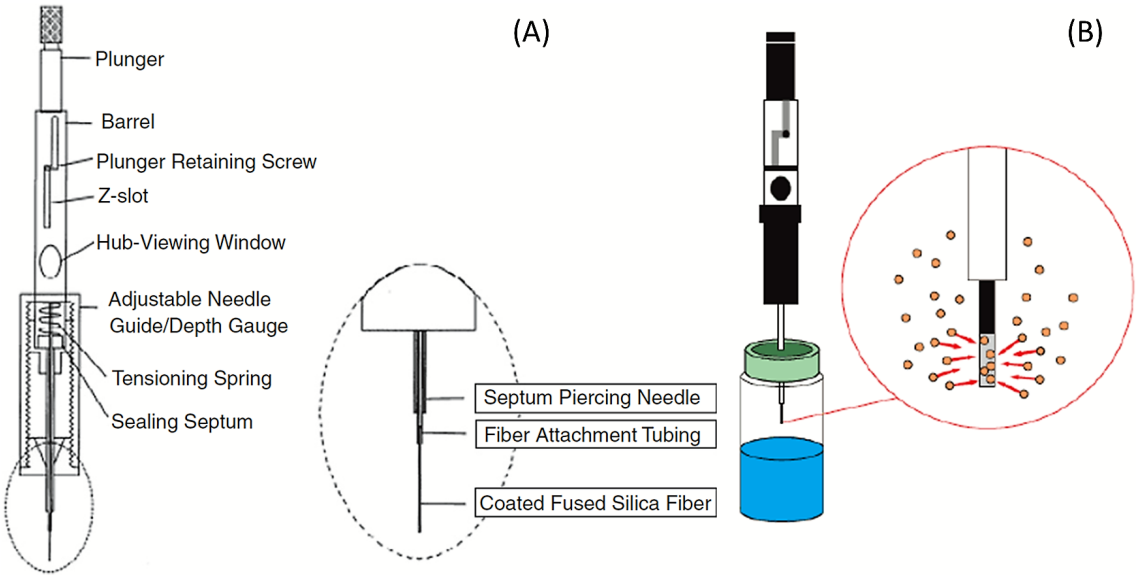


Figure 16 – Schematic overview of a (A) commercial SPME with manual fiber assembly holder, and (B) representation of a headspace-solid-phase extraction. Adapted from [173, 189].

The selection of the fiber coating is important since it influences the selectivity and sensitivity of the extraction. Different fibers are commercially available consisting of different materials such as fused silica and metal alloy coated with several stationary phases composed by one, two or three polymers with different film thickness (7 to 100 µm) and lengths (1 to 2 cm), which adsorb/absorb and concentrate the metabolites by partition between the matrix and the coating during the extraction. Fibers coated by a single-polymer (liquid) have absorption as the extraction mechanism. They may be nonpolar such as polydimethylsiloxane (PDMS), suitable for the extraction of nonpolar analytes, or polar such as polyacrylate (PA) and carbowax-polyethylene glycol (PEG) suitable for the extraction of highly polar compounds. Those of mixed-polymer (solid) have the adsorption as extraction mechanism, have bipolar properties, are available for the extraction of low molecular weight analytes possessing a wide range of physicochemical properties and include the polydimethylsiloxane/divinylbenzene

(PDMS/DVB), carboxen/polydimethylsiloxane (CAR/PDMS), and the divinylbenzene/ carboxen/polydimethylsiloxane (DVB/CAR/PDMS) [173].

Agitation of sample by ultrasound and/or magnetic stirring, promotes the diffusion of the analytes from the bulk matrix, by mass transfer, to the fiber, thereby reducing the time to reach equilibrium. The more efficient the agitation, the shorter the time required to reach the equilibrium and the greater the amount of analyte extracted under non-equilibrium conditions.

Extraction time is one of the most important parameters since it is important to reach the equilibrium between the analytes in the matrix and in the fiber coating without degrading the sample. In HS-SPME mode, incubation time is also important since the equilibrium between the matrix phase and the headspace needs to be reached [173, 180, 187, 188].

Sample pre-treatment parameters

The sample pre-treatment with influence in the extraction efficiency include the sample temperature, volume, pH and ionic strength [173, 180, 187, 188].

A high temperature improves the extraction rate by decreasing the metabolites fiber coating–sample matrix distribution constant even though it can decrease the amount of extracted metabolite, sensitivity, precision and lead to sample degradation.

Higher sample volumes lead to an increase on the total amount of extracted analytes. It is generally compromised by the size of the vial or by the amount of sample. SPME fibers only extract analytes in their neutral form. pH adjustments of the sample, between the limits of the fiber, provide the selectivity of the technique by interfering with the ionization state of the compounds. A low pH promotes the extraction of the acidic compounds while a high pH promotes the extraction of the basic compounds. The ionic strength of the sample is higher with the addition of soluble salts – salting-out effect. This causes a decrease in the solubility of many organic compounds, except for those highly polar, simplifying their diffusion from the sample to the headspace.

SPME has been applied in the study of asthma. Table 4 describes some studies of SPME-asthma related. Furthermore, this strategy has been adapted and applied in pharmacotherapeutic analysis, forensics, clinical diagnosis and environmental pollutants exposure, among others, as reviewed by Kataoka and Saito in 2011 [188].

(iii) Recent microextraction techniques with high potential application to asthma field

The challenge for more efficient and environmentally friendly techniques is a continuous process. Recently, it has given rise to modifications of already existent techniques including the dispersive "quick, easy, cheap, effective, rugged and safe" (QuEChERS) with μ-QuEChERS [191], SPE upgraded to MEPS [178, 192] and more recently to μSPEed® [193]. The concept of fiber SPME gave rise to other extraction techniques suitable for the extraction of metabolites present in a gaseous phase. Regarding this, a needle-based extraction technique named needle trap device (NTD) emerged as an exhaustive active sampling procedure with the simplicity of SPME [173].

QuEChERS is an extraction and clean-up technique developed in 2003 by Anastassiades et al. [194] for the characterization of pesticide residues in vegetables and fruits. Briefly, in their work, they used a single-phase extraction of 10 g of sample with 10 mL of acetonitrile (ACN) followed by a salting-out extraction step with $MgSO_4$ and NaCl and a dispersive SPE clean-up step with primary-secondary amine (PSA) and $MgSO_4$. Since QuEChERS is a versatile extraction technique, it can be optimized essentially in terms of salts (e.g. citrate and acetate buffers), extraction solvents and extraction type (e.g. ultrasound and vortex) in order to fulfill the different analytical needs. More recently, this technique has suffered miniaturization in terms of amount of sample, salts and solvent from g and mL to mg and μL allowing the analysis of small sample amounts – μQuEChERS. This MET has not been used in pathological studies but has been used for the analysis of pesticide residues present in different matrices such as gastropods and arthropods [195], tissues such as rat brain and liver [196], biofluids such as rat blood [196], micotoxins in cereals [191], organic pollutants in human urine [197], among others.

MEPS is a multipurpose technique that had a technological upgrade with the arose of μSPEed® [193]. This downscaled MEPS technique distinguishes itself by the direct flow through the cartridge into the syringe barrel by means of a micro one-way valve. Under controlled conditions, the sample is then pushed through the sorbent allowing the analytes to be concentrated onto the top of the sorbent bed. Even though it has a strong potential since it allowed the reduction of the dead volume, the elution solvent needed and the reduction of the particle size from 30-50 μm, used in SPE and MEPS, to less than 3 μm which is an improvement in terms of extraction and elution efficiency and

sensitivity. This strategy was recently used by Porto-Figueira et al. for the analysis of phenolic compounds in teas [193].

Over the years, different types of needle-based sampling techniques were developed. Although the first report about a needle filled with a sorbent bed for trapping volatile compounds from air was described in 1978 by Raschdorf [198], needle-based extraction devices have only recently become popular due to SPME and SPE. The demand for a more robust technique, gave rise to an in needle packed sorbent format, the needle trap device (NTD) introduced by Pawliszyn et al. in 2001 [199]. While SPME has a higher efficiency extracting less or semi-volatile free metabolites, NTDs are suitable for the extraction of highly volatile and particle-bound metabolites [173, 200]. This strategy is a promising sampling platform distinguished by having an exhaustive active extraction nature that results in a total concentration information rather than free concentration information provided by other METs such as SPME. The NTD technique allows us to collect, prepare and introduce the analytes in just one step simplifying all the process maintaining the sensitivity and eliminating the time and expendable large volumes [200, 201]. Different variants of NTDs are commercialized by PAS Technology (Magdala, Germany) and Shinwa Chemical Industries Ltd. (Kyoto, Japan) thus, NTDs with different combinations of sorbent, lengths, gauges, side hole and tips can be acquired for a particular purpose. This MET has been used in the analysis of volatile analytes present in the breath of heart failure patients [202], breath of healthy, smoking and nonsmoking individuals [203], air pollutants (aldehydes) [204], headspace VOMs of whole blood [205], among others.

2.1.4 Chromatographic techniques – metabolite characterization strategies

An appropriate analytical platform must be selected in order to perform the characterization of the extracted metabolites. With the advance of technology, detection techniques, including mass spectrometry (MS), nuclear magnetic resonance (NMR), ultraviolet-visible (UV-Vis) and fluorescence detections (FD), required to detect, identify, and quantify metabolites, had made remarkable improvements regarding analytical sensitivity along with the development of the separation techniques such as LC with the emergence of ultra-high performance liquid chromatography (UHPLC) and GC, both with the possibility of automation ease of use and costs reduction [125, 145] leading

to the possible analysis of metabolites in trace concentrations in non-invasive samples such as urine, saliva and exhaled breath.

Even though NMR being a powerful detection technique that allows the simultaneous analysis of several metabolites without comprising the integrity of the sample/extract, it is considered of low sensitivity relatively to its counterparts and thus, used to detect and quantify the main metabolites of an extract allowing its recovery for further analysis. Therefore, NMR is used as a mandatory complement to more sensitive techniques such as MS detection [121, 125].

Below it is described the most commonly chromatographic approaches used in metabolomics: (*i*) LC and (*ii*) GC.

(i) Liquid chromatography

LC is usually chosen to characterize more polar and less volatile or non-volatile metabolites [206]. LC-based approaches are among the most widely used analytical platforms in metabolomics and one of the most important tools regarding detection, identification and validation of disease biomarkers [207].

In order to obtain short analysis times and high analyte separation, over the past years we have seen an evolution in terms of particle size (< 2 μm), and particle technology such as porous, solid and particles with a solid core and a porous shell particle. Considering this, instruments capable of tolerating the extreme pressures generated were required, due to the increased theoretical plates (van Deemter equation), which could reach around 18000 psi. With this in mind, UHPLC emerged in order to respond to the most recent needs of analytical chemistry and to make an upgrade to its ancestor, the HPLC, in terms of resolution, sensitivity and speed of analysis, which is particularly important when developing a method [208]. A schematic of a UHPLC apparatus is shown in Figure 17.

In the 1970's, a few years after LC hyphenated with a ultraviolet (UV) detector being in use for metabolite studies [209], liquid chromatography-mass spectrometry (LC-MS) begun to be used [210]. Currently, due to its higher sensitivity (i.e. limit of detection, LOD < 1 pg) above other detection techniques [211], MS is the metabolomics detection tool by excellence after separation by LC [207]. The miniaturization of the sample treatment techniques and their improvement in terms of sensitivity has allowed the use of

absorbance detection techniques [178] such as photodiode array (PDA) and UV at a relative reduce cost compared to MS.

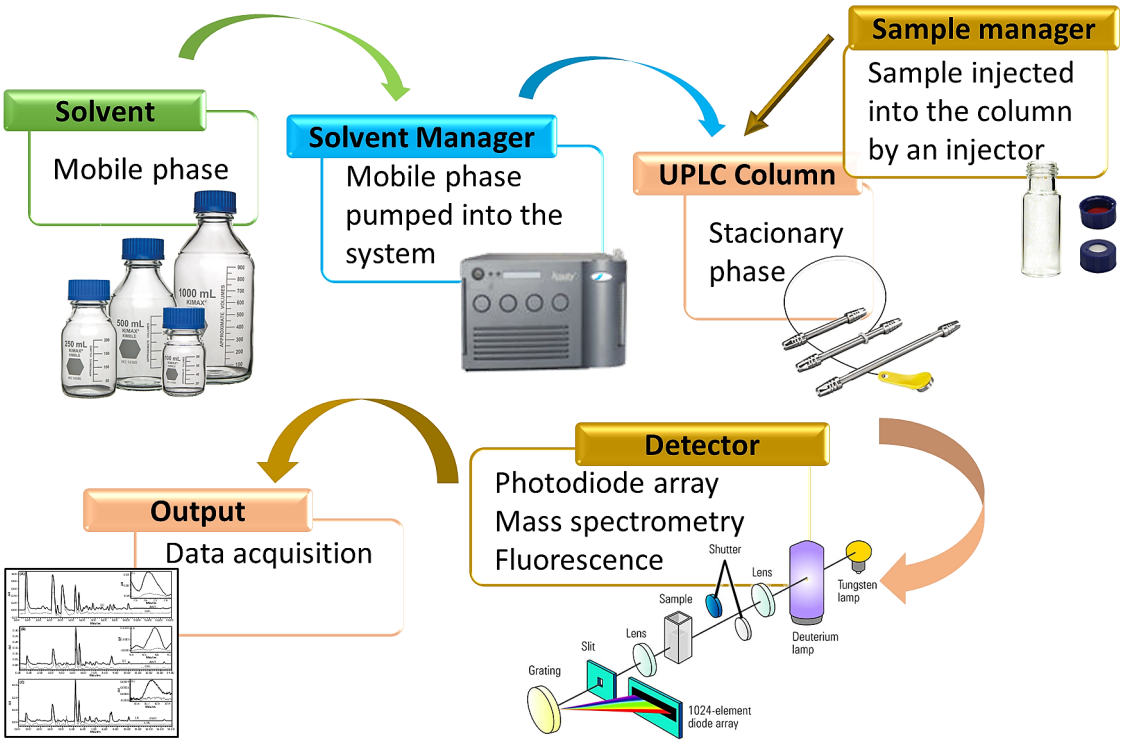


Figure 17 – Schematic diagram of an UHPLC system.

After isolation, extracts usually do not need further treatment, even though sometimes, they can be reduced or completely evaporated under a gentle nitrogen stream and resuspended in mobile phase [156]. Depending on the nature of the extracted metabolites, different parameters could be optimized in order to obtain the best resolution and analytical performance possible. Among these parameters, it is highlighted the column selection, column temperature, flow rate of the elution solvents and gradient.

(ii) Gas chromatography

GC is an analytical strategy frequently selected to characterize volatile or "volatizable" metabolites [206]. Compared to LC, this separation technique has a higher chromatographic resolution being an advantage on the analysis of trace amounts of analytes [121].

Even though there are different GC detectors, in this section it will be given special attention to the gas chromatography-mass spectrometry (GC-MS) configuration due to its wide applicability. Basically, all GC-MS share the same essential components: an injector, to allow the sample to get into the column system; a detector; and a gas

chromatograph, which connects a column to the injector and to the detector. Metabolites are separated by their interaction with the column (stationary phase), housed into a thermostated oven, which regulates the temperature on which the separation is made (e.g. isothermal or programmed). The elution is further made by means of a carrier inert gas (mobile phase) such as helium or hydrogen controlled through a flow/pressure regulator. The interface module connects the column to the detector where an ion source ionizes and fragments the metabolites subsequently detected by a mass analyzer before its output, data is selected, controlled and processed into a chromatogram [212]. A schematic diagram of a GC-MS apparatus is shown in Figure 18.

The GC-MS begun to be used in the middle 1960's for metabolite studies [213]. Currently, this platform is the most widely used chromatographic technique for the characterization of VOMs due to its high resolution and cost comparatively to its liquid counterpart. Metabolites are obtained from a volatile fraction of the sample (i.e. HS) or through derivatization of non-volatile metabolites. In order to improve the number of detected metabolites (i.e. number of peaks) and sensitivity, two-dimensional gas chromatography (GC×GC) appeared as an upgrade to the analytical platform. Metabolites separated on a column are separated a second time on a second column with different properties [125].

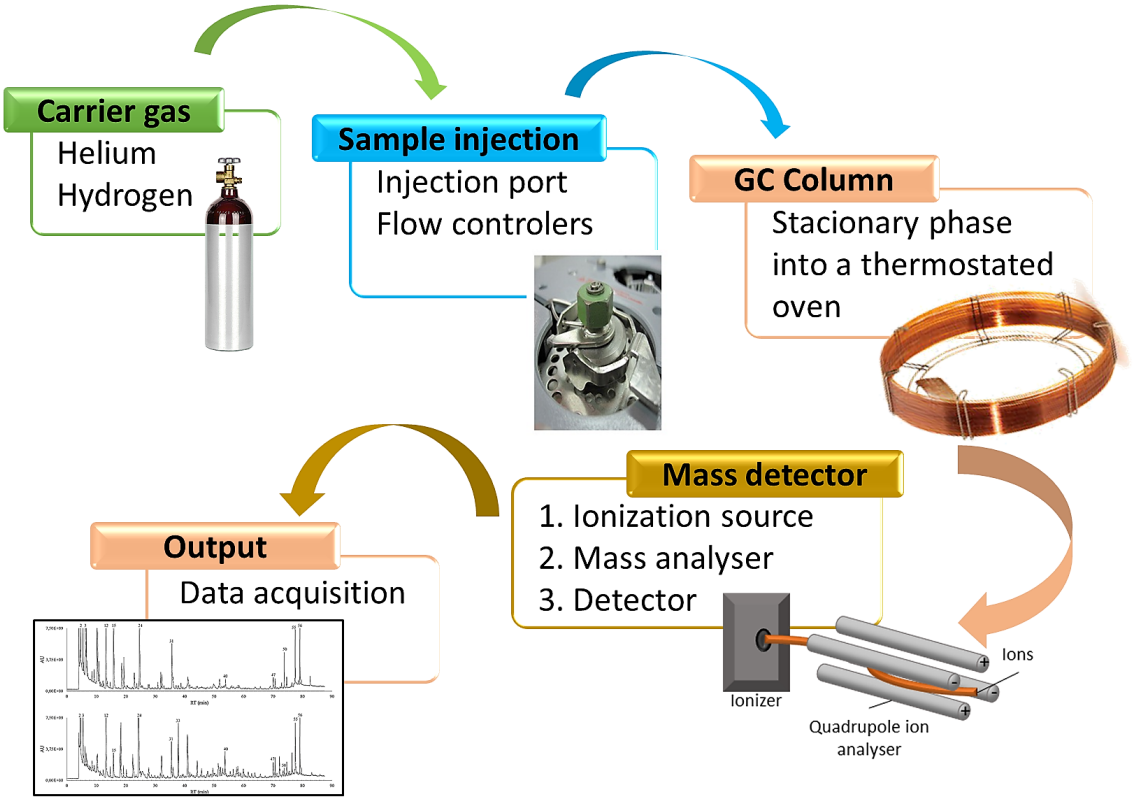


Figure 18 – Schematic diagram of a GC-MS system.

In addition to the advances that have been made in terms of sensitivity and resolution of the analytical platforms, the most recent improvements in chromatography include the coupling of two separation techniques such as LC to GC greatly increasing the separation efficiency at two different states at the same time large amounts of solvents are introduced into a GC column [214].

2.1.5 Statistical methods in metabolomics analysis

Data analysis comprises the statistical treatment of the data obtained from the previous experimental steps. Depending on the purpose, it can be performed either on normalized metabolite values including their areas or concentration values obtained through the application of the regression equation (data preprocessing), generally used in MVSA, or directly on the values obtained without normalization frequently used in UVSA.

Statistic data analysis can be branched mainly in UVSA and MVSA. UVSA is the statistical analysis of one variable at once while MVSA analyzes many variables at once. UVSA tests are performed in order to group differences such as metabolite mean values (e.g. healthy control group versus asthmatic group) for instance. It includes both parametric tests such as dependent/independent *t*-test and analysis of variance (ANOVA) tests, and non-parametric tests such as Mann-Whitney, Wilcoxon signed-rank, Kruskal-Wallis and Friedman's, which can be easily applied to data obtained from the analytical platform [215]. MVSA is an essential tool for the analysis and interpretation of high-throughput metabolomics generated from untargeted and multitargeted analysis [216]. As part of the metabolomics, MVSA analysis can be applied through two different strategies, chemometrics and quantitative metabolomics. Chemometrics is used in untargeted analysis and can be automated, statistical analysis is performed directly on spectral patterns and signal intensity, and metabolites are identified at the end while in quantitative metabolomics, metabolites are identified first, and data is analyzed after its preprocessing. Even though they serve two different purposes, they share the same statistical methods [140].

Statistical models can be unsupervised or supervised. Unsupervised models (unlabeled data) allow us to discover patterns, groups and outliers within data and include principal component analysis (PCA), a powerful statistical technique that has the

capability to reduce a high-dimensional dataset into principal components keeping the maximum of variability. By plotting the first two or three principal components that explain the total variance (scores plot), it is given a graphical visualization of the separation between groups and by plotting variables (loadings plot) on the same diagram, it can be concluded which variables accounted for the separation and to which group they belong [217]. Other unsupervised statistical models include clustering methods such as K-means and Hierarchical clustering and Self-organizing map [134, 140]. Supervised models (labeled data) are useful to explore which metabolites have discriminatory power regarding disease, disease phenotypes/endotypes and prediction, allowing the identification of biomarkers and include stepwise linear discriminant analysis (SLDA), which forms a number of orthogonal linear discriminant functions equal to the number of dependent variables minus one. This method has the ability to minimize the variance within groups and maximize the variance among groups [218]. Other supervised statistical strategies include partial least squares (PLS) methods (e.g. PLS-discriminant analysis, PLS-DA; and orthogonal PLS-DA) [219] and support vector machine [134, 140].

Chapter III – Looking for new contributions in asthma biomarkers – a MEPS/UHPLC-based approach

Submitted to Journal of Chromatography A

3.1 Introduction

Asthma, one of the major noncommunicable diseases, is a potentially serious chronic disease usually characterized by chronic airway inflammation, bronchial hyperresponsiveness and recurrent episodes of reversible airway obstruction. It is defined by the history of respiratory symptoms such as cough and wheezing, shortness of breath and chest tightness that vary over time in their occurrence, frequency and intensity, with variable expiratory airflow limitation due to bronchoconstriction (airway narrowing), airway wall thickening and increased mucus [220]. The symptoms may be triggered or worsened by a combination of host factors including genetic predisposition, gender and ethnicity, with environmental factors such as viral infections, indoor and outdoor allergens, tobacco smoke, air pollution, food additives, exercise, stress and emotions, among others. According to the Global Asthma Report (2014) [2], is estimated that 300 million people of all ages in all parts of the world currently are affected. Its prevalence is increasing in many countries and especially in children. Asthma is associated with a high disease burden at both social and economic level. Adverse social impacts include: (*i*) reduced self-confidence, (*ii*) decreased quality of life, (*iii*) time lost from work or education, and (*iv*) social stigma, whereas the economic burden is due to direct costs (hospital admissions and medication) and indirect medical expenses, including productivity losses and school absenteeism [3]. Asthma is particularly problematic given that the disease consists of many variants, each with its own etiologic and pathophysiologic factors, including different cellular responses and inflammatory phenotypes. Thus, asthma is not considered a single disease, but a multifaceted syndrome which turns its diagnosis extremely difficult [221].

Its diagnosis is based on history and patterns of symptoms; physical examination; measurements of lung function and measurements of allergic status to identify risk factors. In order to improve the diagnosis and treatment of the different asthma subtypes, especially those which are more severe and therapy resistant, considerable research efforts has been made in recent years aimed at revealing biomarkers capable to discriminate different phenotypes of the disease [222].

Currently, biomarkers for asthma are found in several biological fluids which can be assessed by distinct sampling methodologies of the lower airways, meeting at least, some of the above mentioned criteria: invasive sampling (BAL, submucosal or transbronchial biopsies), semi-invasive (induced sputum), minimally invasive (blood)

and totally non-invasive (saliva, exhaled breath and urine) [223]. Invasive sampling techniques have been largely substituted by other less invasive as they may require complicated and extremely invasive techniques, hard to use as a routine diagnostic tool, particularly in children. In turn, sputum induction is a validated method for an asthma diagnosis, corresponding to a safer procedure, cheaper and generally easier to perform than bronchial biopsy or BAL, although more troublesome than exhaled breath. Further, the interest in saliva studies in relation to asthma is still preliminary and the role of many possible confounders needs to be considered [223]. On the other hand, potential biomarkers have been measured and characterized in EBC. However, this sampling technique still awaits standardization before it can be reliably implemented into clinical practice [224]. In fact, the lack of validation of such sampling techniques and the heterogeneous nature of the inflammatory response has boosted the development of new non-invasive airways sampling methods, and the determination of adequate biomarkers [86, 221].

Asthma biomarkers obtained using non-invasive sampling procedures are especially appealing for young patients, despite issues with dilution and sensitivity [86]. For body fluids such as urine, samples are easily obtained and possess an array of molecular biomarkers related to airway inflammation. Urinary levels of arachidonic acid metabolism end products, have revealed potential phenotype-specific biomarkers in asthma [225]. Oxidation of arachidonic acid is a fundamental enzymatic lipid peroxidation reaction that leads to the formation of bioactive eicosanoids such as PGs and LTs, which are key mediators of bronchial inflammation and response modulation in airways [226]. These metabolites can not only be used as asthma biomarkers, but also in the evaluation of disease progression and follow of the therapy efficiency [86, 222].

The major asthma-related PGs and LTs metabolites were previously measured in urine by means of radioimmunoassay or enzyme immunoassay [227] and then, by reversed phase LC after pre-column extraction combined with radioimmunoassay [228]. Still, spectroscopic techniques corresponded to the standard technological approach to measure the urine metabolome [229, 230], which, in combination with LC is useful to distinguish asthma severity subtypes [231, 232].

Subsequent studies performed on patients with persistent asthma also assayed urinary LTs levels by MS for disease risk assessments and therapy purposes [233, 234]. In 2016, Loureiro et al. [147] examined the relationship between oxidative stress

extension, eosinophilic inflammation, and disease severity using SPME followed by a two-dimensional gas chromatography coupled to mass spectrometry with a high-resolution time-of-flight analyzer (GC×GC-ToFMS). Other approaches to screen urinary LTs based on LC tandem MS (LC-MS/MS) method were also reported [235].

The detection of eicosanoid metabolites in urine has provided insights into the inflammatory and oxidative stress status of asthmatic patients. However, constraints related with population sample sizes and intrinsic sample variability of urine samples have affected the accuracy of the analytical approaches so far.

METs emerged in the 90's with SPME [173, 174] and up to date, a noteworthy number of different METs are continuously being developed including needle-based sampling techniques such as MEPS [177], a miniaturized SPE technique consisting of a removable cartridge containing a sorbent, and a syringe-like sampler device, which can be manual, semi-automatic or fully automatic [178].

Taking into consideration the most recent trends in LC, UHPLC has been widely spread due to its increased sensitivity, ultra-resolution and ultra-fast analysis. Therefore, the present work aimed to develop an improved and highly sensitive MEPS/UHPLC-based approach, to identify and quantify lipid peroxidation biomarkers related with asthma – LTs E_4 and B_4 and $11\beta PGF_{2\alpha}$ – present in urine of asthmatic patients and healthy individuals. The most extraction efficiency-influencing experimental parameters, including the sorbent material, the number and mode (extract-discard and extract-eject) of sample loading cycles, the pH, the volume of elution solvent and the conditioning solvent, were evaluated and optimized by means of a univariate experimental design. In addition, important instrumental parameters with influence on the chromatographic resolution, such as composition of the mobile phase, the nature of the stationary phase of capillary column and the flow rate of mobile phase, were also optimized. The stability of eicosanoids extracts was checked over a period of 24 hours with sampling throughout a single day. The proposed methodology was applied to the analysis of children urine with allergic asthma and healthy children, used as control group.

3.2 Materials and methods

3.2.1 Reagents and materials

All standards and reagents were of analytical grade with a purity higher than 98%. ACN (99.9%) and MeOH (99.9%) were obtained from Fisher Scientific (Leics, UK). Formic acid (FA; 98%), EtOH (99.5%), calcium chloride-2-hydrate, sodium sulfate anhydrous, sodium carbonate anhydrous, potassium di-hydrogen phosphate and sodium hydroxide (NaOH) were acquired from Panreac (Barcelona, Spain). Magnesium chloride-6-hydrate and potassium chloride were purchased from Honeywell Riedel-de-Haën AG (Seelze, Germany). Tri-sodium citrate-2-hydrate and ammonium chloride were acquired from Merck (Darmstadt, Germany) and sodium chloride from Sigma-Aldrich (Buchs, Switzerland). Ultrapure H_2O (18 MΩ cm at 23 °C) was prepared using a Milli-Q water purification system (Millipore, Milford, MA, USA). Kaempferol, used as internal standard (IS), was acquired from Fluka Biochemica AG (Buchs, Switzerland). LTB_4, LTE_4 and $11\beta PGF_{2\alpha}$ standards were acquired from Cayman Chemical Co. (MI, USA).

The eVol® XR hand-held automated analytical syringe (500 µL) and the BINs: C18, C8, C2, SIL, M1, HLB-DVB, R-AX and R-CX, containing the sorbent used in the microextraction procedure were purchased from SGE Analytical Science Europe, Ltd. (Milton Keynes, United Kingdom). The polymeric sorbents PEP, VAX and PGC were from Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

The capillary columns and pre-columns: Acquity UPLC® BEH C18 (2.1 × 50 mm) protected with an Acquity UPLC® BEH C18 VanGuard™ Pre-column (2.1 × 5 mm), Acquity UPLC® HSS T3 (2.1 × 100 mm) protected with an Acquity UPLC® HSS T3 VanGuard™ Pre-column (2.1 × 5 mm), Acquity UPLC® CSH C18 (2.1 × 150 mm) protected with an Acquity UPLC® CSH C18 VanGuard™ Pre-column (2.1 × 5 mm) and CORTECS UPLC® C18 (2.1 × 100 mm) protected with a CORTECS UPLC® C18 VanGuard™ Pre-column (2.1 × 5 mm), were acquired from Waters Portugal (Lisboa, Portugal). The Kinetex® HILIC (2.1 × 100 mm) protected with SecurityGuard™ ULTRA cartridge for HILIC UHPLC (2.1 × 2 mm) was supplied by Tecnocroma – Técnicas analíticas, LDA (Caldas da Rainha, Portugal).

3.2.2 Standard solutions and synthetic urine

Individual solutions of all standards were prepared in EtOH. The LTB_4 and LTE_4 were prepared at 10 µg mL^{-1} directly as working solutions and the $11\beta PGF_{2\alpha}$ and the IS at 1 mg mL^{-1} as stock solutions. The $11\beta PGF_{2\alpha}$ and IS working solutions were diluted in EtOH at concentrations of 100 µg mL^{-1} and 1 µg mL^{-1}, respectively. All stocks and working solutions were aliquoted in 2-mL amber glass vials and stored at -80 °C.

A synthetic urine (SU) solution was prepared according to Wilsenach et al. [236] with slight modifications (Table 1A, *Annex I*). Urea was not used due to possible degradation (interferent peaks). Creatinine was not used due to a possible normalization of the targeted analytes to this compound. Prior to its use, the pH of the SU was adjusted to 5.1 (Mettler Toledo™ EL20 Benchtop pH Meter for Teaching and Learning, Mettler Toledo AG, Switzerland) with 10% FA or 1M NaOH, and filtered through a 0.2 µm membrane PTFE filter (Merck Millipore, Milford, MA, USA). The pH of real urine samples including an unspiked real urine (USRU), were adjusted to 5.1, centrifuged at 5000 rpm (2739 × g; Sigma 3K30, Sigma Laborzentrifugen GmbH, Germany) for 15 min at 5 °C, and the supernatants filtered through 0.22 µm PTFE syringe filters (BGB Analytik, VA, USA).

For method development, optimization and validation, the assays were carried out using fortified SU with LTB_4, LTE_4 and $11\beta PGF_{2\alpha}$ at different concentrations according to the sensitivity of the UHPLC-PDA and the amount of eicosanoids present in urine.

The stability of the extracts was evaluated in extracts of spiked SU at low level (LL) and medium level (ML) of concentrations. A set of 4 replicates were taken and analyzed at 0, 8, 16 and 24 hours after extraction. This parameter, evaluated by comparing the concentration at each time compared to the concentration at time 0, was calculated as follows:

$$Stability\ (\%) = (A_{ti}/A_{t0}) \times 100$$

where A_{ti} represents the analyte concentration measured at the specific time and A_{t0} represents analyte concentration measured immediately after MEPS procedure (0 hour), time point zero. This parameter allowed us to know how long the eluates can be in the sample manager chamber before analysis without compromising the integrity of the target analytes.

3.2.3 Sample collection and preparation

This study was performed in accordance with the principles contained in the Declaration of Helsinki and approved by the Ethical Committee for Health of Funchal Hospital (SESARAM, EPE; *Annex II*) and by Regional Direction of Education (DRE; *Annex III*). All tutors of the subjects were informed about the purpose of the study (*Annex IV*) and signed an informed consent to participate in the study (*Annex V*).

Asthmatic children diagnosed with uncontrolled symptoms (N = 27, age = 8.8 ± 4.6Y) were recruited in the emergency and allergology service of Funchal Hospital, while urine samples from healthy children (N = 17, age = 7.8 ± 1.4Y) were collected at two schools, EB1/PE Tanque – Monte and EB1/PE Visconde Cagongo in Funchal, Portugal. Relevant information for the study including gender, age and other information such as familiar diseases, home characteristics, type of disease including clinical data (controlled or not controlled disease, and clinical symptoms), therapies, among others, was obtained at sample collection (Table 5) through a brief questionnaire (*Annex VI* for children from hospital and *Annex VII* for children from schools).

No restrictions in terms of diet and urine sample (e.g. first morning sample) were applied to the individuals. Each individual, healthy or asthmatic patient, provided a urine sample in a 100-mL urine beaker with integrated transfer device (Greiner Bio-One GmbH, Austria). Samples were transported to the laboratory in a portable cooling box, suitable for this kind of biological samples, aliquoted in 8-mL glass vials and immediately frozen at -80 ºC in order to quench any rapid degradation activity such as oxidation of labile metabolites as well as various enzymatic reactions. Compared with other biofluids, urine offers several advantages since it is obtained through an easy and totally non-invasive sampling procedure, may be collected across all ages even in severe patients, is a rich source of metabolites and its composition reflects the imbalances of all biochemical pathways within the body.

3.2.4 Isolation of targeted analytes by MEPS

The targeted analytes, LTB_4, LTE_4 and $11\beta PGF_{2\alpha}$, were isolated from urine using a semi-automatic miniaturization of SPE, the MEPS. This off-line procedure combines a digital and programmable semi-automatic device (eVol® XR) that controls the aspiration/dispensing volume and flow rate with minimal user interference allowing

greater precision and accuracy and consequently greater reproducibility; a 500-µL gas-tight analytical syringe; and a removable MEPS cartridge, known as "barrel insert and needle" (BIN), containing 1-4 mg of a solid packed sorbent bed. This experimental procedure includes: (*i*) an activation process of the sorbent with H_2O and MeOH, (*ii*) the conditioning of the sorbent usually performed with the elution solvent, (*iii*) the sample loading where the sample is forced to pass through the sorbent bed several times, in order to efficiently retain the analytes of interest, (*iv*) a washing step to remove unwanted analytes not retained in the sorbent; and finally (*v*) the elution where the analytes were eluted using an appropriate solvent [178]. A SU sample spiked at 40 ng mL^{-1} for the LTB_4 and LTE_4, and 160 ng mL^{-1} for the $11\beta PGF_{2\alpha}$, was used in the optimization of the experimental parameters with influence on the MEPS efficiency. The chemical nature of the sorbent material, the number and mode (extract-discard and extract-eject) of sample loading cycles, the pH, the volume of elution solvent and the conditioning solvent, were evaluated and optimized by means of a univariate experimental design.

Table 5 – Characteristics of the asthmatic patients and healthy individuals (controls).

Subjects	**Asthmatic patients**	**Healthy individuals**
n	27	17
Age (years; mean ± std)	8.8 ± 4.6	7.8 ± 1.4
Gender (male:female)	16:11	10:07
Pathology		
Allergic asthma	26 (96%)	0
Allergic asthma + allergic rhinitis	25 (93%)	0
Allergic asthma + sinusitis	2 (7%)	0
Allergic asthma + conjunctivitis	7 (26%)	0
Allergic asthma + eczema	14 (52%)	0
Allergens		
House dust mites	19 (70%)	0
Pollens	10 (37%)	0
Animal epithelium	15 (56%)	0
Moulds	3 (11%)	0
Cockroaches	5 (19%)	0
Initial diagnose (intermittent:mild:moderate:severe:n/a[a])	6:3:12:0:4	-
Uncontrolled asthma	27 (100%)	0
Exacerbation in last 12 months	23 (85%)	0
Missed school in last 12 months because of illness[b]	23 (85%)	8 (47%)
Parent/Tutor missed work in last 12 months because of the child[b]	14 (52%)	7 (41%)
Smoker	3 (11%)	0
Have family members with asthma	22 (81%)	10 (59%)
Relatives with asthma (only paternal:only maternal)	4:13	0:4
Both relatives with asthma	5 (19%)	6 (35%)
Without relatives with asthma	5 (19%)	7 (41%)

[a] n/a – no answer.

[b] Because of asthma, in the case of the asthmatic patients and because of any illness, in the case of normal controls.

The extraction efficiency of 11 commercially available MEPS sorbents including, C18 (octadecyl-silica), C8 (octyl-silica), C2 (ethyl-silica), SIL (silica), M1 (80% C8 + 20% SCX – strong cation exchange), PEP (polar enhanced polymer), VAX (verify AX; C8 + SAX – strong anion exchange), HLB-DVB (hydrophilic-lipophilic-balanced reversed-phase sorbent, divinylbenzene), PGC (porous graphitic carbon), R-AX (retain anion exchange) and R-CX (retain cation exchange), was evaluated and optimized in order to select the sorbent that offers the best performance for isolation of targeted eicosanoids. The main characteristics of the MEPS sorbents used in this study are represented in Table 6.

The number of loading cycles was optimized by drawing up 250 µL of sample, 3, 5 and 10 times, while the mode of the loading cycle was evaluated by drawing up the sample and ejecting in the same vial (extract-eject) and by drawing up the sample and discarding as waste (extract-discard).

The number of loading cycles was optimized by drawing up 250 µL of sample, 3, 5 and 10 times, while the mode of the loading cycle was evaluated by drawing up the sample and ejecting in the same vial (extract-eject) and by drawing up the sample and discarding as waste (extract-discard).

The influence of pH was evaluated by testing the extraction efficiency at three different sample pH values (2.8, 5.1 and 8.5), adjusted with NaOH at 1M or FA at 10%, in two opposite ion exchange MEPS sorbents: R-AX and R-CX.

Eluates were eluted into 100-µL micro-inserts (VWR International - Material de Laboratório, Lda., Carnaxide, Portugal), suitable for 2-mL vials. In addition, the total dryness of the eluate and its resuspension in 50 µL and 100 µL of mobile phase (95% H_2O at 0.1% FA and 5% ACN at 0.1% FA) and the concentration of the eluate until half of its volume (without dryness) were also investigated.

In order to investigate the best elution conditions, the impact of different elution solvents was investigated with different solvent solutions based on pure ACN, pure MeOH, acidified ACN and acidified MeOH, and different proportions of ACN and MeOH (50:50, 75:25 and 25:75). The washing solution, 0.1% FA, was also used to investigate if any of our target analytes were washed away in the washing step. After solvent selection, the elution volume (50 µL and 100 µL) and the number of cycles (1, 2 and 3 times) was also investigated.

Table 6 – Properties of the MEPS sorbents used in this study. Adapted from Pereira et al [178].

Type		Chemistry	Name	Properties and purpose
Silica	Unmodified	Normal phase	SIL	Highly polar sorbent Polar analytes
	Modified	Reverse phase	C2	Fairly non-polar sorbent Analytes highly retained to C8 and C18
			C8	Non-polar sorbent Non-polar analytes with diverse structures
			C18	Highly non-polar sorbent Least selective sorbent Retains most organic analytes from aqueous matrices Analytes of diverse structures
	Functionalized	Mixed-mode (reverse phase, ion exchange)	M1	Dual retention mechanism Neutral, basic, acidic and zwitterionic analytes Higher selectivity for basic analytes
			VAX	Non-polar and cationic sorbent Acidic analytes Moderately polar to non-polar and ionized and charged analytes
Carbon	Unmodified	Reverse phase	PGC	Pure porous graphitic carbon material Highly polar compounds from different matrices
PS-DVB	Modified	Reverse phase	HLB-DVB	Polar and non-polar sorbent Retain a wide spectrum of both polar and nonpolar analytes
	Functionalized	Mixed-mode (reverse phase, ion exchange)	PEP	Urea functional groups Retention of polar and non-polar analytes Analytes of diverse structures in biological matrices
		Mixed-mode (normal phase, ion exchange)	R-AX	Partially functionalized sorbent with quaternary amine groups Balance retention of acidic and non-polar analytes
			R-CX	Partially functionalized sorbent with sulfonic acidic groups Balance retention of basic and non-polar analytes Basic and neutral analytes

Abbreviations: PS-DVB – Polymeric polystyrene – divinylbenzene, SIL – silica, C2 – ethyl-silica, C8 – octyl-silica, C18 – octadecyl-silica, M1 – mixed mode sorbent with 80% C8 + 20% strong cation exchange, VAX – verify AX (C8 + strong anion exchange), PGC – porous graphitic carbon, HLB-DVD – hydrophilic-lipophilic-balanced reversed-phase sorbent – DVB, PEP – polar enhanced polymer, R-AX – retain anion exchange, R-CX retain cation exchange.

An important step in MEPS is the conditioning of the sorbent since it can influence the performance of the extraction. It consists on the clean up to avoid carry-over, and regeneration of the active sites in the sorbent for its reuse. According to Pereira et al. [180], different organic solvents can be used to perform this step. In this regard, the effect of the conditioning solvent was performed by testing two common organic solvents, 250 µL of ACN and 250 µL of MeOH, followed by an equilibration with 250 µL of FA at 0.1%. The number of conditioning-equilibration cycles (1, 2 and 3 times) were tested in order to suppress the carry-over effect.

In all steps of the MEPS procedure the aspiration and dispensing flow rate was limited to the minimum provided by the device (20 µL s^{-1}) in order to increase the interaction time of the analytes with the sorbent and to minimize the occurrence of cavitation. All extractions were made in duplicate (n = 2).

3.2.5 Chromatographic conditions. Optimization

The analysis of the target analytes was carried out on a Waters Ultra Performance Liquid Chromatographic Acquity system (UPLC Acquity H-Class, Waters Corporation, Milford, MA, USA) equipped with a 2996 PDA detector, a Waters Acquity quaternary solvent manager (QSM), an Acquity sample manager, a column heater and a degassing system. The whole configuration was driven by Empower software v2.0 (Waters Corporation). In order to achieve the best performance, important instrumental parameters with influence on the chromatographic resolution, composition of mobile phase and nature of the stationary phase of capillary column, were optimized. Several types of columns with different dimensions including Acquity UPLC® BEH C18 protected with an Acquity UPLC® BEH C18 VanGuard™ Pre-column, Acquity UPLC® HSS T3 protected with an Acquity UPLC® HSS T3 VanGuard™ Pre-column, Acquity UPLC® CSH C18 protected with an Acquity UPLC® CSH C18 VanGuard™ Pre-column, CORTECS UPLC® C18 protected with a CORTECS UPLC® C18 VanGuard™ Pre-column, and Kinetex® HILIC protected with SecurityGuard™ ULTRA cartridge for HILIC UHPLC, were evaluated in terms of peak resolution, shape and measurable areas of the target analytes (Table 7).

Table 7 – Properties of the UPLC columns used in this study.

Chromatographic mode	Name	Chemistry	Particle technology	Particle size (µm)	surface area (m^2/g)	pH range	Pressure Stability (psi)	Temperature limits (°C)		Properties and purpose
								Low pH	High pH	
Reversed phase	Acquity UPLC® BEH C18	C18	Fully porous	1.7	185	1 - 12	18000	80	60	Ethylene Bridged hybrid particle technology
										Universal column
										Wide variety of compounds
										Separations at high temperatures and extremes of pH
	Acquity UPLC® HSS T3	Tri-functionally bonded C18 ligand	Fully porous	1.8	230	2 - 8	18000	45	45	High strength silica particle technology
										High retention of polar organic analytes
										Balanced retention of hydrophilic and hydrophobic analytes
	Acquity UPLC® CSH C18	C18	Fully porous	1.7	185	1 - 11	18000	60	45	Charged surface hybrid particle technology
										Separation of basic analytes at low pHs
										Fast pH transitions and equilibration
										Fast method development
	CORTECS UPLC® C18	C18	Solid core	1.6	100	2 - 8	18000	45	20	Solid-core particle technology
										High efficiency and increased sensitivity
Normal phase	Kinetex® HILIC	Unbonded silica (silanol groups)	Solid core with a porous shell	1.7	200	2 - 7.5	14500	60	< 60	Core-shell particle technology: 1.25 µm solid core with a 0.23 µm porous coating
										Hydrophilic interaction chromatography
										High selectivity and increased retention of highly polar analytes

These parameters were developed with spiked SU and real urine at the same concentration as described in a previous section. The optimized gradient was as follow: (A) 0.1% FA and (B) ACN acidified with FA (0.1%): 95% A (0 min), 47% A (9.5 min), 24% A (11 min), 0% A (11.1-12.1 min) and 95% A (14 min) followed by a re-equilibration time of 2 min. The vials containing the extracts were kept inside the sample manager at a temperature of 15 ºC. The flow rate was of 300 µL min^{-1}, the injection volume was set at 5 µL and the capillary columns thermostatted at 30 ºC. For quantification purposes, the detector was set to 4 distinct channels corresponding to the maximum absorbance wavelength of each target analyte and IS (*see* Table 8).

The identification of the target analytes was made by comparison of the retention time and UV-Vis spectral characteristics with those of pure standards and confirmed by the standard addition to the eluates. All UPLC injections were carried out in triplicate (n = 3).

3.2.6 Method validation

MEPS/UHPLC-PDA method was validated according to the International Union of Pure and Applied Chemistry (IUPAC) guidelines, being assessed the following parameters: selectivity, linearity, instrumental sensitivity, precision, accuracy, extraction efficiency and matrix effect.

The method selectivity was assessed by analyzing the absence of interfering components at the retention time of the eicosanoids.

Linearity was evaluated by spiking SU at different concentrations. A seven-point calibration curve for LTE_4 and $11\beta PGF_{2\alpha}$, and nine concentration levels for the LTB_4 were constructed from the least square linear regression model with a weighing factor of $1/\times^2$ by plotting the peak area ratio between each eicosanoid and the IS ($Area_{analyte}/Area_{IS}$) against the eicosanoid concentration. Since the evaluation of the coefficient of determination (r^2) as a measure of linearity may be misleading, the proportional ratio using chromatographic signal and eicosanoid concentration was confirmed by means of the *F*-test through the lack-of-fit, goodness-of-fit and Mandel's fitting tests.

The sensitivity of the method was expressed in terms of LOD and limit of quantification (LOQ). LOD is defined as the lowest concentration of analyte which can be reliably detectable and identified with an acceptable precision and accuracy (commonly within 20% RSD and within 20% bias), and LOQ is defined as the lowest

concentration of analyte which can be identified and quantified with a satisfactory precision and trueness [237]. These parameters were calculated from the least squares linear regression analysis being LOD defined as: $a + 3.3S_{a/b}$ and LOQ as: $a + 10S_{a/b}$, where *a* represents the origin ordinate, *Sa* represents the standard deviation of the experimental value closest to the intercept, and *b* the slope of the calibration curve.

The matrix effect was calculated by the ratio between the slope of the standards in USRU, obtained in the least squares linear regression analysis, and the slope of the standards in SU:

$$Matrix\ effect\ (\%) = (\text{slope}_{USRU}/\text{slope}_{SU}) \times 100$$

where slope_{USRU} and slope_{SU} are the slopes obtained from the analytical curve of the USRU and SU respectively.

In order to determine precision, accuracy and recovery percentage, the SU was spiked at three concentration levels corresponding to the LL, ML and high level (HL), covering the entire calibration range. Precision (expressed as percentage of relative standard deviation, % RSD) was evaluated in terms of intraday repeatability obtained from the analyzes of 4 replicates of spiked SU performed at the same day, and analyzed in the same analytical run, assayed in three different runs ($n = 9$) over a period of three days (reproducibility).

Accuracy refers to how close the concentration values obtained experimentally are to the tabulated values and is calculated as follows:

$$Accuracy\ (\%) = (x_i/x) \times 100$$

where x_i represents the experimental concentration values and x the tabulated concentration values. This parameter was calculated with the data obtained in the least squares linear regression analysis.

The recovery study allowed the evaluation of the extraction efficiency carried out in duplicate (n = 2) for each level of concentration. Briefly, one set of aliquots of SU was spiked with the analytes before the MEPS procedure and another set of unspiked SU went through the process and the respective eluates were spiked with the different

concentration levels of the target analytes. This parameter is calculated by means of the following formula:

$$Recovery\ (\%) = (S_{SU}/S_{Eluate}) \times 100$$

where S_{SU} represents the obtained concentration of the analytes in the spiked SU when added before the MEPS procedure, and S_{Eluate} represents the concentration obtained from the analysis of the spiked eluates.

3.2.7 Statistical analysis

Further statistical data analysis was performed by means of the IBM SPSS Statistics Version 22 (IBM Corp., Armonk, NY, USA). The independent samples *t*-test is used in situations with two experimental conditions with different participants in each condition [215]. Regarding this, the *t*-test was performed on the target analytes values obtained from the samples in order to characterize the significant differences among both groups under study: the asthmatic patients and normal controls, in terms of mean values.

3.3 Results and discussion

With this work, it was intended to develop an easy and quick analytical method able to detect and quantify simultaneously three eicosanoids present in urine that are biomarkers of inflammation and potentially of asthma *per se*.

3.3.1 Individuals characterization

The main characteristics of the subjects of this study are shown in Table 5.

Family history is strongly related to asthma. Most of the asthmatic patients (81%) that took part of this study, had a family member with asthma being the majority (66.7%), maternal-related. This trend has been reported by Valerio et al. [238] where children with relatives with asthma are 2 to 4 times more likely to have an asthma diagnosis than children with no asthmatic relatives. Even though family history is strongly related to the asthmatic diagnosis in children, many of our asthmatic patients did not have any relative with asthma (19%) and many of the normal controls (59%) had a relative with an asthmatic diagnosis during their lifetime but no asthma or any allergen sensitization was

reported by this group. In the present work, it was interesting to find that most of the asthmatic patients were boys (59% of total population of asthmatic patients and 74% of asthmatic patients until 13 years old) being in accordance with the pattern of other countries [26]. Although the prevalence of asthma is higher in boys at this age (< 13 years old), the risk of developing asthma increases rapidly in women after puberty [239]. It is believed that these changes might have a relationship to the endocrine system, specifically to leptin, an hormone produced by adipocytes, related to inflammation [240] and asthma in children [241].

The main comorbidity found throughout this study was allergic rhinitis with 93% of the asthmatic patients with allergic asthma having also rhinitis. Although higher, this value is in agreement with the literature [242] which stated that allergic rhinitis coexists in 60 to 80% of allergic asthmatic children probably because of their similar pathophysiology. This comorbidity has also been found in general asthmatic population and not only in children but in lower values (52.3%) [243]. The most common allergen sensitization, among the allergic population under study, was sensitivity to house dust mites (70% of the allergic population) followed by animal epithelium and pollens, with 56% and 37% respectively. A similar trend was previously reported by Boulet et al. [244].

3.3.2 MEPS optimization

The efficiency of eleven commercial sorbents suitable for eVol®-MEPS format (Figure 19) was evaluated. Each sorbent has specific chemical properties that make them suitable for the extraction of a particular class of compounds (Table 6). As shown in Figure 19, R-AX was the most favorable for the isolation of all targeted eicosanoids and thus, was selected as the extraction sorbent for the following experiments.

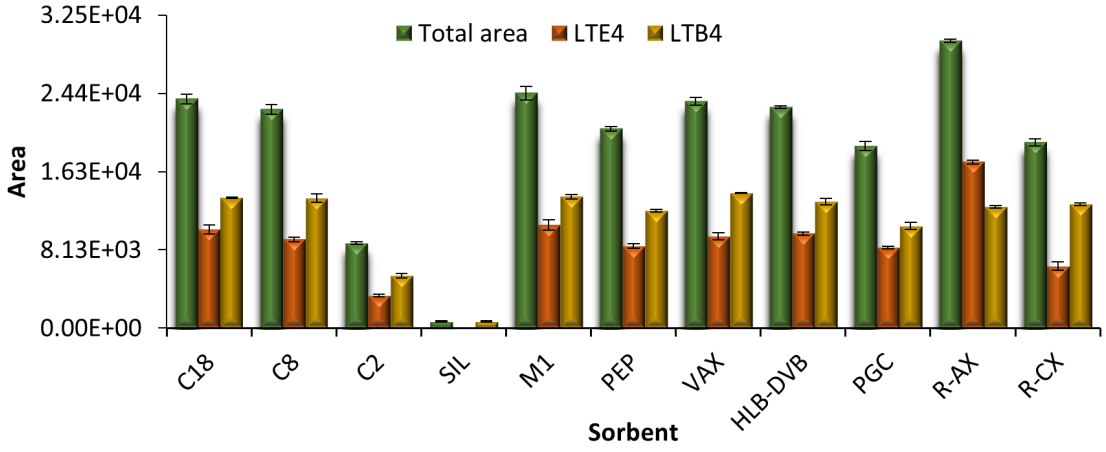


Figure 19 – Comparison of the efficiency of eleven commercial MEPS sorbents. Values are expressed as mean ± standard deviation (n = 6).

Although R-CX and VAX sorbents, show best efficiency for LTB_4, the difference is not significant when compared to the overall of the R-AX that had a better extraction efficiency for the LTE_4. Furthermore, the target analytes, $11\beta PGF_{2\alpha}$, LTE_4 and LTB_4, have an acidic nature due to the presence of a carboxylic group making them suitable to be extracted by the R-AX. This sorbent is chemically functionalized with quaternary amine groups that gives to it a balanced retention capacity between acidic and non-polar compounds [178].

The number of loading cycles is determinant when developing a MEPS procedure. This parameter was optimized by drawing up 250 µL of sample 3, 5 and 10 times (Figure 20A). The results showed good extraction efficiencies for the LTs, in addition to detection of the $11\beta PGF_{2\alpha}$ when the sample is drawn up and down 10 times through the sorbent. It has not been tested more cycles because of sorbent saturation that was reflected on cavitation which started to become more prominent with the increase of loading cycles from 5 cycles. Consequently, more time was needed for each loading cycle and thus, a reduction on the sorbent lifetime was expected. Additionally, the mode of the loading cycles was also tested. The extract-discard mode consists on the drawn up of the sample from an aliquot and the waste is discarded, the extract-eject mode involves the drawn-up of the sample from an aliquot and the waste is not discarded, returning to the aliquot. The extract-discard mode was selected because the obtained performance was slightly better (Figure 20B) for all target analytes.

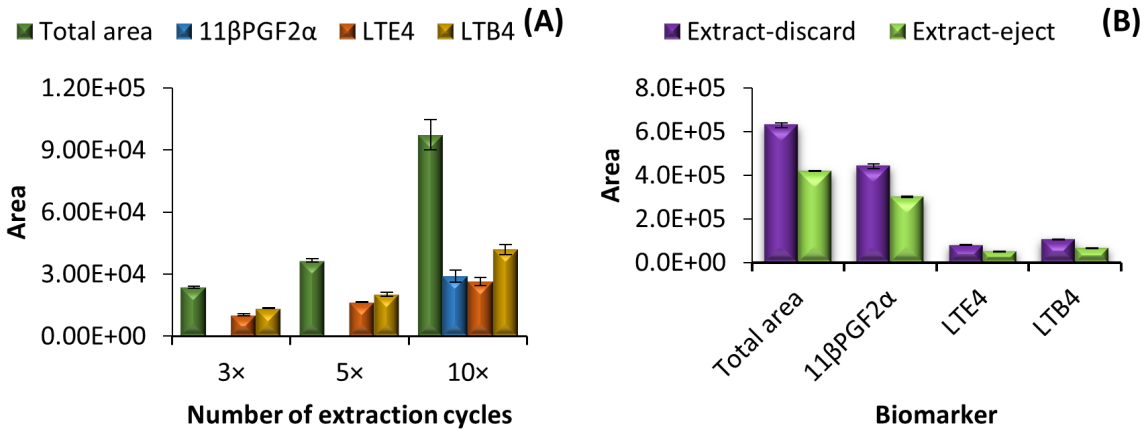


Figure 20 – Effect of the (A) number and (B) mode of loading cycles of the sample on the extraction efficiency. Values are expressed as mean ± standard deviation (n = 6).

The quaternary amine groups present on the surface of the R-AX are strong bases with a great acid dissociation constant ($pK_a > 14$), positively-charged at all pHs that, as

the name indicates, exchange or attracts anionic compounds in solution. R-CX is another ion exchange sorbent, functionalized with sulfonic acid groups that have very low pK_a (< 1), which make it negatively-charged at all pHs [245]. The targeted eicosanoids have low strongest acidic pK_a ($11\beta PGF_{2\alpha}$ = 4.36, LTE_4 = 2.39 and LTB_4 = 4.65; obtained from "The Human Metabolome Database" version 4.0 – http://www.hmdb.ca/ on November 2017). For this reason, at a pH higher than their pK_as, most of the analytes would be deprotonated, being able to be adsorbed through the surface of a basic sorbent. Although R-CX had the best results toward $11\beta PGF_{2\alpha}$ and in terms of total area, the extraction of LTE_4 was not so efficient. Hence, R-AX at pH = 5.1 was chosen as the best pH for subsequent experiments (Figure 21).

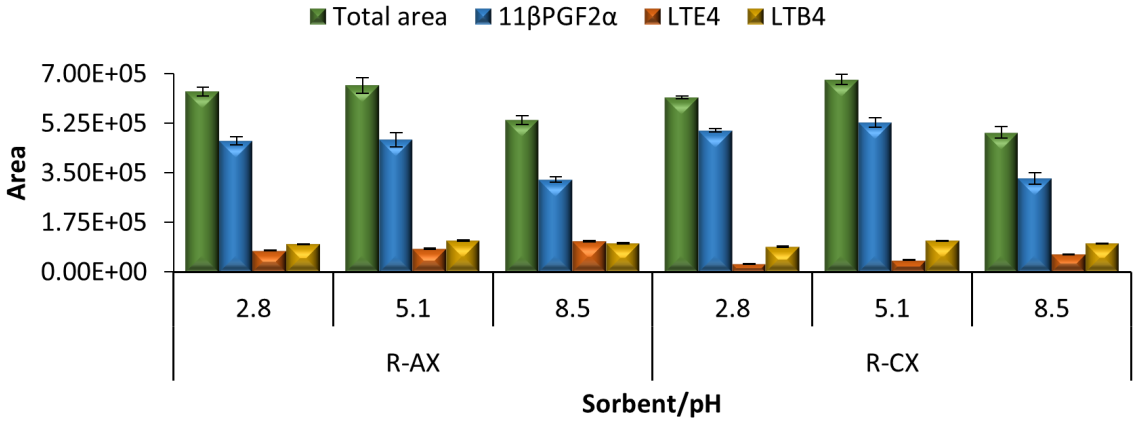


Figure 21 – Influence of the pH of the sample in the extraction efficiency with two ion exchange sorbents: R-AX and R-CX. Values are expressed as mean ± standard deviation (n = 6).

The investigation of the eluate concentration is shown in Figure 22.

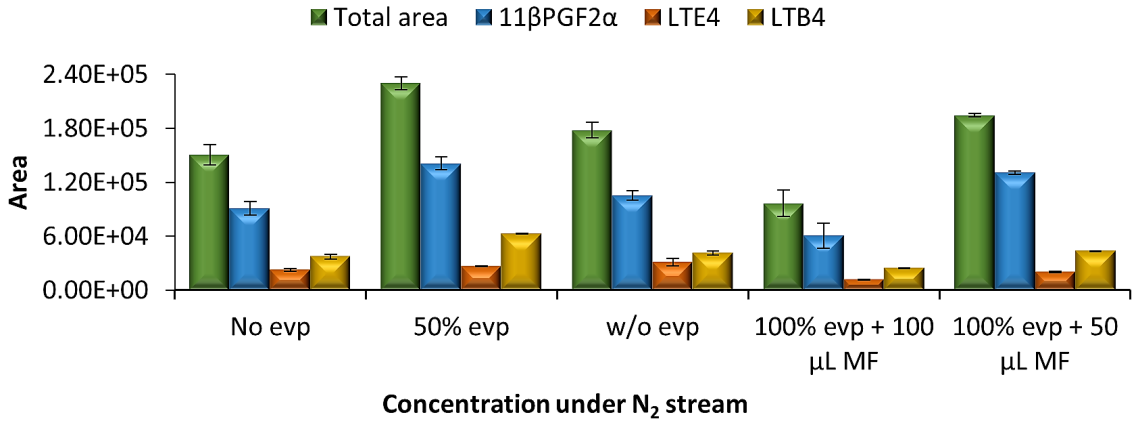


Figure 22 – Influence of the eluate concentration (under N_2 stream) on the extraction efficiency. Abbreviations: evp – evaporation; MF – mobile phase. Values are expressed as mean ± standard deviation (n = 6).

Comparing the obtained areas from 50% evaporation of the eluates (until 50 µL) with the areas obtained from complete dryness and resuspension in mobile phase (50 and

100 µL), it can be understood that complete dryness of the eluates is not appropriate for the target analytes once there are losses when resuspended. Evaporation of the eluates until half of its volume was the best option not only in terms of areas but also in terms of peak resolution and analyte detection, and therefore were used for further experiments.

Other important steps in MEPS procedure are washing and elution. The washing step allows us to remove undesired matrix interferences that are weakly retained on the sorbent while the elution step consists on the use of the best solvent and the least volume possible that is able to elute the target analytes retained on the surface of the sorbent [178, 179]. In this study, the best elution solvent was MeOH followed by ACN (Figure 23A). The elution volume was also considered and no substantial differences were found between eluting 2 or 3 times with 50 µL of MeOH (Figure 23B), therefore, in subsequent experiments, the elution was carried out using 2×50 µL of MeOH. The washing solvent, 0.1% FA, was also investigated and none of the target analytes were eluted with this solvent making it suitable for this step (Figure 23A).

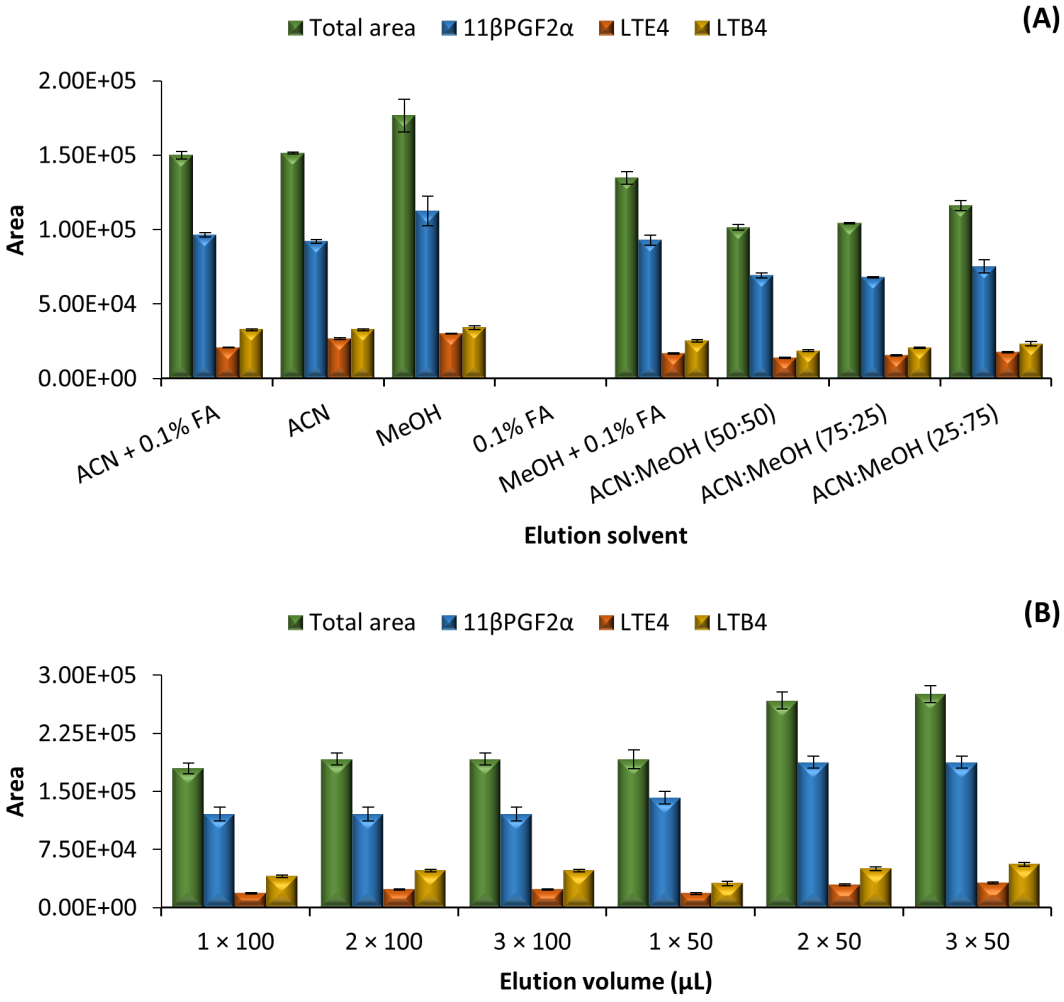


Figure 23 – (A) Influence of the elution solvent and (B) volume on the extractions. Values are expressed as mean ± standard deviation (n = 6).

An important and final/beginning step of the MEPS procedure is the conditioning/regeneration of the fiber. Each MEPS procedure starts with the conditioning of the fiber with an organic solvent followed by its equilibration before the loading of the sample and ends with the regeneration of the fiber that match with conditioning step if further extractions are made [180]. Even though the best elution solvent was MeOH, it was remarkable to find out that ACN is a better surfactant, allowing a greater conditioning of the fiber (Figure 24). ACN is less viscous than MeOH (0.37 centipoise, cp *versus* 0.55 cp at 20 ℃ (Physical Properties of Solvents – https://www.sigmaaldrich.com on November 2017) which can be a determinant factor that allows it to be more efficient involving the sorbent micro-particles reaching all spaces between them and consequently increasing the number of functionalized groups that are conditioned, improving the number of analytes bound to the surface of the sorbent. To the best of our knowledge, no previous report was made regarding the optimization of the conditioning/regeneration step with different solvents. To avoid carry-over, the number of conditioning-equilibration cycles were optimized. The best results, which are the less number of cycles enough to remove the carry-over, was 3×250 µL of ACN - 250 µL of 0.1% FA.

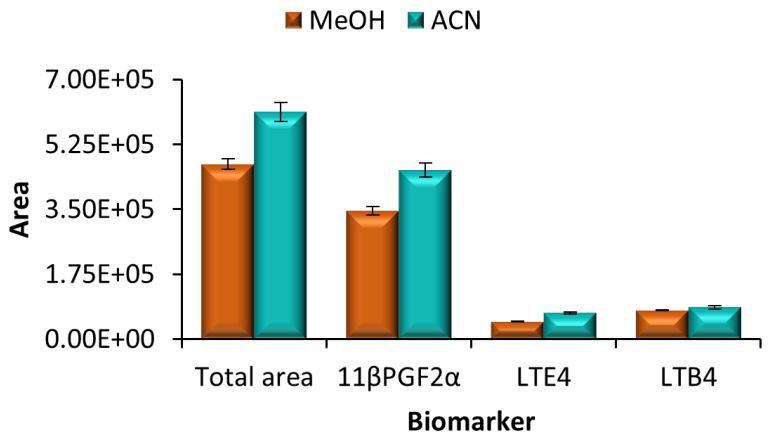


Figure 24 – Influence of the conditioning step solvent on the extraction performance. Values are expressed as mean ± standard deviation (n = 6).

3.3.3 Optimization of chromatographic conditions

The gradient of the mobile phase, the flow rate and several types of columns with different dimensions and particle sizes were tested and optimized.

The influence of five commercial columns suitable for UPLC® analyzes were evaluated (Figure 25 and Figure 26A). Each column has specific chemical and physical properties that makes them suitable for the separation of a wide range of compounds at

diverse conditions (Table 7). The BEH C18 column was selected for its narrow and sharp peaks, reproducible retention times and good chromatographic resolution of the target analytes (Figure 26A) and by the areas obtained (Figure 27). This column was previously used by Perestrelo et al. [246] and Chappell et al. [78] for the analysis of LTs in urine and sputum samples.

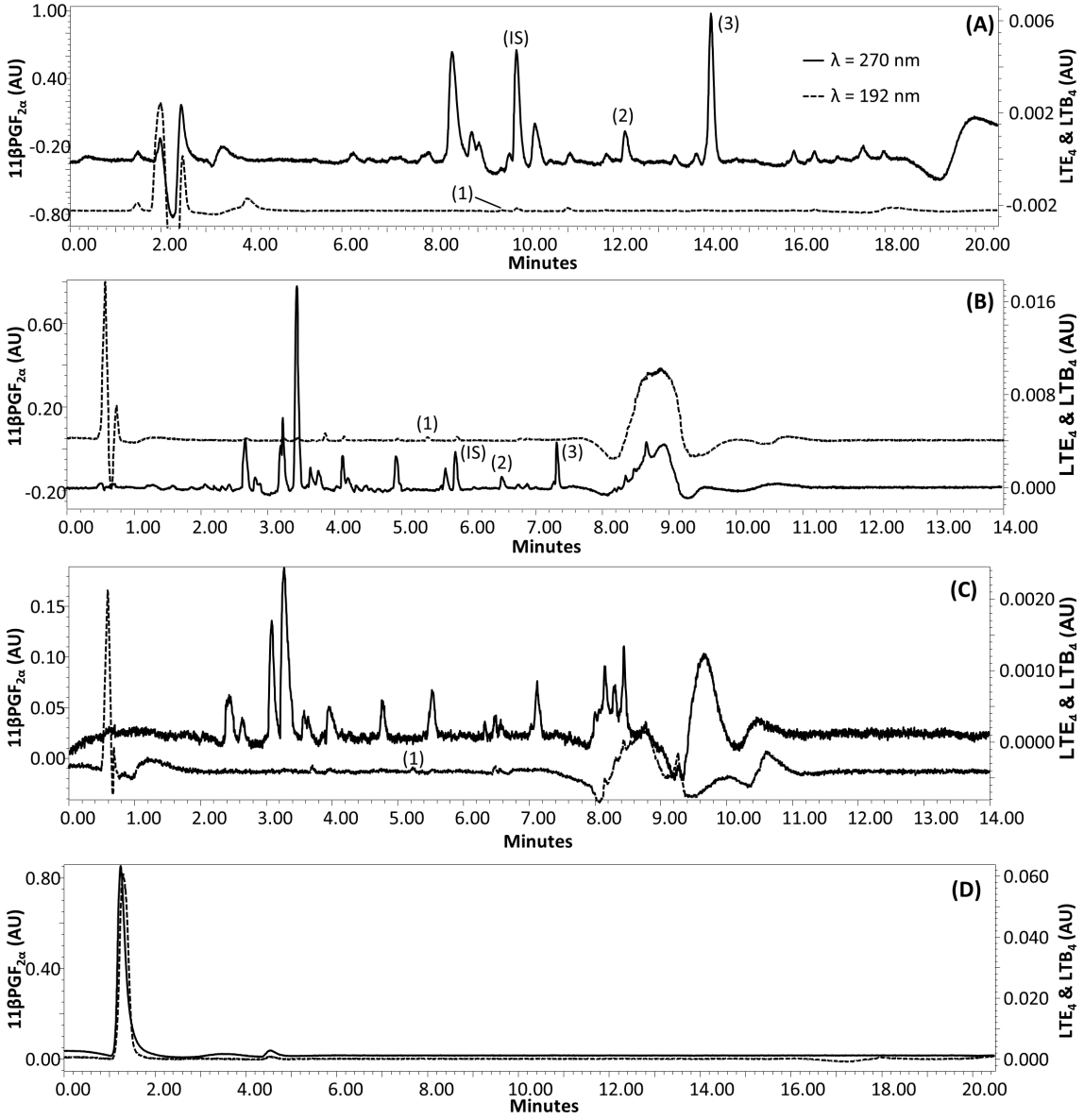


Figure 25 – Representative chromatograms of the evaluation of the different columns in synthetic urine. (A) CSH C18; (B) HSS T3; (C) CORTECS C18; and (D) HIILIC. Abbreviations: (1) – 11β-prostaglandin $F_{2\alpha}$; (2) – leukotriene E_4; (3) – leukotriene B_4; IS – internal standard.

In terms of gradient and flow rate, the initial conditions selected were a modification of Perestrelo el al. [246] and were tested with spiked SU. It consisted of a BEH C18 column and by a gradient profile composed by (A) 0.1% FA and (B) ACN acidified with FA (0.1%), at a flow rate of 500 µL min^{-1}: 95% A (0 min), 47% A (9.5

min), 24% A (11 min), 0% A (11.1-12.1 min) and 95% A (14 min) followed by a re-equilibration time of 2 min. The chromatogram obtained is shown in Figure 26A. The best gradient with spiked SU was obtained with a flow rate of 300 µL min^{-1} as follows: 95% A (0 min), 85% A (2 min), 60% A (2.5 min), 45% A (5 min); 95% A (6-7 min) (Figure 26B) followed by a re-equilibration time of 2 min.

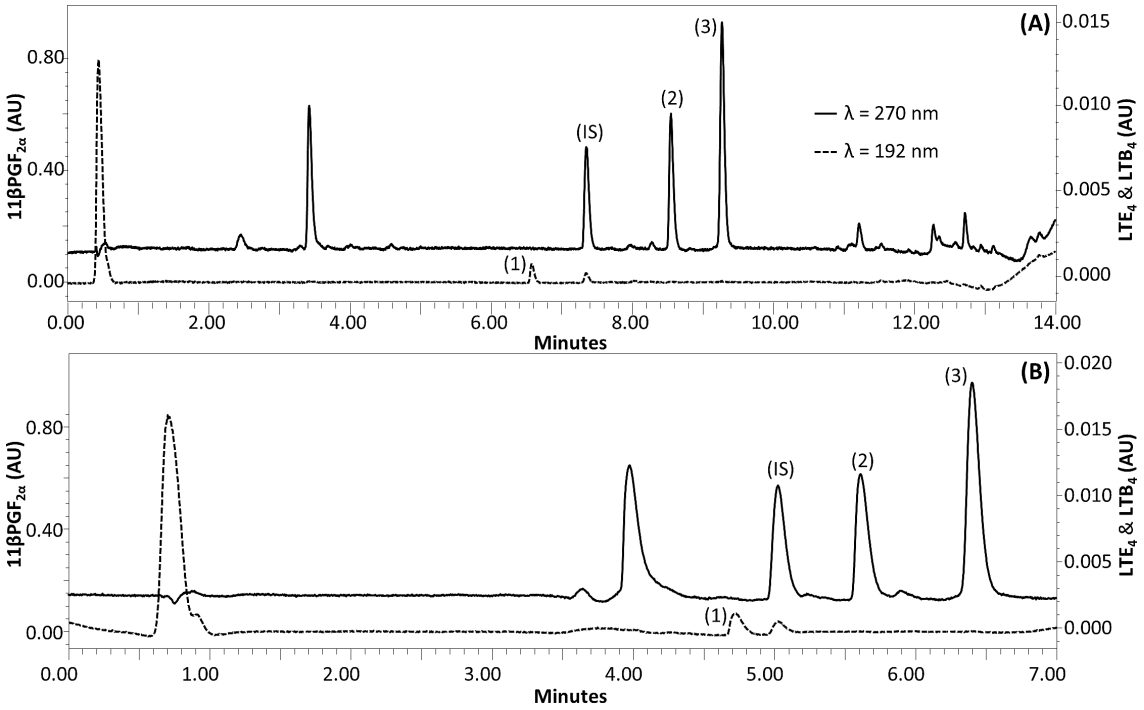


Figure 26 – Chromatograms obtained during gradient and flow rate optimization with a BEH C18 column with spiked synthetic urine samples. (A) Initial gradient at a flow rate of 500 µL min^{-1} as follows (A – 0.1% FA and B – ACN acidified with FA – 0.1%): 95% A (0 min), 47% A (9.5 min), 24% A (11 min), 0% A (11.1-12.1 min) and 95% A (14 min); (B) Optimized gradient at a flow rate of 300 µL min^{-1} as follows: 95% A (0 min), 85% A (2 min), 60% A (2.5 min), 45% A (5 min); 95% A (6-7 min). Abbreviations: (1) – 11β-prostaglandin $F_{2\alpha}$; (2) – leukotriene E_4; (3) – leukotriene B_4; IS – internal standard.

The best conditions obtained with SU were tested using spiked real urine. As shown in Figure 28A, these conditions were not effective once the analytes were co-eluted with urine interferents. A modification of the initial conditions in terms of flow rate (300 µL min^{-1}) were successfully tested with spiked real urine (Figure 28B) and spiked SU (Figure 29). With this optimization, even though we were not able to reduce the run time of analysis, a considerable decrease of 40% on the amount of solvent was achieved which is important regarding environmental issues and cost of analysis. Regarding the gradient of the mobile phase and the flow rate, they were optimized for the best results within a total run time of 16 min, including column equilibration. A representative chromatogram obtained with the final optimized conditions in SU is shown in Figure 29.

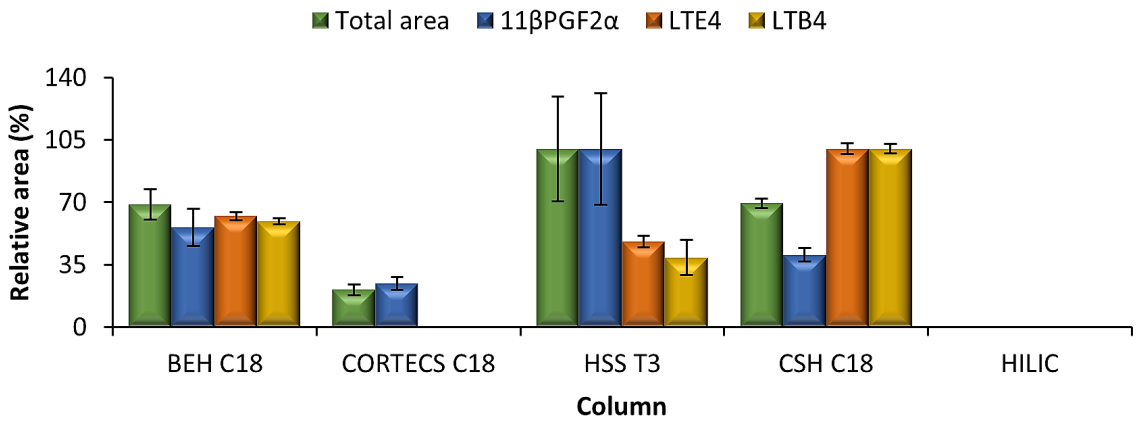


Figure 27 – Influence of five commercial columns suitable for UPLC® analyzes. Values are expressed as mean ± standard deviation (n = 3).

Considering the obtained data, the best chromatographic conditions were obtained using a gradient elution mode, with a flow rate of 300 µL min^{-1}, as follows: 95% A (0 min), 47% A (9.5 min), 24% A (11 min), 0% A (11.1-12.1 min) and 95% A (14 min) followed by a re-equilibration time of 2 min using an UHPLC-PDA equipped with a BEH C18 capillary column.

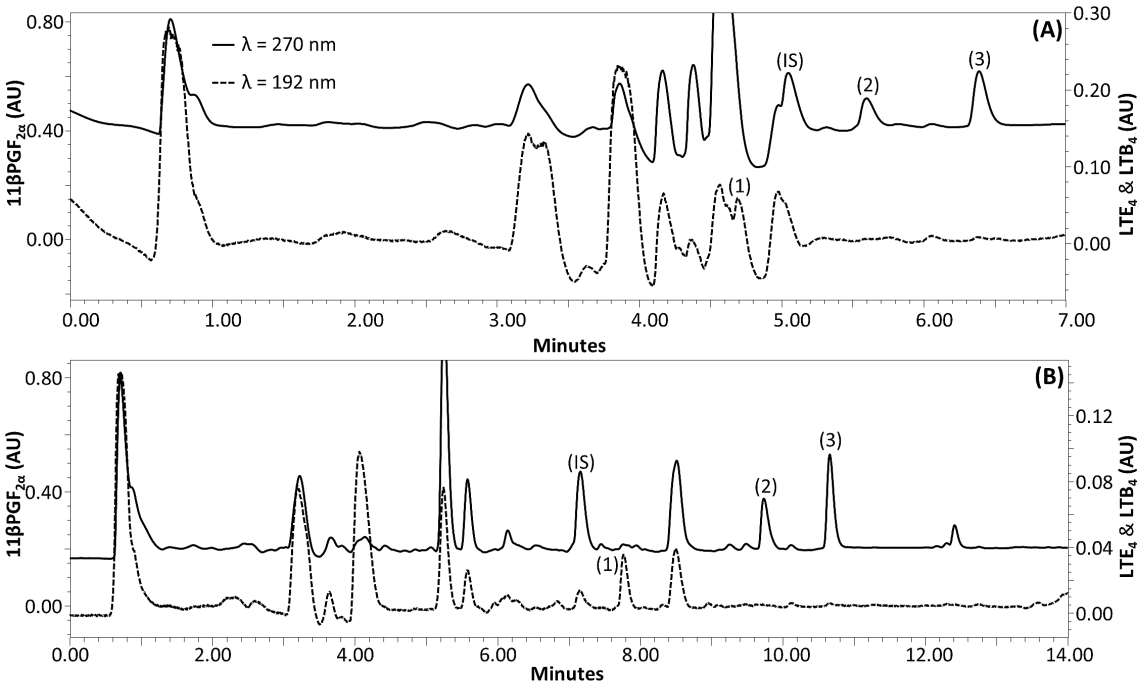


Figure 28 – Chromatograms obtained during gradient and flow rate optimization with a BEH C18 column with spiked real urine. (A) Best gradient and flow rate obtained with spiked synthetic urine samples applied to spiked real urine; (B) Optimized gradient in spiked real urine samples at a flow rate of 300 µL min^{-1} as follows: 95% A (0 min), 47% A (9.5 min), 24% A (11 min), 0% A (11.1-12.1 min) and 95% A (14 min). Abbreviations: (1) – 11β-prostaglandin $F_{2\alpha}$; (2) – leukotriene E_4; (3) – leukotriene B_4; IS – internal standard.

3.3.4 Method validation

In order to demonstrate the practicability of the developed analytical strategy and its ability to quantify the target analytes in urine samples, the method was fully validated in terms of selectivity, linearity, instrumental sensitivity, precision, accuracy, extraction efficiency and matrix effect for each eicosanoid. The method figures of merit are described in Table 8 and Table 9.

Table 8 – Analytical performance of the optimized methodology.

Analytes	**IS**	**11βPGF$_{2\alpha}$**	**LTE$_4$**	**LTB$_4$**
Peak number	-	1	2	3
RT[a] (min)	7.43	7.65	9.61	10.75
λ_{max}[b] (nm)	366	192	281	270
LDR[c] (ng mL $^{-1}$)	-	5 - 300	0.5 - 30	0.1 - 30
Calibration in solvent				
Regression equation	-	y = 0.1304x + 0.6418	y = 0.1225x + 0.0627	y = 0.1708x + 0.0105
r^{2}[d]	-	0.9988	0.9989	0.9997
r[e]	-	0.9994	0.9995	0.9998
Calibration in matrix				
Regression equation	-	y = 0.0951x - 2.6893	y = 0.0932x + 0.0224	y = 0.1385x - 0.0640
r^{2}[d]	-	0.9841	0.9884	0.9989
r[e]	-	0.9920	0.9942	0.9994
Matrix effect (%)	-	73	76	81
Sensitivity				
LOD[f] (ng mL^{-1})	-	1.12	0.16	0.04
LOQ[g] (ng mL^{-1})	-	2.11	0.35	0.10
Lack-of-fit test				
$F_{calculated}$:$F_{tabulated}$[h]	-	1.48:2.49[h]	0.67:2.49[h]	0.32:2.22[i]
Goodness-of-fit test				
$F_{calculated}$:$F_{tabulated}$	-	0.00:2.49[h]	0.06:2.49[h]	0.11:2.22[i]
Mandel's fitting test				
$F_{calculated}$:$F_{tabulated}$	-	2.50:7.71[j]	0.12:7.71[j]	0.08:5.99[k]

[a] RT – retention time.
[b] λ_{max} – maximum absorbance values obtained in the PDA system detection.
[c] LDR – linear dynamic range.
[d] r^2 – coefficient of determination.
[e] r – coefficient of correlation (Person's r).
[f] LOD – limit of detection.
[g] LOQ – limit of quantification.
[h] $F_{tabulated}$ – numerator degrees of freedom (df1) = 5; denominator degrees of freedom (df2) = 35; p = 95%.
[i] $F_{tabulated}$ – df1 = 7; df2 = 45; p = 95%.
[j] $F_{tabulated}$ – df1 = 1; df2 = 4; p = 95%.
[k] $F_{tabulated}$ – df1 = 1; df2 = 6; p = 95%.

Selectivity was demonstrated by the absence of interfering peaks at the retention times of the target analytes in blank SU. Figure 29 clearly demonstrates the absence of interfering peaks in the retention times demonstrating the ability of the analytical method to unequivocally identify and quantify the analytes of interest.

Table 9 – Precision, accuracy and extraction efficiency obtained for the 11β-prostaglandin $F_{2\alpha}$ ($11\beta PGF_{2\alpha}$), leukotriene E_4 (LTE_4) and leukotriene B_4 (LTB_4) at different levels of concentration.

Analytes	Level of concentration[a] (ng mL^{-1})		Precision (%)		Accuracy (%)	Extraction efficiency
			Intraday repeatability	Intermediate precision		
$11\beta PGF_{2\alpha}$	LL	25	3.17	4.81	93.62	95.52
	ML	100	3.17	3.89	100.50	99.71
	HL	200	3.73	5.40	103.02	98.87
LTE_4	LL	2.5	6.63	10.43	96.73	91.13
	ML	10	4.70	2.86	95.85	83.86
	HL	20	3.11	1.29	103.51	74.99
LTB_4	LL	2.5	6.17	6.54	101.08	84.53
	ML	10	4.82	3.57	96.32	92.25
	HL	20	4.58	3.68	100.04	88.14

[a] Levels of concentration in the validation studies: LL – low level, ML – medium level and HL – high level.

The linearity was assayed using different calibration curves of each analyte obtained by least squares linear regression analysis as described in section *3.1.6 Method validation*. All of three regression equations of the target analytes showed a coefficient of determination higher than 0.9980 but these values alone, do not guarantee the linearity of the signal. Therefore, linearity was assessed by means of the *F*-test at different approaches: lack-of-fit test, based on the analysis of the residual variance; goodness-of-fit test, based on the ratio between the mean sum of squares of the factors and the residuals; and Mandel's fitting test, based on the difference between the residual standard deviation of the obtained first-order calibration model and a potential second-order calibration model, as described by Kruve et al. [139]. The signal is considered linear when the calculated *F*, by means of the experimental data, is lower than the respective tabulated *F*. As show in Table 8, all signal linearities (Lack-of-fit, Godness-of-fit and Mandel's tests) calculated by the previous mentioned approaches, are confirmed ($F_{calculated} < F_{tabulated}$) with a 95% confidence level.

The sensitivity can be described as the lowest value of concentration at which the method is able to detect (LOD) and quantify (LOQ) the target analytes (Table 8) with satisfactory accuracy and precision. As previously reported, the developed method showed low values of LOD which ranged between 0.04 ng mL^{-1} for LTB_4 and 1.12 ng mL^{-1} for $11\beta PGF_{2\alpha}$ while LOQ values ranged between 0.10 ng mL^{-1} for the LTB_4 and 2.11 ng mL^{-1} for $11\beta PGF_{2\alpha}$.

Matrix effect was also evaluated in order to determine which calibration curve should be used during the real sample analysis. The values obtained with this test is represented in Table 8. All the target analytes have revealed considerable matrix effects

with 73% for the 11βPGF$_{2\alpha}$, 76% for the LTE$_4$ and 81% for the LTB$_4$. Consequently, the regression equation obtained with real urine would be used for further real sample analyzes.

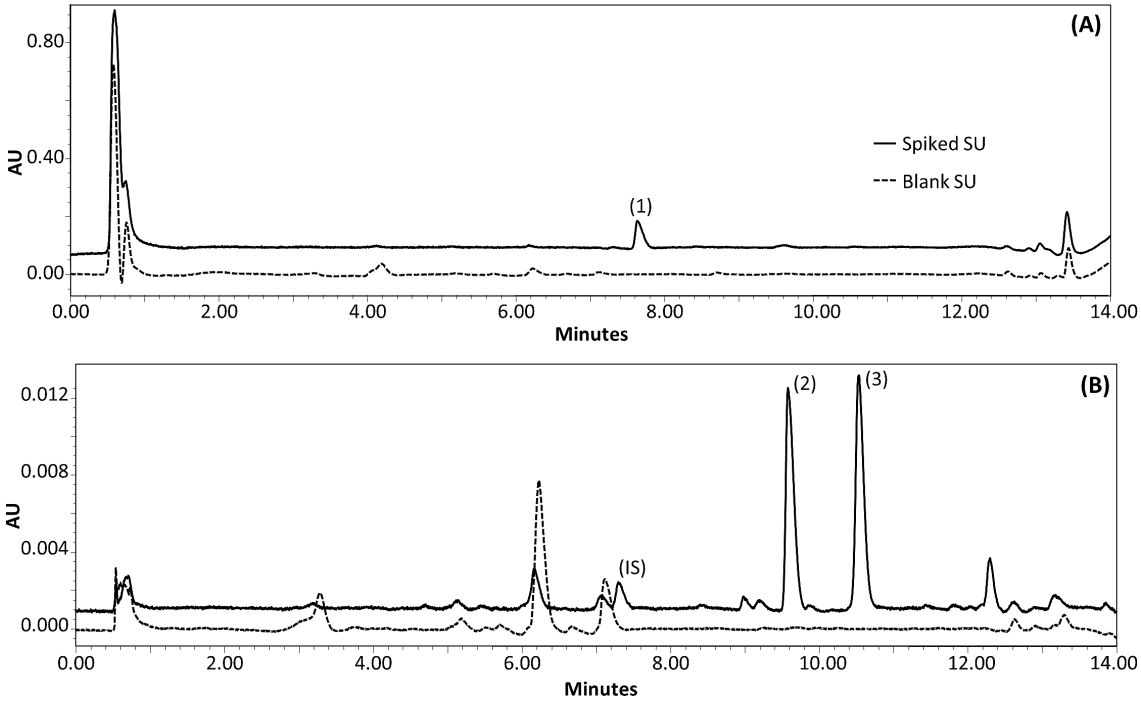


Figure 29 – Representative chromatograms of an unspiked synthetic urine and synthetic urine spiked with the investigated standards: (1) 11β-prostaglandin F$_{2\alpha}$, (2) leukotriene E$_4$ and (3) leukotriene B$_4$ – at the best optimization parameters obtained at (A) 192 nm and (B) 281 nm.

An important parameter when validating a method is precision. This parameter can be defined as repeatability at which the % RSD is calculated in order to assess the error associated between replicates performed in the same day, and as reproducibility at which the % RSD is calculated in order to assess the error associated between replicates performed over a certain period of days. The developed method proved to have repeatability with values that ranged from 3.11% for LTE$_4$ at HL and 6.63% at LL for the same analyte. As for reproducibility, values ranged from 1.29% for LTE$_4$ at HL and 10.43% at LL for the same compound being LTE$_4$ on the extremes of both precision approaches (Table 9).

The performance of the method can also be assayed as accuracy. In general, all experimental values have shown to be accurate (approximately 100%) during the study of this parameter with less than 7% deviation from the tabulated value as shown in Table 9. The least accurate was 11βPGF$_{2\alpha}$ at LL with 93.62%, and LTB$_4$ the most accurate with 100.04%.

The extraction efficiency was evaluated in order to know how much of the target analytes is lost during extractions. The obtained recovery percentage for 11βPGF$_{2\alpha}$ ranged between 95.52% at a LL and 99.71% at a ML; LTE$_4$ ranged between 74.99% and 91.13% at HL and LL respectively; and LTB$_4$ ranged from 84.53% at LL and 92.25% at ML (Table 9).

3.3.5 Stability of the extracts

The stability of the extracts was tested with the target analytes at different concentrations (LL and ML), over a 24-hour period with analyzes running each 8 hours.

It was found out that eluates were stable for 8 hours, with less than 6% lost during the tested time, but after that time, the LTs stability decrease, being the 11βPGF$_{2\alpha}$ the most stable with less than 7% lost during the assay as observed in Figure 30. All the analyzes were performed taking into account the stability of the extracts.

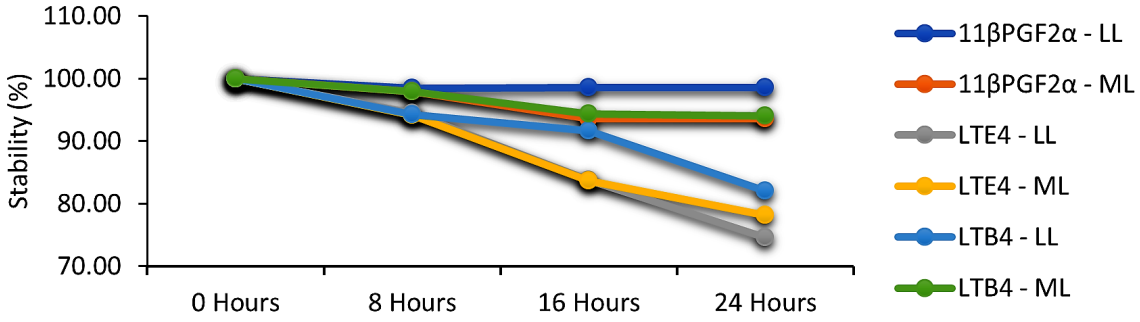


Figure 30 – Stability of extracts at 0, 8, 16 and 24 hours after extraction. Target analytes were at low level (LL) and medium level (ML) of concentration.

3.3.6 Application of the developed MEPS/UHPLC-PDA methodology

In order to evaluate the applicability of the method to urine samples, R-AX sorbent and the developed method proposed in this study were applied to detect the three eicosanoids – 11βPGF$_{2\alpha}$, LTE$_4$ and LTB$_4$, in urine of 27 asthmatic and 17 healthy children. The concentration values obtained for the urine of each asthmatic patient and healthy individual (control) is represented in the Table 2A, *Annex VIII*. Figure 31 represents a chromatogram of an asthmatic patient and another from a normal control. Even though these eicosanoids are at a very low concentration, in a complex matrix, it is clear the difference on peaks intensity between controls and asthmatic groups.

One third of the normal controls had values bellow the limit of quantification or detection, except for the 11βPGF$_{2\alpha}$ that was not detected in 2 asthmatic patients. In both groups, the major analyte was 11βPGF$_{2\alpha}$ with greater values when compared to the two

LTs of this study. 11βPGF$_{2\alpha}$ values varied from bellow the LOD (< LOD) until 310.32 ± 9.59 ng mL^{-1} for the asthmatic patients group, and from 32.16 ± 1.00 until 167.29 ± 17.75 ng mL^{-1} for the normal controls. LTE$_4$ values varied from < LOD until 4.94 ± 0.22 ng mL^{-1} for the asthmatic group, and from 0.47 ± 0.06 until 2.53 ± 0.41 ng mL^{-1} for the normal controls. The trend for the LTB$_4$ was the same with values between < LOD and 2.64 ± 0.05 ng mL^{-1} for asthmatics and < LOD and 1.74 ± 0.05 ng mL^{-1} for normal controls.

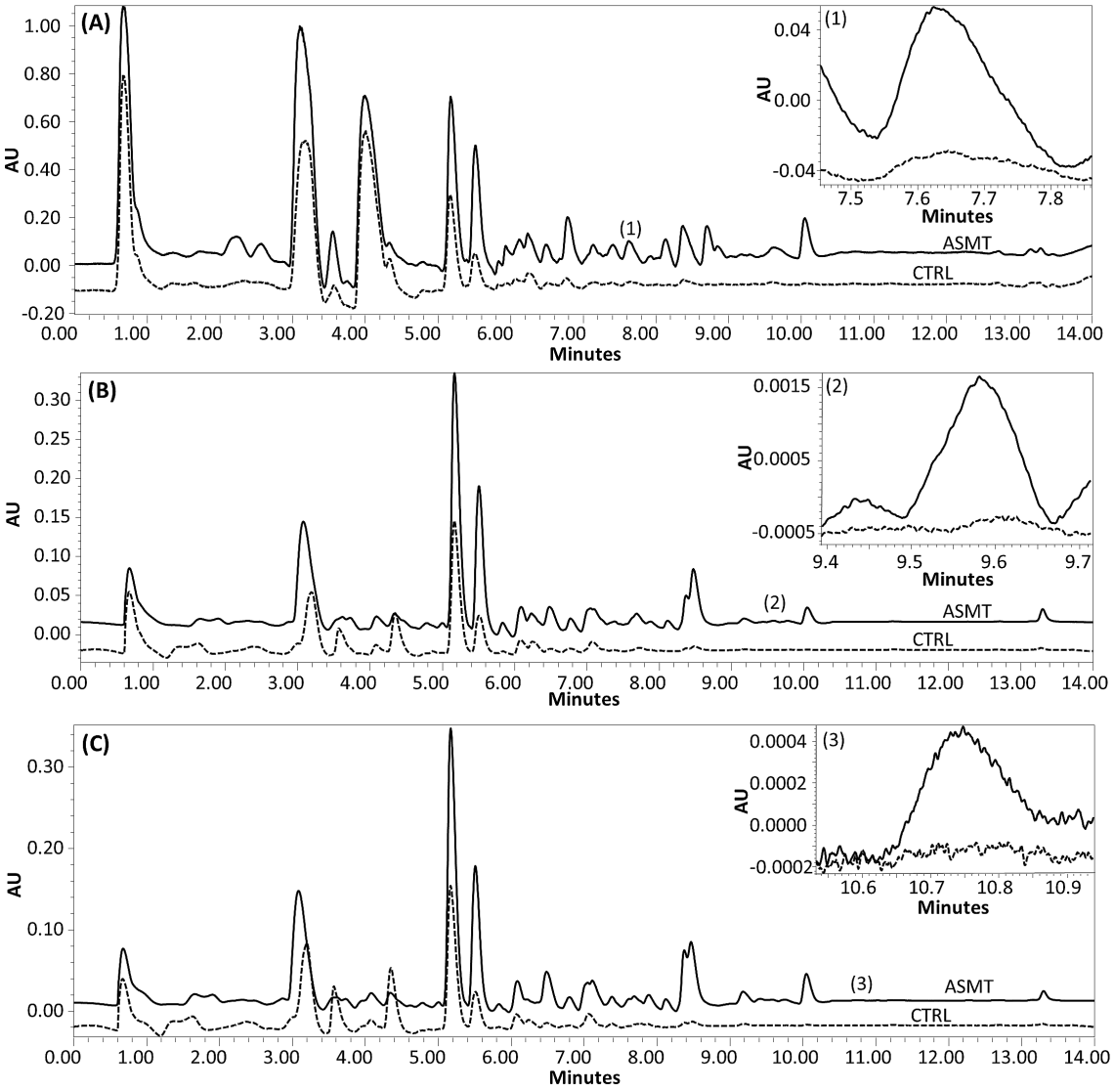


Figure 31 – Representative chromatograms of urine from a healthy individual (CTRL) and urine from an asthmatic patient (ASMT) at (A) 192, (B) 281, and (C) 270 nm. Numbers represent the target analytes: (1) 11β-prostaglandin F$_{2\alpha}$, (2) leukotriene E$_4$ and (3) leukotriene B$_4$. Boxes on the up-right corner of each chromatogram represent the magnification of the target analyte peak.

Before running the *t*-test some specific assumptions were evaluated, including the type of the variables, independence of observations, normal distribution, outliers and homogeneity of variances [215]. The results showed that there are significant differences

between groups. For this procedure, the logarithmic values of concentrations were used in order to obtain values closer to the normal pattern. For the $11\beta PGF_{2\alpha}$, the *t*-test value was 7.415 with 289.6 degrees of freedom (df; $p < 0.001$); the LTE_4 had a *t*-test value of 3.653 (df = 217, $p < 0.001$) and LTB_4 had 5.437 (df = 198, $p < 0.001$). The mean values calculated by means of the *t*-test procedure of each target analyte and investigated group, are shown in Table 2A, *Annex VIII*.

PGD_2 is a major COX metabolite produced during an inflammatory response by mast cells and leukocytes as dendritic cells and Th2 cells with a pro-inflammatory role. This unstable eicosanoid is rapidly transformed into more stable products, PGs of the J series and $11\beta PGF_{2\alpha}$ [77]. As PGD_2, $11\beta PGF_{2\alpha}$ also has biological activity as bronchoconstrictor and contraction of coronary arteries [247], and can be found in increased values in the urine of asthmatic patients after allergen-induced bronchoconstriction [227] and after exercise challenge [81]. On average, the asthmatic patients had higher concentrations of $11\beta PGF_{2\alpha}$ (112.96 ng mL^{-1}) than normal controls (62.56 ng mL^{-1}; Figure 32) with a mean difference of about 50.40 ng mL^{-1} between groups. This significant difference ($p < 0.001$) represent a medium-sized effect ($r = 0.40$). The difference among the total population was between 35.97 and 64.83 ng mL^{-1} for this eicosanoid, with a confidence level of 95%.

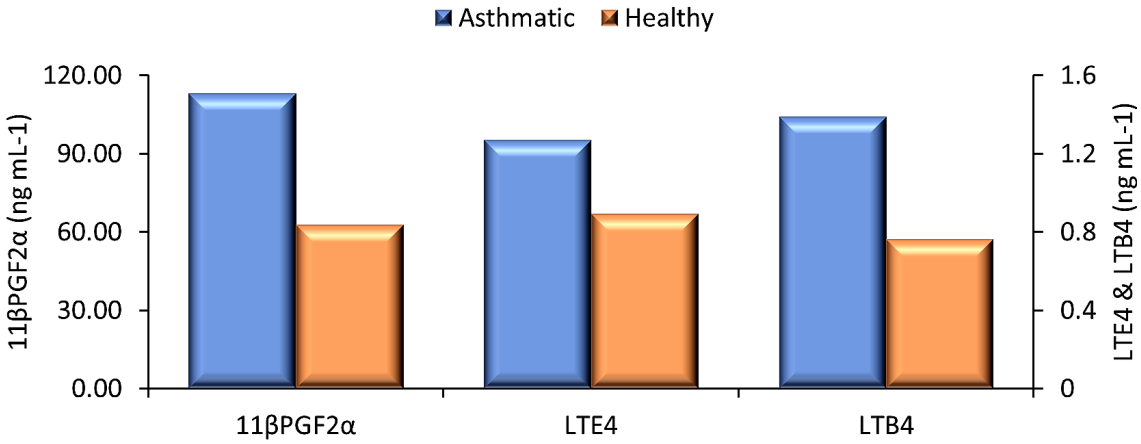


Figure 32 – 11β-prostaglandin $F_{2\alpha}$ ($11\beta PGF_{2\alpha}$), leukotriene E_4 (LTE_4) and leukotriene B_4 (LTB_4) mean concentrations obtained for the urine of asthmatic patients and urine of healthy individuals.

Cys-LTs, powerful bronchoconstrictors, 100 to 1000 times more powerful than histamine [59], play an important role in the pathophysiology of asthma. They also have a role in the vascular permeability allowing the exudation of inflammatory cells from plasma to the airway mucosa, stimulate mucus secretion and inhibit mucociliar clearance – characteristics of the pathophysiology of asthma [59]. Since LTC_4 and LTD_4 are

biologically converted into LTE_4, they are not excreted in measurable concentrations in urine. Therefore, LTE_4 is considered a biomarker of the total of cys-LTs levels in urine [72]. Studies suggest that LTE_4 is dependent on allergen dose inhaled during allergen challenge in asthmatic patients [66]. Having this into account, in the evaluation of the groups present in this study, it was found increased mean levels of LTE_4 in the urine of asthmatic patients with 1.27 ng mL^{-1} against 0.89 ng mL^{-1} in normal controls (Figure 32) with a mean difference of 0.38 ng mL^{-1}. This difference was significant ($p < 0.001$) and represented a small-sized effect ($r = 0.24$). Regarding the total population, the difference was of 0.11-0.64 ng mL^{-1} with a confidence level of 95%.

LTB_4 is produced by a diversity of cells including neutrophils and macrophages and it is a powerful pro-inflammatory chemoattractant whose main targets are neutrophils promoting their activation, adhesion to the endothelium and chemotaxis. This LT also activates neutrophils degranulation of additional mediators and it is also involved in inflammatory pain by neutrophils dependent processes [59]. LTB_4 tend to be in higher concentrations in diverse matrices like EBC [248], sputum [249] and urine [246]. The asthmatic patients of our investigations had a trend for higher values of LTB_4 with a mean concentration of 1.39 ng mL^{-1} in the urine of asthmatics patients against 0.76 ng mL^{-1} in the normal controls (Figure 32; mean difference = 0.63 ng mL^{-1}). This difference was significant ($p < 0.001$) and represented a medium-sized effect ($r = 0.36$). In terms of total population, the difference varied between 0.47 and 0.78 ng mL^{-1} with a confidence level of 95%.

Chapter IV – Conclusions and future perspectives

Throughout this work, it was developed a powerful methodology able to simultaneously detect and quantify eicosanoid asthma biomarkers present in urine of asthmatic patients and healthy individuals.

During the development of the method, eleven commercial sorbents with different chemical properties were evaluated with SU. The number of sample loading cycles as well as their mode (extract-discard and extract-eject) were tested. Three different sample pHs were studied (acid pH, physiological pH and basic pH) with two ion exchange sorbents (R-AX and R-CX). The elution was studied in terms of solvents, based on ACN and MeOH (pure and acidified), and volume. The conditioning solvent (MeOH and ACN) was also evaluated and optimized in terms of extraction efficiency. In addition, the influence of eluate concentration under N_2 stream was investigated. The best extraction conditions for the targeted analytes were obtained using R-AX as sorbent, 3 conditioning-equilibration cycles with 250 µL of ACN-H_2O at 0.1% FA, 10 extract-discard cycles of 250 µL of sample at a pH of 5.1, an elution with 2 times 50 µL of MeOH and the concentration of the eluate until half of initial volume.

In terms of chromatographic conditions, a set of five commercial capillary columns with different dimensions and stationary phases were evaluated and optimized. BEH C18 proved to be the best column in terms of peak resolution, shape and measurable areas for the targeted analytes. The gradient and flow rate of mobile phase were also evaluated. The optimized gradient of mobile phase, for both SU and real urine, was obtained with 0.1% FA and ACN acidified with FA (0.1%) with a run time of 14 min at a flow rate of 300 µL min^{-1} followed by a re-equilibration of 2 min.

The validation of the method revealed compatible values for each parameter in SU. The selectivity evaluation reveled no interfering peaks at the retention times of the target analytes. All signals proved to be linear by means of the three different fitting tests (lack-of-fit, goodness-of-fit and Mandel's fitting tests). The instrumental sensitivity showed low values of LOD (0.04 ng mL^{-1} for LTB_4 and 1.12 ng mL^{-1} for $11\beta PGF_{2\alpha}$) and LOQ (0.10 ng mL^{-1} for the LTB_4 and 2.11 ng mL^{-1} for $11\beta PGF_{2\alpha}$). The developed method revealed to be precise in terms of repeatability (3.11% for LTE_4 at HL and 6.63% at LL for the same analyte) and reproducibility (1.29% for LTE_4 at HL and 10.43% at LL for the same analyte); accurate, with less than 7% of deviation from the tabulated value; and efficient (> 74.99%). All targeted analytes revealed matrix effect. The extracts were

analyzed within a period of 8 hours since the stability test revealed they were stable for this period of time.

Therefore, it was possible to successfully apply the method to the samples and discriminate between asthmatics and healthy individuals. On average, the urine of asthmatic patients presented significantly higher concentrations of all eicosanoids. The obtained values for the 11β$PGF_{2\alpha}$ and LTB_4 were about 1.8 times higher and for the LTE_4 were about 1.4 times higher in asthmatic patients than in healthy individuals.

The developed MEPS/UHPLC-PDA approach proved to be an ultra-fast, accurate, precise and easy analytical strategy. The extraction procedure is fast (a total analysis time of 31 min). Furthermore, The MEPS/UHPLC-PDA procedure revealed to be environmental friendly due to the reduce levels of solvents consumed during both extraction and analytical procedures.

The developed MEPS/UHPLC-PDA method reveled to be a promising strategy regarding the study of these and other eicosanoids present in other biological matrices such as plasma, sputum and EBC of patients from inflammatory illnesses other than asthma.

The results obtained during the experimental work suggest the potential of these eicosanoids on asthma diagnosis. However, a larger and more extensive study would be necessary, using a bigger number of cases, to confirm the data obtained, guarantee a greater robustness to the approach and to obtain a stratification of the patients in terms of age, level of control (well-controlled, partly controlled and uncontrolled) and their therapeutic response using the developed method.

In the future, I would also be interested in learning other extractive or chromatographic techniques as well as I would like to continue in the health field studying other diseases.

References

1. Asher I, Pearce N. Global burden of asthma among children. Int J Tuberc Lung Dis 2014;18:1269-1278.

2. Global Asthma Network. The global asthma report 2014. 2014. Accessed on February 2016. available from: www.globalasthmanetwork.org.

3. Lai CKW, Beasley R, Crane J, Foliaki S, et al. Global variation in the prevalence and severity of asthma symptoms: Phase three of the international study of asthma and allergies in childhood (ISAAC). Thorax 2009;64:476-483.

4. Mallol J, Crane J, von Mutius E, Odhiambo J, et al. The international study of asthma and allergies in childhood (ISAAC) phase three: a global synthesis. Allergol Immunopathol (Madr) 2013;41:73-85.

5. Masoli M, Fabian D, Holt S, Beasley R, et al. The global burden of asthma: executive summary of the GINA Dissemination Committee Report. Allergy 2004;59:469-478.

6. Soriano JB, Abajobir AA, Abate KH, Abera SF, et al. Global, regional, and national deaths, prevalence, disability-adjusted life years, and years lived with disability for chronic obstructive pulmonary disease and asthma, 1990–2015: a systematic analysis for the Global Burden of Disease Study 2015. Lancet Respir Med 2017;5:691-706.

7. Jarvis D, Newson R, Lotvall J, Hastan D, et al. Asthma in adults and its association with chronic rhinosinusitis: the GA2LEN survey in Europe. Allergy 2012;67:91-98.

8. Sa-Sousa A, Morais-Almeida M, Azevedo L, Carvalho R, et al. Prevalence of asthma in Portugal - The Portuguese National Asthma Survey. Clin Transl Allergy 2012;2:15.

9. Pinto JR. ISAAC - 20 anos em Portugal. Acta Pediatr Port 2011;S35-S40.

10. Bahadori K, Doyle-Waters MM, Marra C, Lynd L, et al. Economic burden of asthma: a systematic review. BMC Pulm Med 2009;9:24.

11. Loftus PA, Wise SK. Epidemiology and economic burden of asthma. Int Forum Allergy Rhinol 2015;5 Suppl 1:S7-10.

12. Lötvall J, Akdis CA, Bacharier LB, Bjermer L, et al. Asthma endotypes: A new approach to classification of disease entities within the asthma syndrome. J Allergy Clin Immunol 2011;127:355-360.

13. Hargreave FE. Asthma is not a syndrome. J Allergy Clin Immunol 2011;128:904.

14. Yang C-W, Hojer Caroline D, Zhou M, Wu X, et al. Regulation of T cell receptor signaling by DENND1B in TH2 cells and allergic disease. Cell 2016;164:141-155.

15. Moffatt MF, Kabesch M, Liang L, Dixon AL, et al. Genetic variants regulating ORMDL3 expression contribute to the risk of childhood asthma. Nature 2007;448:470-473.

16. National Heart, Lung, and Blood Institute. National asthma education and prevention program. Expert panel report 3: guidelines for the diagnosis and management of asthma - full report 2007. 2007. Accessed on February 2016. available from: http://www.nhlbi.nih.gov/health-pro/guidelines/current/asthma-guidelines.

17. Osman M, Tagiyeva N, Wassall HJ, Ninan TK, et al. Changing trends in sex specific prevalence rates for childhood asthma, eczema, and hay fever. Pediatr Pulmonol 2007;42:60-65.

18. Farrokhi S, Gheybi MK, Movahed A, Tahmasebi R, et al. Common aeroallergens in patients with asthma and allergic rhinitis living in southwestern part of iran: based on skin prick test reactivity. Iran J Allergy Asthma Immunol 2015;6.

19. Darveaux JI, Lemanske Jr RF. Infection-related asthma. J Allergy Clin Immunol Pract 2014;2:658-663.

20. Zhou C, Baïz N, Banerjee S, Charpin DA, et al. The relationships between ambient air pollutants and childhood asthma and eczema are modified by emotion and conduct problems. Ann Epidemiol 2013;23:778-783.e773.

21. Allan K, Devereux G. Diet and Asthma: Nutrition implications from prevention to treatment. J Am Diet Assoc 2011;111:258-268.

22. Olin JT. Exercise-Induced Asthma: Strategies to improve performance. In: Leung DYM, Szefler SJ, Bonilla FA, Akdis CA and Sampson HA, editors. Pediatric allergy: principles and practice (Third Edition). London: Elsevier, 2016. p. 336-342.e2.

23. Villeneuve PJ, Leech J, Bourque D. Frequency of emergency room visits for childhood asthma in Ottawa, Canada: the role of weather. Int J Biometeorol 2005;50:48-56.

24. Global Initiative for Asthma. Global strategy for asthma management and prevention. 2017. Accessed on December 2017. available from: http://www.ginasthma.org/.

25. Lockey RF. Asthma Phenotypes: An approach to the diagnosis and treatment of asthma. J Allergy Clin Immunol Pract 2014;2:682-685.

26. Bacharier LB, Boner A, Carlsen KH, Eigenmann PA, et al. Diagnosis and treatment of asthma in childhood: a PRACTALL consensus report. Allergy 2008;63:5-34.

27. Magnan A, Botturi K, Pipet A, Cavaillès A, et al. Asthma exacerbations: a paradigm of synergy between allergens, pollutants and viruses. Allergens and respiratory pollutants. Biohealthcare Publishing (Oxford) Ltd. Marc A. Williams, 2011. p. 89-116.

28. Rogers DF. Airway goblet cell hyperplasia in asthma: hypersecretory and anti-inflammatory? Clin Exp Allergy 2002;32:1124-1127.

29. Rogers DF. Airway mucus hypersecretion in asthma and COPD: not the same?. In: Barnes PJ, Drazen JM, Rennard SI, Thomson NC, editors. Asthma and COPD: basic mechanisms and clinical management. USA: Elsevier, 2009. p. 211-223.

30. Papadopoulos NG, Arakawa H, Carlsen KH, Custovic A, et al. International consensus on (ICON) pediatric asthma. Allergy 2012;67:976-997.

31. Jeffery P, Holgate S, Wenzel S. Methods for the assessment of endobronchial biopsies in clinical research: application to studies of pathogenesis and the effects of treatment. Am J Respir Crit Care Med 2003;168:S1-17.

32. Global Initiative for Asthma. Global Strategy for Asthma Management and Prevention. 2006. Accessed on February 2016. available from: http://www.ginasthma.org/.

33. Rackemann FM. A working classification of asthma. Am J Med 1947;3:601-606.

34. Nieves A, Magnan A, Boniface S, Proudhon H, et al. Phenotypes of asthma revisited upon the presence of atopy. Respir Med 2005;99:347-354.

35. Humbert M, Menz G, Ying S, Corrigan CJ, et al. The immunopathology of extrinsic (atopic) and intrinsic (non-atopic) asthma: more similarities than differences. Immunol Today 1999;20:528-533.

36. de Blic J, Tillie-Leblond I, Tonnel AB, Jaubert F, et al. Difficult asthma in children: an analysis of airway inflammation. J Allergy Clin Immunol 2004;113:94-100.

37. Lin T-Y, Poon AH, Hamid Q. Asthma phenotypes and endotypes. Curr Opin Pulm Med 2013;19:18-23.

38. Anderson GP. Endotyping asthma: new insights into key pathogenic mechanisms in a complex, heterogeneous disease. Lancet 2008;372:1107-1119.

39. Agache I, Akdis C, Jutel M, Virchow JC. Untangling asthma phenotypes and endotypes. Allergy 2012;67:835-846.

40. Murdoch JR, Lloyd CM. Chronic inflammation and asthma. Mutat Res-Fund Mol 2010;690:24-39.

41. Janeway CA, Jr., Medzhitov R. Innate immune recognition. Annu Rev Immunol 2002;20:197-216.

42. Janeway CA, Jr., Bottomly K. Signals and signs for lymphocyte responses. Cell 1994;76:275-285.

43. Baky AA. Inflammation in asthma. Egypt J Pediatr Allergy Immunol 2003;1:68-70.

44. Todo-Bom A, Mota-Pinto A. Fisiopatologia da asma grave. Rev Port Imunoalergologia 2006;14:43-48.

45. Taher YA, Henricks PA, van Oosterhout AJ. Allergen-specific subcutaneous immunotherapy in allergic asthma: immunologic mechanisms and improvement. Libyan J Med 2010;5.

46. Holgate ST, Sly PD. 50 - Asthma pathogenesis. In: Adkinson NF, Bochner BS, Burks AW, Busse WW, Holgate ST, Lemanske RF, O'Hehir RE, editors. Middleton's allergy: principles and practice (Vol. 1). London: Elsevier Health Sciences, 2014. p. 812-841.

47. Valenta R. The future of antigen-specific immunotherapy of allergy. Nat Rev Immunol 2002;2:446.

48. Shi HZ. Eosinophils function as antigen-presenting cells. J Leukoc Biol 2004;76:520-527.

49. Lamkhioued B, Gounni AS, Aldebert D, Delaporte E, et al. Synthesis of yype 1 (IFNyγ) and type 2 (IL-4, IL-5, and IL-10) cytokines by human eosinophils. Ann NY Acad Sci 1996;796:203-208.

50. Ying S, Khan LN, Meng Q, Barnes NC, et al. Cyclosporin A, apoptosis of BAL T-cells and expression of Bcl-2 in asthmatics. Eur Respir J 2003;22:207-212.

51. Kuhn H, Borchert A. Regulation of enzymatic lipid peroxidation: the interplay of peroxidizing and peroxide reducing enzymes. Free Radic Biol Med 2002;33:154-172.

52. Brink C, Dahlen SE, Drazen J, Evans JF, et al. International union of pharmacology XXXVII. Nomenclature for leukotriene and lipoxin receptors. Pharmacol Rev 2003;55:195-227.

53. Simmons DL, Botting RM, Hla T. Cyclooxygenase isozymes: the biology of prostaglandin synthesis and inhibition. Pharmacol Rev 2004;56:387-437.

54. Smith WL, Murphy RC. Chapter 9 - The Eicosanoids: Cyclooxygenase, Lipoxygenase and Epoxygenase Pathways A2 - Ridgway, Neale D. in: RS McLeod (Ed.) Biochemistry of Lipids, Lipoproteins and Membranes (Sixth Edition). Boston: Elsevier, 2016. p. 259-296.

55. Spector AA. Arachidonic acid cytochrome P450 epoxygenase pathway. J Lipid Res 2009;50:S52-S56.

56. Basu S. The enigma of in vivo oxidative stress assessment: isoprostanes as an emerging target. Scand J Food Nutr 2007;51:48-61.

57. Korotkova M, Lundberg IE. The skeletal muscle arachidonic acid cascade in health and inflammatory disease. Nat Rev Rheumatol 2014;10:295-303.

58. Hata AN, Breyer RM. Pharmacology and signaling of prostaglandin receptors: multiple roles in inflammation and immune modulation. Pharmacol Ther 2004;103:147-166.

59. Sharma JN, Mohammed LA. The role of leukotrienes in the pathophysiology of inflammatory disorders: is there a case for revisiting leukotrienes as therapeutic targets?. Inflammopharmacology 2006;14:10-16.

60. Seki K, Hisada T, Kawata T, Kamide Y, et al. Oxidative stress potentially enhances FcεRI-mediated leukotriene C4 release dependent on the late-phase increase of intracellular glutathione in mast cells. Biochem Biophys Res Commun 2013;439:357-362.

61. Perestrelo R, Silva CL, Camara JS. Determination of urinary levels of leukotriene B(4) using ad highly specific and sensitive methodology based on automatic MEPS combined with UHPLC-PDA analysis. Talanta 2015;144:382-389.

62. Jian W, Edom RW, Xue X, Huang M-Q, et al. Quantitation of leukotriene B4 in human sputum as a biomarker using UPLC–MS/MS. J Chromatogr B 2013;932:59-65.

63. Trischler J, Müller C-M, Könitzer S, Prell E, et al. Elevated exhaled leukotriene B4 in the small airway compartment in children with asthma. Ann Allergy Asthma Immunol 2015;114:111-116.

64. Zanconato S, Carraro S, Corradi M, Alinovi R, et al. Leukotrienes and 8-isoprostane in exhaled breath condensate of children with stable and unstable asthma. J Allergy Clin Immunol 2004;113:257-263.

65. Pavord ID, Ward R, Woltmann G, Wardlaw AJ, et al. Induced sputum eicosanoid concentrations in asthma. Am J Respir Crit Care Med 1999;160:1905-1909.

66. Bancalari L, Conti I, Giannessi D, Lazzerini G, et al. Early increase in urinary leukotriene E4 (LTE4) is dependent on allergen dose inhaled during bronchial challenge in asthmatic subjects. Allergy 1999;54:1278-1285.

67. O'Byrne PM, Gauvreau GM, Murphy DM. Efficacy of leukotriene receptor antagonists and synthesis inhibitors in asthma. J Allergy Clin Immunol 2009;124:397-403.

68. Capra V, Thompson MD, Sala A, Cole DE, et al. Cysteinyl-leukotrienes and their receptors in asthma and other inflammatory diseases: critical update and emerging trends. Med Res Rev 2007;27:469-527.

69. Bisgaard H. Pathophysiology of the cysteinyl leukotrienes and effects of leukotriene receptor antagonists in asthma. Allergy 2001;56 Suppl 66:7-11.

70. Shin TR, Kim JH, Kim CH, Hyun IG, et al. Urinary excretion of 9alpha,11beta-prostaglandin F2 and leukotriene E4 in patients with exercise-induced bronchoconstriction. J Thorac Dis 2015;7:1198-1204.

71. Rabinovitch N. Urinary Leukotriene E4. Immunol Allergy Clin North Am 2007;27:651-664.

72. Rabinovitch N. Urinary leukotriene E4 as a biomarker of exposure, susceptibility and risk in asthma. Immunol Allergy Clin North Am 2012;32:433-445.

73. Samitas K, Chorianopoulos D, Vittorakis S, Zervas E, et al. Exhaled cysteinyl-leukotrienes and 8-isoprostane in patients with asthma and their relation to clinical severity. Respir Med 2009;103:750-756.

74. Christie PE, Tagari P, Ford-Hutchinson AW, Black C, et al. Increased urinary LTE4 excretion following inhalation of LTC4 and LTE4 in asthmatic subjects. Eur Respir J 1994;7:907-913.

75. Dal Negro RW, Visconti M, Micheletto C, Tognella S, et al. Reference urinary LTE4 levels in normal individuals: a pilot study. Eur Ann Allergy Clin Immunol 2011;43:22-28.

76. Kalayci O, Birben E, Sackesen C, Keskin O, et al. ALOX5 promoter genotype, asthma severity and LTC production by eosinophils. Allergy 2006;61:97-103.

77. Ricciotti E, FitzGerald GA. Prostaglandins and Inflammation. Arterioscler Thromb Vasc Biol 2011;31:986-1000.

78. Chappell GP, Xiao X, Pica-Mendez A, Varnell T, et al. Quantitative measurement of cysteinyl leukotrienes and leukotriene B4 in human sputum using ultra high pressure liquid chromatography–tandem mass spectrometry. J Chromatogr B 2011;879:277-284.

79. O'Sullivan S, Dahlén B, Dahlén S-E, Kumlin M. Increased urinary excretion of the prostaglandin D2 metabolite 9α,11β-prostaglandin F2 after aspirin challenge supports mast cell activation in aspirin-induced airway obstruction. J Allergy Clin Immunol 1996;98:421-432.

80. Bochenek G, Nagraba K, Nizankowska E, Szczeklik A. A controlled study of 9alpha,11beta-PGF2 (a prostaglandin D2 metabolite) in plasma and urine of patients with bronchial asthma and healthy controls after aspirin challenge. J Allergy Clin Immunol 2003;111:743-749.

81. Baek H-S, Choi J-H, Oh J-W, Lee H-B. Leptin and urinary leukotriene E4 and 9α,11β-prostaglandin F2 release after exercise challenge. Ann Allergy Asthma Immunol 2013;111:112-117.

82. Cap P, Chladek J, Pehal F, Maly M, et al. Gas chromatography/mass spectrometry analysis of exhaled leukotrienes in asthmatic patients. Thorax 2004;59:465-470.

83. Chen LC, Tseng HM, Kuo ML, Chiu CY. A composite of exhaled LTB4 , LXA4 , FeNO, and FEV1 as an "asthma classification ratio" characterizes childhood asthma. Allergy 2018;73:627-634.

84. Ugajin T, Satoh T, Kanamori T, Aritake K, et al. FcεRI, but Not FcγR, signals induce prostaglandin D2 and E2 production from basophils. Am J Pathol 2011;179:775-782.

85. Dahlén S, Kumlin M. Monitoring mast cell activation by prostaglandin D2 in vivo. Thorax 2004;59:453-455.

86. Fitzpatrick AM. Biomarkers of asthma and allergic airway diseases. Ann Allergy Asthma Immunol 2015;115:335-340.

87. Wood LG, Gibson PG, Garg ML. Biomarkers of lipid peroxidation, airway inflammation and asthma. Eur Respir J 2003;21:177-186.

88. Voynow JA, Kummarapurugu A. Isoprostanes and asthma. Biochim Biophys Acta 2011;1810:1091-1095.

89. Riedl MA, Nel AE. Importance of oxidative stress in the pathogenesis and treatment of asthma. Curr Opin Allergy Clin Immunol 2008;8:49-56.

90. Cho H-Y, Jedlicka AE, Reddy SPM, Kensler TW, et al. Role of NRF2 in protection against hyperoxic lung injury in mice. Am J Respir Cell Mol Biol 2002;26:175-182.

91. Sahiner UM, Birben E, Erzurum S, Sackesen C, et al. Oxidative stress in asthma. World Allergy Organ J 2011;4:1-8.

92. Heffner JE, Repine JE. Pulmonary strategies of antioxidant defense. Am Rev Respir Dis 1989;140:531-554.

93. Sackesen C, Ercan H, Dizdar E, Soyer O, et al. A comprehensive evaluation of the enzymatic and nonenzymatic antioxidant systems in childhood asthma. J Allergy Clin Immunol 2008;122:78-85.

94. Comhair SA, Erzurum SC. Redox control of asthma: molecular mechanisms and therapeutic opportunities. Antioxid Redox Signal 2010;12:93-124.

95. Barnes PJ. Reactive oxygen species and airway inflammation. Free Radic Biol Med 1990;9:235-243.

96. Comhair SA, Erzurum SC. Antioxidant responses to oxidant-mediated lung diseases. Am J Physiol Lung Cell Mol Physiol 2002;283:L246-255.

97. Eiserich JP, Hristova M, Cross CE, Jones AD, et al. Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. Nature 1998;391:393-397.

98. Van Der Vliet A, Eiserich JP, Halliwell B, Cross CE. Formation of reactive nitrogen species during peroxidase-catalyzed oxidation of nitrite a potential additional mechanism of nitric oxide-dependent toxicity. J Biol Chem 1997;272:7617-7625.

99. Moncada S, Higgs A. The L-arginine-nitric oxide pathway. N Engl J Med 1993;329:2002-2012.

100. Barnes PJ, Dweik RA, Gelb AF, Gibson PG, et al. Exhaled nitric oxide in pulmonary diseases: a comprehensive review. Chest 2010;138:682-692.

101. Zuo L, Koozechian MS, Chen LL. Characterization of reactive nitrogen species in allergic asthma. Ann Allergy Asthma Immunol 2014;112:18-22.

102. Guo FH, Comhair SAA, Zheng S, Dweik RA, et al. Molecular mechanisms of increased nitric oxide (NO) in asthma: evidence for transcriptional and post-translational regulation of NO synthesis. J Immunol 2000;164:5970-5980.

103. Hollá LI, Bučková D, Kuhrová V, Stejskalová A, et al. Prevalence of endothelial nitric oxide synthase gene polymorphisms in patients with atopic asthma. Clin Exp Allergy 2002;32:1193-1198.

104. Maarsingh H, Leusink J, Bos IST, Zaagsma J, et al. Arginase strongly impairs neuronal nitric oxide-mediated airway smooth muscle relaxation in allergic asthma. Respir Res 2006;7:6.

105. Saleh D, Ernst P, Lim S, Barnes PJ, et al. Increased formation of the potent oxidant peroxynitrite in the airways of asthmatic patients is associated with induction of nitric oxide synthase: effect of inhaled glucocorticoid. FASEB J 1998;12:929-937.

106. Ghosh S, Erzurum SC. Nitric oxide metabolism in asthma pathophysiology. BBA-Gen Subjects 2011;1810:1008-1016.

107. Dröge W. Free radicals in the physiological control of cell function. Physiol Rev 2002;82:47-95.

108. Iijima H, Duguet A, Eum S-Y, Hamid Q, et al. Nitric oxide and protein nitration are eosinophil dependent in allergen-challenged mice. Am J Respir Crit Care Med 2001;163:1233-1240.

109. Osoata GO, Hanazawa T, Brindicci C, Ito M, et al. Peroxynitrite elevation in exhaled breath condensate of COPD and its inhibition by fudosteine. Chest 2009;135:1513-1520.

110. Miekisch W, Schubert JK, Noeldge-Schomburg GFE. Diagnostic potential of breath analysis—focus on volatile organic compounds. Clin Chim Acta 2004;347:25-39.

111. Montuschi P, Corradi M, Ciabattoni G, Nightingale J, et al. Increased 8-isoprostane, a marker of oxidative stress, in exhaled condensate of asthma patients. Am J Respir Crit Care Med 1999;160:216-220.

112. Wu W, Samoszuk MK, Comhair SA, Thomassen MJ, et al. Eosinophils generate brominating oxidants in allergen-induced asthma. J Clin Invest 2000;105:1455-1463.

113. Ercan H, Birben E, Dizdar EA, Keskin O, et al. Oxidative stress and genetic and epidemiologic determinants of oxidant injury in childhood asthma. J Allergy Clin Immunol 2006;118:1097-1104.

114. Li N, Sioutas C, Cho A, Schmitz D, et al. Ultrafine particulate pollutants induce oxidative stress and mitochondrial damage. Environ Health Perspect 2003;111:455-460.

115. Tredaniel J, Boffetta P, Saracci R, Hirsch A. Exposure to environmental tobacco smoke and adult non-neoplastic respiratory diseases. Eur Respir J 1994;7:173-185.

116. Gutteridge JM. Lipid peroxidation and antioxidants as biomarkers of tissue damage. Clin Chem 1995;41:1819-1828.

117. Morrow JD, Awad JA, Boss HJ, Blair IA, et al. Non-cyclooxygenase-derived prostanoids (F2-isoprostanes) are formed in situ on phospholipids. Proc Natl Acad Sci U S A 1992;89:10721-10725.

118. Baraldi E, Carraro S, Alinovi R, Pesci A, et al. Cysteinyl leukotrienes and 8-isoprostane in exhaled breath condensate of children with asthma exacerbations. Thorax 2003;58:505-509.

119. Adamko DJ, Sykes BD, Rowe BH. The metabolomics of asthma: novel diagnostic potential. Chest 2012;141:1295-1302.

120. Johnson CH, Ivanisevic J, Siuzdak G. Metabolomics: beyond biomarkers and towards mechanisms. Nat Rev Mol Cell Biol 2016;17:451-459.

121. Gomase VS, Changbhale SS, Patil SA, Kale KV. Metabolomics. Curr Drug Metab 2008;9:89-98.

122. Carraro S, Giordano G, Reniero F, Perilongo G, et al. Metabolomics: a new frontier for research in pediatrics. J Pediatr 2009;154:638-644.

123. Ryan D, Robards K. Metabolomics: The greatest omics of them all? Anal Chem 2006;78:7954-7958.

124. Villasenor A, Rosace D, Obeso D, Perez-Gordo M, et al. Allergic asthma: an overview of metabolomic strategies leading to the identification of biomarkers in the field. Clin Exp Allergy 2017;47:442-456.

125. Wolfender J-L, Marti G, Thomas A, Bertrand S. Current approaches and challenges for the metabolite profiling of complex natural extracts. J Chromatogr A 2015;1382:136-164.

126. Khamis MM, Adamko DJ, El-Aneed A. Mass spectrometric based approaches in urine metabolomics and biomarker discovery. Mass Spectrom Rev 2017;36:115-134.

127. Judenhofer MS, Wehrl HF, Newport DF, Catana C, et al. Simultaneous PET-MRI: a new approach for functional and morphological imaging. Nat Med 2008;14:459-465.

128. Miura D, Fujimura Y, Wariishi H. In situ metabolomic mass spectrometry imaging: Recent advances and difficulties. J Proteomics 2012;75:5052-5060.

129. Andrew Clayton T, Lindon JC, Cloarec O, Antti H, et al. Pharmaco-metabonomic phenotyping and personalized drug treatment. Nature 2006;440:1073.

130. Fens N, Douma RA, Sterk PJ, Kamphuisen PW. Breathomics as a diagnostic tool for pulmonary embolism. J Thromb Haemost 2010;8:2831-2833.

131. Caldeira M, Barros AS, Bilelo MJ, Parada A, et al. Profiling allergic asthma volatile metabolic patterns using a headspace-solid phase microextraction/gas chromatography based methodology. J Chromatogr A 2011;1218:3771-3780.

132. Caldeira M, Perestrelo R, Barros AS, Bilelo MJ, et al. Allergic asthma exhaled breath metabolome: A challenge for comprehensive two-dimensional gas chromatography. J Chromatogr A 2012;1254:87-97.

133. Schmidt K, Podmore I. Current challenges in volatile organic compounds analysis as potential biomarkers of cancer. J Biomark 2015;2015:16.

134. Turi KN, Romick-Rosendale L, Ryckman KK, Hartert TV. A review of metabolomics approaches and their application in identifying causal pathways of childhood asthma. J Allergy Clin Immunol 2017.

135. Kohler I, Verhoeven A, Derks RJ, Giera M. Analytical pitfalls and challenges in clinical metabolomics. Bioanalysis 2016;8:1509-1532.

136. Rotter M, Brandmaier S, Prehn C, Adam J, et al. Stability of targeted metabolite profiles of urine samples under different storage conditions. Metabolomics 2017;13:4.

137. Sahu PK, Ramisetti NR, Cecchi T, Swain S, et al. An overview of experimental designs in HPLC method development and validation. J Pharm Biomed Anal 2018;147:590-611.

138. Kruve A, Rebane R, Kipper K, Oldekop M-L, et al. Tutorial review on validation of liquid chromatography–mass spectrometry methods: Part I. Anal Chim Acta 2015;870:29-44.

139. Kruve A, Rebane R, Kipper K, Oldekop M-L, et al. Tutorial review on validation of liquid chromatography–mass spectrometry methods: Part II. Anal Chim Acta 2015;870:8-28.

140. Ren S, Hinzman AA, Kang EL, Szczesniak RD, et al. Computational and statistical analysis of metabolomics data. Metabolomics 2015;11:1492-1513.

141. Mayeux R. Biomarkers: potential uses and limitations. NeuroRx 2004;1:182-188.

142. Agache I, Akdis CA. Endotypes of allergic diseases and asthma: an important step in building blocks for the future of precision medicine. Allergol Int 2016;65:243-252.

143. Vijverberg SJ, Hilvering B, Raaijmakers JA, Lammers JW, et al. Clinical utility of asthma biomarkers: from bench to bedside. Biologics 2013;7:199-210.

144. Zhang A, Sun H, Wang P, Han Y, et al. Recent and potential developments of biofluid analyses in metabolomics. J Proteomics 2012;75:1079-1088.

145. Bouatra S, Aziat F, Mandal R, Guo AC, et al. The human urine metabolome. PLoS One 2013;8:e73076.

146. Pauling L, Robinson AB, Teranishi R, Cary P. Quantitative analysis of urine vapor and breath by gas-liquid partition chromatography. Proc Natl Acad Sci U S A 1971;68:2374-2376.

147. Loureiro CC, Oliveira AS, Santos M, Rudnitskaya A, et al. Urinary metabolomic profiling of asthmatics can be related to clinical characteristics. Allergy 2016;71:1362-1365.

148. Loureiro CC, Duarte IF, Gomes J, Carrola J, et al. Urinary metabolomic changes as a predictive biomarker of asthma exacerbation. J Allergy Clin Immunol 2014;133:261-263.e265.

149. Adamko DJ, Nair P, Mayers I, Tsuyuki RT, et al. Metabolomic profiling of asthma and chronic obstructive pulmonary disease: a pilot study differentiating diseases. J Allergy Clin Immunol 2015;136:571-580.e3.

150. Saude EJ, Skappak CD, Regush S, Cook K, et al. Metabolomic profiling of asthma: Diagnostic utility of urine nuclear magnetic resonance spectroscopy. J Allergy Clin Immunol 2011;127:757-764.e756.

151. Fukuhara K, Ohno A, Ota Y, Senoo Y, et al. NMR-based metabolomics of urine in a mouse model of Alzheimer's disease: identification of oxidative stress biomarkers. J Clin Biochem Nutr 2013;52:133-138.

152. Michell AW, Mosedale D, Grainger DJ, Barker RA. Metabolomic analysis of urine and serum in Parkinson's disease. Metabolomics 2008;4:191.

153. Zheng H, Cai A, Zhou Q, Xu P, et al. Optimal preprocessing of serum and urine metabolomic data fusion for staging prostate cancer through design of experiment. Anal Chim Acta 2017;991:68-75.

154. Ganti S, Weiss RH. Urine metabolomics for kidney cancer detection and biomarker discovery. Urol Oncol-Semin O I 2011;29:551-557.

155. Mendes B, Silva P, Mendonça I, Pereira J, et al. A new and fast methodology to assess oxidative damage in cardiovascular diseases risk development through eVol-MEPS–UHPLC analysis of four urinary biomarkers. Talanta 2013;116:164-172.

156. Gonçalves JL, Alves VL, Conceição CJF, Teixeira HM, et al. Development of MEPS–UHPLC/PDA methodology for the quantification of clozapine, risperidone and their major active metabolites in human urine. Microchem J 2015;123:90-98.

157. Alves V, Gonçalves J, Conceição C, Teixeira HM, et al. An improved analytical strategy combining microextraction by packed sorbent combined with ultra high pressure liquid chromatography for the determination of fluoxetine, clomipramine and their active metabolites in human urine. J Chromatogr A 2015;1408:30-40.

158. Dodds MWJ, Johnson DA, Yeh C-K. Health benefits of saliva: a review. J Dent 2005;33:223-233.

159. Liu J, Duan Y. Saliva: A potential media for disease diagnostics and monitoring. Oral Oncol 2012;48:569-577.

160. Lima DP, Diniz DG, Moimaz SA, Sumida DH, et al. Saliva: reflection of the body. Int J Infect Dis 2010;14:e184-188.

161. Chiappin S, Antonelli G, Gatti R, De Palo EF. Saliva specimen: a new laboratory tool for diagnostic and basic investigation. Clin Chim Acta 2007;383:30-40.

162. Wang Z, Zhang J, Wei W, Zhou D, et al. Identification of saliva using microRNA biomarkers for forensic purpose. J Forensic Sci 2015;60:702-706.

163. Gonzalez M, Banderas JA, Baez A, Belmont R. Salivary lead and cadmium in a young population residing in Mexico city. Toxicol Lett 1997;93:55-64.

164. Cavaco C, Perestrelo R, Silva CL, Aveiro F, et al. Establishment of the saliva volatomic profile as an exploratory and non-invasive strategy to find potential breast cancer biomarkers. International Labmate 2014;4-5.

165. Popov TA. Human exhaled breath analysis. Ann Allergy Asthma Immunol 2011;106:451-456.

166. Kubáň P, Foret F. Exhaled breath condensate: Determination of non-volatile compounds and their potential for clinical diagnosis and monitoring. A review. Anal Chim Acta 2013;805:1-18.

167. Lourenço C, Turner C. Breath analysis in disease diagnosis: methodological considerations and applications. Metabolites 2014;4:465-498.

168. Neerincx AH, Vijverberg SJH, Bos LDJ, Brinkman P, et al. Breathomics from exhaled volatile organic compounds in pediatric asthma. Pediatr Pulmonol 2017;52:1616-1627.

169. Fens N, Zwinderman AH, van der Schee MP, de Nijs SB, et al. Exhaled breath profiling enables discrimination of chronic obstructive pulmonary disease and asthma. Am J Respir Crit Care Med 2009;180:1076-1082.

170. Phillips M, Gleeson K, Hughes JMB, Greenberg J, et al. Volatile organic compounds in breath as markers of lung cancer: a cross-sectional study. Lancet 1999;353:1930-1933.

171. Peng G, Hakim M, Broza YY, Billan S, et al. Detection of lung, breast, colorectal, and prostate cancers from exhaled breath using a single array of nanosensors. Br J Cancer 2010;103:542-551.

172. Samara MA, Tang WHW, Cikach F, Gul Z, et al. Single exhaled breath metabolomic analysis identifies unique breathprint in patients with acute decompensated heart failure. J Am Coll Cardiol 2013;61:1463-1464.

173. Risticevic S, Vuckovic D, Lord HL, Pawliszyn J. 2.21 - Solid-phase microextraction. In: Pawliszyn J, editor. Comprehensive sampling and sample preparation. Oxford: Academic Press, 2012. p. 419-460.

174. Arthur CL, Pawliszyn J. Solid phase microextraction with thermal desorption using fused silica optical fibers. Anal Chem 1990;62:2145-2148.

175. Liu H, Dasgupta PK. Analytical chemistry in a drop. Solvent extraction in a microdrop. Anal Chem 1996;68:1817-1821.

176. Filippou O, Bitas D, Samanidou V. Green approaches in sample preparation of bioanalytical samples prior to chromatographic analysis. J Chromatogr B 2017;1043:44-62.

177. Ocaña-González JA, Fernández-Torres R, Bello-López MÁ, Ramos-Payán M. New developments in microextraction techniques in bioanalysis. A review. Anal Chim Acta 2016;905:8-23.

178. Pereira J, Gonçalves J, Alves V, Câmara JS. Microextraction using packed sorbent as an effective and high-throughput sample extraction technique: recent applications and future trends. Sample Prep 2013;1:38-53.

179. Abdel-Rehim M. Recent advances in microextraction by packed sorbent for bioanalysis. J Chromatogr A 2010;1217:2569-2580.

180. Pereira J, Silva CL, Perestrelo R, Goncalves J, et al. Re-exploring the high-throughput potential of microextraction techniques, SPME and MEPS, as powerful strategies for medical diagnostic purposes. Innovative approaches, recent applications and future trends. Anal Bioanal Chem 2014;406:2101-2122.

181. Abdel-Rehim M. New trend in sample preparation: on-line microextraction in packed syringe for liquid and gas chromatography applications. I. Determination of local anaesthetics in human plasma samples using gas chromatography–mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 2004;801:317-321.

182. Mendes B, Silva P, Aveiro F, Pereira J, et al. A micro-extraction technique using a new digitally controlled syringe combined with UHPLC for assessment of urinary biomarkers of oxidatively damaged DNA. PLoS One 2013;8:9.

183. Moein MM, Jabbar D, Colmsjö A, Abdel-Rehim M. A needle extraction utilizing a molecularly imprinted-sol–gel xerogel for on-line microextraction of the lung cancer biomarker bilirubin from plasma and urine samples. J Chromatogr A 2014;1366:15-23.

184. Ares AM, Fernández P, Regenjo M, Fernández AM, et al. A fast bioanalytical method based on microextraction by packed sorbent and UPLC–MS/MS for determining new psychoactive substances in oral fluid. Talanta 2017;174:454-461.

185. Amiri A, Ghaemi F. Microextraction in packed syringe by using a three-dimensional carbon nanotube/carbon nanofiber-graphene nanostructure coupled to dispersive liquid-liquid microextraction for the determination of phthalate esters in water samples. Microchimica Acta 2017;184:3851-3858.

186. Adam M, Pavlíková P, Čížková A, Bajerová P, et al. Microextraction by packed sorbent (MEPS) as a suitable selective method for l-ascorbic acid determination in beverages. Food Chem 2012;135:1613-1618.

187. Risticevic S, Lord H, Gorecki T, Arthur CL, et al. Protocol for solid-phase microextraction method development. Nat Protoc 2010;5:122-139.

188. Kataoka H, Lord HL, Pawliszyn J. Applications of solid-phase microextraction in food analysis. J Chromatogr A 2000;880:35-62.

189. Schmidt K, Podmore I. Solid phase microextraction (SPME) method development in analysis of volatile organic compounds (VOCS) as potential biomarkers of cancer. J Mol Biomark Diagn 2015;6:253.

190. Couto M, Barbosa C, Silva D, Rudnitskaya A, et al. Oxidative stress in asthmatic and non-asthmatic adolescent swimmers-A breathomics approach. Pediatr Allergy Immunol 2017;28:452-457.

191. Porto-Figueira P, Camacho I, Câmara JS. Exploring the potentialities of an improved ultrasound-assisted quick, easy, cheap, effective, rugged, and safe-based extraction technique combined with ultrahigh pressure liquid chromatography-fluorescence detection for determination of Zearalenone in cereals. J Chromatogr A 2015;1408:187-196.

192. Moein MM, Abdel-Rehim A, Abdel-Rehim M. Microextraction by packed sorbent (MEPS). Trends Anal Chem 2015;67:34-44.

193. Porto-Figueira P, Figueira JA, Pereira JAM, Câmara JS. A fast and innovative microextraction technique, μSPEed, followed by ultrahigh performance liquid chromatography for the analysis of phenolic compounds in teas. J Chromatogr A 2015;1424:1-9.

194. Anastassiades M, Lehotay SJ, Stajnbaher D, Schenck FJ. Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce. J AOAC Int 2003;86:412-431.

195. Stockelhuber M, Muller C, Vetter F, Mingo V, et al. Determination of pesticides adsorbed on arthropods and gastropods by a micro-QuEChERS approach and GC-MS/MS. Chromatographia 2017;80:825-829.

196. Brandhonneur N, Mendes M, Lepvrier E, Esseiva E, et al. A micro-QuEChERS method coupled to GC–MS for the quantification of pesticides in specific maternal and fetal tissues. J Pharm Biomed Anal 2015;104:90-96.

197. Correia-Sá L, Norberto S, Delerue-Matos C, Calhau C, et al. Micro-QuEChERS extraction coupled to GC–MS for a fast determination of Bisphenol A in human urine. J Chromatogr B 2018;1072:9-16.

198. Raschdorf F. Rapid measurements in the ppm and ppb region. Chimia 1978;32:478-483.

199. Koziel JA, Odziemkowski M, Pawliszyn J. Sampling and analysis of airborne particulate matter and aerosols using in-needle trap and SPME fiber devices. Anal Chem 2001;73:47-54.

200. Lord HL, Zhan W, Pawliszyn J. Fundamentals and applications of needle trap devices: A critical review. Anal Chim Acta 2010;677:3-18.

201. Lord HL, Zhan W, Pawliszyn J. 2.30 - Fundamentals and applications of needle trap devices. In: Pawliszyn J, editor. Comprehensive Sampling and Sample Preparation. Oxford: Academic Press, 2012. p. 677-697.

202. Cheng WH, Huang HL, Chen KS, Chang YJ. Quantification of VOC emissions from paint spraying on a construction site using solid phase microextraction devices. J Environ Sci Health A Tox Hazard Subst Environ Eng 2017;52:1158-1163.

203. Kleeblatt J, Schubert JK, Zimmermann R. Detection of gaseous compounds by needle trap sampling and direct thermal-desorption photoionization mass spectrometry: concept and demonstrative application to breath gas analysis. Anal Chem 2015;87:1773-1781.

204. Azari MR, Barkhordari A, Zendehdel R, Heidari M. A novel needle trap device with nanoporous silica aerogel packed for sampling and analysis of volatile aldehyde compounds in air. Microchem J 2017;134:270-276.

205. Alonso M, Castellanos M, Besalú E, Sanchez JM. A headspace needle-trap method for the analysis of volatile organic compounds in whole blood. J Chromatogr A 2012;1252:23-30.

206. Jiménez-Díaz I, Vela-Soria F, Rodríguez-Gómez R, Zafra-Gómez A, et al. Analytical methods for the assessment of endocrine disrupting chemical exposure during human fetal and lactation stages: a review. Anal Chim Acta 2015;892:27-48.

207. Denoroy L, Zimmer L, Renaud B, Parrot S. Ultra high performance liquid chromatography as a tool for the discovery and the analysis of biomarkers of diseases: a review. J Chromatogr B 2013;927:37-53.

208. Swartz ME. UPLC™: an introduction and review. J Liq Chromatogr Relat Technol 2005;28:1253-1263.

209. Inaba T, Brien JF. Determination of the major urinary metabolite of diphenylhydantoin by high-performance liquid chromatography. J Chromatogr A 1973;80:161-165.

210. Arpino PJ. On-line liquid chromatography/mass spectrometry? An odd couple! Trends Analyt Chem 1982;1:154-158.

211. Skoog DA, West DM, Holler FJ, Crouch SR. High-performance liquid chromatography. In: Simpson C, editor. Fundamentals of analytical chemistry (9th edition). USA: Mary Finch, 2014. p. 912-934.

212. Skoog DA, West DM, Holler FJ, Crouch SR. Gas chromatography. In: Simpson C, editor. Fundamentals of analytical chemistry (9th edition). USA: Mary Finch, 2014. p. 887-911.

213. Dalgliesh CE, Horning EC, Horning MG, Knox KL, et al. A gas-liquid-chromatographic procedure for separating a wide range of metabolites occuring in urine or tissue extracts. Biochem J 1966;101:792.

214. Purcaro G, Moret S, Conte L. Hyphenated liquid chromatography–gas chromatography technique: recent evolution and applications. J Chromatogr A 2012;1255:100-111.

215. Field A. The independent t-test. In: Discovering statistics using IBM SPSS statistics. London: SAGE Publications Ltd, 2009. p. 334-341.

216. Long FH. Chapter 19 - Multivariate Analysis for Metabolomics and Proteomics Data. In: Issaq HJ, Veenstra TD, editors. Proteomic and metabolomic approaches to biomarker discovery. Boston: Academic Press, 2013. p. 299-311.

217. Jolliffe I. Principal component analysis. In: Everitt BS, Howell DC, editors. Encyclopedia of statistics in behavioral science. Chichester: John Wiley & Sons, Ltd, 2005. p.1580-1584.

218. Câmara JS, Alves MA, Marques JC. Multivariate analysis for the classification and differentiation of Madeira wines according to main grape varieties. Talanta 2006;68:1512-1521.

219. Gromski PS, Muhamadali H, Ellis DI, Xu Y, et al. A tutorial review: metabolomics and partial least squares-discriminant analysis – a marriage of convenience or a shotgun wedding. Anal Chim Acta 2015;879:10-23.

220. Global Initiative for Asthma. Global strategy for asthma management and prevention. 2017. Accessed on October 2017. available from: http://www.ginasthma.org/.

221. Kuang Z, Wilson JJ, Luo S, Zhu SW, et al. Deciphering asthma biomarkers with protein profiling technology. Int J Inflam 2015;2015:630637.

222. James A, Hedlin G. Biomarkers for the phenotyping and monitoring of asthma in children. Curr Treat Options Allergy 2016;3:439-452.

223. Pite H, Morais-Almeida M, Mensinga T, Diamant Z. Non-invasive biomarkers in asthma: promises and pitfalls. In: Pereira C, editor. Asthma - from childhood asthma to ACOS phenotypes. InTech, 2016. p. 15-39.

224. Diamant Z, Boot JD, Mantzouranis E, Flohr R, et al. Biomarkers in asthma and allergic rhinitis. Pulm Pharmacol Ther 2010;23:468-481.

225. Balgoma D, Larsson J, Rokach J, Lawson JA, et al. Quantification of lipid mediator metabolites in human urine from asthma patients by electrospray ionization mass spectrometry: controlling matrix effects. Anal Chem 2013;85:7866-7874.

226. Matacuta I. Is there an ideal biomarker for pediatric asthma? Int J Recent Sci Res 2015;6:5561-5568.

227. O'Sullivan S, Dahlen B, Dahlen SE, Kumlin M. Increased urinary excretion of the prostaglandin D2 metabolite 9 alpha, 11 beta-prostaglandin F2 after aspirin challenge supports mast cell activation in aspirin-induced airway obstruction. J Allergy Clin Immunol 1996;98:421-432.

228. Green SA, Malice MP, Tanaka W, Tozzi CA, et al. Increase in urinary leukotriene LTE4 levels in acute asthma: correlation with airflow limitation. Thorax 2004;59:100-104.

229. Szefler SJ, Wenzel S, Brown R, Erzurum SC, et al. Asthma outcomes: biomarkers. J Allergy Clin Immunol 2012;129:S9-23.

230. Leung TF, Ko FW, Wong GW. Recent advances in asthma biomarker research. Ther Adv Respir Dis 2013;7:297-308.

231. Di Gangi IM, Pirillo P, Carraro S, Gucciardi A, et al. Online trapping and enrichment ultra performance liquid chromatography-tandem mass spectrometry method for sensitive measurement of "arginine-asymmetric dimethylarginine cycle" biomarkers in human exhaled breath condensate. Anal Chim Acta 2012;754:67-74.

232. Mattarucchi E, Baraldi E, Guillou C. Metabolomics applied to urine samples in childhood asthma; differentiation between asthma phenotypes and identification of relevant metabolites. Biomed Chromatogr 2012;26:89-94.

233. Rabinovitch N, Reisdorph N, Silveira L, Gelfand EW. Urinary leukotriene E(4) levels identify children with tobacco smoke exposure at risk for asthma exacerbation. J Allergy Clin Immunol 2011;128:323-327.

234. Rabinovitch N, Mauger DT, Reisdorph N, Covar R, et al. Predictors of asthma control and lung function responsiveness to step-3 therapy in children with uncontrolled asthma. J Allergy Clin Immunol 2014;133:350-356.

235. Divekar R, Hagan J, Rank M, Park M, et al. Diagnostic utility of urinary LTE4 in asthma, allergic rhinitis, chronic rhinosinusitis, nasal polyps, and aspirin sensitivity. J Allergy Clin Immunol Pract 2016;4:665-670.

236. Wilsenach JA, Schuurbiers CAH, van Loosdrecht MCM. Phosphate and potassium recovery from source separated urine through struvite precipitation. Water Res 2007;41:458-466.

237. Magnusson B, Örnemark U, editors. Eurachem guide: the fitness for purpose of analytical methods – a laboratory guide to method validation and related topics. 2014. Accessed on October 2017. available from: www.eurachem.org.

238. Valerio MA, Andreski PM, Schoeni RF, McGonagle KA. Examining the association between childhood asthma and parent and grandparent asthma status: implications for practice. Clin Pediatr (Phila) 2010;49:535-541.

239. de Marco R, Locatelli F, Sunyer J, Burney P. Differences in incidence of reported asthma related to age in men and women. Am J Respir Crit Care Med 2000;162:68-74.

240. Fernández-Riejos P, Najib S, Santos-Alvarez J, Martín-Romero C, et al. Role of leptin in the activation of immune cells. Mediators Inflamm 2010;2010:568343.

241. Guler N, Kirerleri E, Ones U, Tamay Z, et al. Leptin: does it have any role in childhood asthma? J Allergy Clin Immunol 2004;114:254-259.

242. de Groot EP, Duiverman EJ, Brand PL. Comorbidities of asthma during childhood: possibly important, yet poorly studied. Eur Respir J 2010;36:671-678.

243. Sa-Sousa A, Morais-Almeida M, Azevedo LF, Carvalho R, et al. Prevalence of asthma in Portugal - The Portuguese national asthma survey. Clin Transl Allergy 2012;2:15.

244. Boulet LP, Turcotte H, Laprise C, Lavertu C, et al. Comparative degree and type of sensitization to common indoor and outdoor allergens in subjects with allergic rhinitis and/or asthma. Clin Exp Allergy 1997;27:52-59.

245. Supelco. Guide to Solid Phase Extraction. 1996. Accessed on November 2017. available from: https://www.sigmaaldrich.com/.

246. Perestrelo R, Silva CL, Câmara JS. Determination of urinary levels of leukotriene B4 using ad highly specific and sensitive methodology based on automatic MEPS combined with UHPLC-PDA analysis. Talanta 2015;144:382-389.

247. Dahlen SE, Kumlin M. Monitoring mast cell activation by prostaglandin D2 in vivo. Thorax 2004;59:453-455.

248. Trischler J, Muller CM, Konitzer S, Prell E, et al. Elevated exhaled leukotriene B(4) in the small airway compartment in children with asthma. Ann Allergy Asthma Immunol 2015;114:111-116.

249. Higham A, Cadden P, Southworth T, Rossall M, et al. Leukotriene B4 levels in sputum from asthma patients. ERJ Open Research 2016;2:00088-02015.

Annexes

Annex I – Composition of the synthetic urine

Table 1A – Composition of the synthetic urine used during the optimization and validation of the method.

Name	**Formula**	**g L^{-1}**
Calcium chloride-2-hydrate	$CaCl_2 \cdot 2H_2O$	0.65
Magnesium chloride-6-hydrate	$MgCl_2 \cdot 6H_2O$	0.65
Sodium chloride	$NaCl$	4.60
Sodium sulfate anhydrous	Na_2SO_4	2.30
tri-Sodium citrate-2-hydrate	$Na_3C_6H_5O_7 \cdot 2H_2O$	0.65
Sodium carbonate anhydrous	Na_2CO_3	0.02
Potassium di-hydrogen phosphate	KH_2PO_4	4.20
Potassium chloride	KCl	1.60
Ammonium chloride	NH_4Cl	1.00

Annex II – SESARAM authorization

SESARAM
Serviço de Saúde da RAMadeira

(CES/ SESARAM, EPE)

PARECER nº 42/2015

Sobre o Pedido/Estudo:
"Implementação de Nova Abordagem Metodológica para a Detecção de Biomarcadores de Doenças Respiratórias de Natureza Alérgica. A Asma como Estudo Caso."

A – RELATÓRIO

A.1 A Comissão de Ética para Saúde (CES) do Serviço de Saúde da Região Autónoma da Madeira, EPE (SESARAM, EPE), analisou o documento Nº 72, pedido do **Professor Doutor José Sousa Câmara**, no âmbito de um projecto de Mestrado de Bioquímica Aplicada, da Universidade da Madeira, e colaboração com a **Dra Susana Oliveira**, médica do Serviço de Alergologia do Hospital Dr. Nélio Mendonça, para realizar estudo sobre "***Implementação de Nova Abordagem Metodológica para a Detecção de Biomarcadores de Doenças Respiratórias de Natureza Alergica. A Asma como Estudo Caso"***. Trata-se de um estudo que pretende estabelecer o perfil metabólico volátil de pacientes com Asma, de modo a identificar um conjunto de biomarcadores da patologia como estratégia para a sua detecção e monitorização.

A.2 O documento em análise é constituído por: ofício dirigido ao Conselho de Administração do SESARAM, EPE (EA578800) datado de 30 de Novembro de 2015, que inclui consentimento informado, projecto do estudo, formulário de inquérito, questionário de submissão e email recebido a 15 de Dezembro de 2015 que inclui consentimento informado alterado e projecto do estudo alterado.

A.3 Trata-se de um estudo que pretende identificar um conjunto de metabólitos voláteis presentes em fluídos (urina e saliva), com potencial para a detecção da asma. O mesmo será recolhido aquando da consulta de acompanhamento de rotina em alergologia. Irá ser aplicado em crianças entre os 6 e 12 anos, portadoras deste tipo de patologia, e em crianças da mesma idade de escolas da RAM devidamente autorizadas pela Direcção Regional de Educação, como grupo

SESARAM, E.P.E.
Serviço de Saúde da RAM, E.P.E.

de controlo e sem a patologia. Serão usadas ferramentas estatísticas com o objectivo de analisar os sinais instrumentais e estabelecer biomarcadores por comparação dos perfis da população alvo e de controlo. O estudo pretende estabelecer as bases metodológicas e químicas que permitam contribuir para um desenvolvimento de um meio de diagnóstico rápido, barato e não invasivo. As amostras serão recolhidas e supervisionadas por um técnico de saúde após autorização dos pais ou tutores, e destruídas uma vez terminado o estudo. Aos participantes no estudo será ainda solicitado o preenchimento de um pequeno formulário inquérito.

B – IDENTIFICAÇÃO DAS QUESTÕES COM EVENTUAIS IMPLICAÇÕES ÉTICAS

B.1 Serão salvaguardados ao longo do estudo, os princípios éticos relativos ao trabalho de investigação, nomeadamente no que se refere à autorização e anonimato dos participantes e confidencialidade dos dados.

B.2 Reconhece-se a pertinência do estudo e o interesse prático nos resultados esperados, sendo que a metodologia utilizada salvaguarda o direito dos participantes.

C – CONCLUSÂO

A CES/SESARAM, EPE deliberou emitir **Parecer Favorável,** por não envolver quaisquer questões de ordem ética.

Aprovado em reunião dia 15 de Dezembro de 2015, por unanimidade.

O Presidente da CES/SESARAM, EPE

(Ricardo Santos)

Annex III – DRE authorization

S. R.

REGIÃO AUTÓNOMA DA MADEIRA
Governo Regional
SECRETARIA REGIONAL DE EDUCAÇÃO
DIREÇÃO REGIONAL DE EDUCAÇÃO

Exmo. Senhor
José Câmara
jsc@uma.pt
camire@uma.pt

C/C. EB1/PE V. Cacongo
EB1/PE Tanque - Monte

ASSUNTO: **Autorização para realização de estudo em escolas da RAM**

Na sequência da vossa solicitação, e por despacho do Exmo. Senhor Diretor Regional de Educação, de 22/04/2016, informa-se V. Exa. que está autorizada a aplicação do estudo aos alunos ou encarregados de educação (inquérito e recolha de fluidos líquidos) das escolas selecionadas, submetidos à respetiva anuência, no âmbito do mestrado em Bioquímica Aplicada, pelo mestrando Pedro Berenguer, promovido pela Universidade da Madeira.

Mais se informa que, para efeitos da concretização do estudo, deverá o mestrando proceder à articulação com as direções escolares.

Com os melhores cumprimentos,

O Diretor de Serviços de Investigação,
Formação e Inovação Educacional

(Bernardo Lage Valério)

Direção Regional de Educação
GGAR

SAÍDA	PROCESSO(s)	DATA
Of: 940	5.67.0.0	27-04-2016

BV/MJM

Na resposta indicar a «Nossa Referência». Em cada ofício tratar só de um assunto

Annex IV – Information to the subject of investigation

Projecto: VOM_ASM

Informação ao Sujeito de Investigação

Por favor, leia com atenção a informação referente à explicação do projeto. Se achar que algo está incorreto ou que não está claro, não hesite em solicitar mais informações.

Responsáveis pelo projecto

Prof. Dr. José Sousa Câmara; Centro de Química da Madeira (CQM/UMa); Faculdade de Ciências Exatas e da Engenharia da Universidade da Madeira

Telefone: 351-291705112 Fax : 351-291705149 Email: jsc@uma.pt

Drª. Susana Oliveira; Hospital Dr. Nélio Mendonça, SESARAM, E.P.E.; Avenida Luís de Camões, nº 57 – 9004-514 Funchal

Título do estudo:

IMPLEMENTAÇÃO DE NOVA ABORDAGEM METODOLÓGICA PARA A DETEÇÃO DE BIOMARCADORES DE DOENÇAS RESPIRATÓRIAS DE NATUREZA ALÉRGICA. A ASMA COMO CASO DE ESTUDO

Pretende-se desenvolver uma metodologia analítica que permita a identificação de biomarcadores associados a patologias respiratórias de natureza alérgica. Este estudo objectiva estabelecer as bases metodológicas e químicas que permitam contribuir para o desenvolvimento de um **meio de diagnóstico rápido, barato e não invasivo**, que possa ser aplicado a um número alargado de indivíduos, mesmo numa fase assintomática, assim como para **monitorizar a evolução dos efeitos terapêuticos.**

Para tal precisamos da **colaboração voluntária do Vosso Educando** no estudo, que se traduz na ***cedência de amostras de urina (jato médio) e saliva***, que serão recolhidas no Hospital Dr. Nélio Mendonça aquando da consulta de rotina e/ou entrada / internamento médico. Esta recolha será supervisionada por um técnico de saúde sob a coordenação da Drª Susana Oliveira. Solicitamos igualmente a **colaboração voluntária dos Pais/Encarregados de Educação/representante legal** para a recolha das amostras, caso seja necessário.

Aos sujeitos, que voluntariamente participarem no estudo, não serão impostas quaisquer restrições alimentares ou de outro tipo, apenas será importante o preenchimento de um pequeno formulário inquérito.

O estudo não acarretará riscos ou encargos para os participantes. Não haverá qualquer tipo de contacto do Investigador com o sujeito dador.

O estudo tem o parecer favorável da Comissão de Ética para a Saúde do (CES) do Serviço Regional de Saúde.

Confidencialidade e anonimato: As amostras serão codificadas pelos responsáveis, e só eles terão acesso ao binómio código / indivíduo, garantindo desta forma a ***máxima confidencialidade e anonimato dos resultados***.

Agradecidos desde já pela V. atenção

Prof. Dr. José Sousa Câmara
Investigador e Professor Auxiliar na Universidade da Madeira,
Centro de Química da Madeira (CQM/UMa); Faculdade de Ciências Exatas e Engenharia da Universidade da Madeira
Telefone: 351-291705112 Fax : 351-291705149
Email: jsc@uma.pt

Drª. Susana Oliveira
Serviço de Alergologia do Hospital Dr. Nélio Mendonça,
SESARAM, E.P.E.; Avenida Luís de Camões, nº 57 – 904-514 Funchal

Annex V – Informed consent

IMPLEMENTAÇÃO DE NOVA ABORDAGEM METODOLÓGICA PARA A DETEÇÃO DE BIOMARCADORES DE DOENÇAS RESPIRATÓRIAS DE NATUREZA ALÉRGICA. A ASMA COMO CASO DE ESTUDO

Projecto: VOM_ASM

Consentimento Informado Livre e Esclarecido para Participação em Investigação
de acordo com a Declaração de Helsínquia e a Convenção de Oviedo

Por favor, leia com atenção a folha de informação referente à explicação do projeto. Se achar que algo está incorreto ou que não está claro, não hesite em solicitar mais informações. Se concorda em participar ou se permite a participação do seu educando de forma voluntária no estudo queira assinar este documento.

Eu, _____,

declaro que após a leitura e compreensão do documento de Informação referente à pesquisa e objetivos do projeto VOM-ASM, autorizo voluntariamente o meu educando a participar no estudo com a cedência de amostras de urina e saliva e autorizo o processamento e a publicação anónima e confidencial dos dados/resultados obtidos. Mais informo que me é facultada a possibilidade de desistência de participação no estudo em qualquer momento.

Funchal, _____ de _____ de 201__

O Investigador **O Declarante/Sujeito**

_____ _____

Annex VI – Questionnaire for asthmatic patients from hospital

Projecto: VOM_ASM

Formulário de inquérito

Data de preenchimento: ___/___/_____

Instituição:	Nº amostra:

Este questionário destina-se **exclusivamente** à recolha de dados de investigação para a elaboração do projeto "IMPLEMENTAÇÃO DE NOVA ABORDAGEM METODOLÓGICA PARA A DETEÇÃO DE BIOMARCADORES DE DOENÇAS RESPIRATÓRIAS DE NATUREZA ALÉRGICA. A ASMA COMO CASO DE ESTUDO".

Os dados recolhidos são **confidenciais**. A veracidade das respostas é de importância crucial na elaboração do estudo.
Caso tenha alguma dúvida nas questões, peça esclarecimento ao Técnico de Saúde presente.

Agradecemos desde já pela atenção e colaboração,
José Sousa Câmara (Universidade da Madeira)
Drª. Susana Oliveira (Hospital Dr. Nélio Mendonça)

Dados Pessoais:

Idade:	Sexo: M ☐ F ☐

Informações gerais acerca de saúde e hábitos de consumo do sujeito

	Sim	**Não**
Estado actual	Totalmente Controlado	Parcialmente controlado
	Não controlado	
Diagnóstico com base na gravidade	Intermitente	Persistente ligeira
	Persistente moderada	Persistente severa
Teve algum ataque de asma nos últimos 12 meses?		
Data da última crise asmática	201_/__/__	
Faltou à escola devido a problemas de asma nos últimos 12 meses?		
O Encarregado de Educação faltou ao trabalho devido a problemas de asma do educando?		
Tem conjuntivite alérgica		

Tem alguma alergia nasal?		
Já lhe foi diagnosticada rinite alérgica?		
Já lhe foi diagnosticado sinusite?		
Já lhe foi diagnosticado bronquite?		
Já teve alergias na pele ou eczema?		
Sensibilizações		
É fumador?		
Quantos membros da sua residência são fumadores?		
Quantos cigarros (em média) fumam por dia?		
Tipo de habitação.		
Quantos quartos tem a sua habitação?		
Quantas pessoas vivem na sua habitação?		
Na sua habitação possui esquentador a gás?		
Dentro ou fora de casa?		
Na sua habitação possui cozinha/churrascaria a lenha?		
Possui chaminé?		
Vive perto de algum sítio que liberte poeiras ou fumos?		
Que sítio?		
Tem familiares com asma?		
Quem?		
Toma medicação para a asma (ex. inaladores ou outros)?		
Qual? _____		

_____

Com que frequência (tomas diárias)?	1	2	≥3

Naturalidade _____

Número do processo clínico _____

Médico _____

Annex VII – Questionnaire for healthy individuals from schools

UNIVERSIDADE da MADEIRA

SESARAM EPE
Serviço de Saúde da RAM EPE

Projecto: VOM_ASM

Amostra_____

Lê com atenção as perguntas. Se tiveres alguma dúvida pergunta aos teus pais ou ao teu professor.

Qual é o nome da tua escola? _____

Qual é o teu número de aluno? _____

Sexo: Masculino ☐ ou Feminino ☐

Quantos anos tens? _____

És natural de onde? _____

Data _____

Por favor assinala com um X a resposta que descreve melhor a pergunta. Algumas perguntas tens de colocar um número.

	Sim	Não
Alguma vez disseram que tinhas asma?	☐	☐
Costumas ter falta de ar por causa de alergias?	☐	☐
Costumas ter pieira por causa de alergias?	☐	☐
Costumas ter tosse por causa de alergias?	☐	☐
Tiveste algum ataque de asma durante o último ano?	☐	☐
Faltaste à escola ou outras actividades durante o último ano?	☐	☐
O teu pai, a tua mãe ou a pessoa que cuida de ti faltaram ao trabalho por tua causa?	☐	☐

	Sim	Não
Costumas ter comichão ou irritação nos olhos por causa de alergias?	☐	☐
Costumas ter comichão no nariz ou na garganta por causa de alergias?	☐	☐
Costumas espirrar por causa de alergias?	☐	☐
Costumas ter ranho ou o nariz a pingar por causa de alergias?	☐	☐
Costumas ter a pele irritada, vermelha, seca, com borbulhas ou a escamar por causa de alguma alergia?	☐	☐
Tens alergia ao pó, poeira?	☐	☐
Tens alergia ao pêlo de animais?	☐	☐
Tens alergia ao pólen ou a flores?	☐	☐
Tens alergia a baratas ou outros insectos?	☐	☐

Quantas pessoas vivem contigo? _____

Alguma(s) das pessoas com quem vives fuma(m)? Quantas? _____

Quantos cigarros fuma(m)/dia (mais ou menos)? _____

	Casa	Apa
Vives numa casa ou apartamento (Apa)?	☐	☐

	Sim	Não
Tens esquentador em casa?	☐	☐
O teu esquentador é a gás?	☐	☐
O teu esquentador fica dentro de casa?	☐	☐
Tens alguma cozinha a lenha ou churrascaria em casa?	☐	☐
E tem chaminé?	☐	☐

Sim	Não

Há alguma oficina de carros que faça fumo perto da tua casa? ☐ ☐

	Sim	Não
Há algum sítio perto da tua casa onde trabalhem com madeira ou pedra e faça poeira?	☐	☐
O teu pai ou alguém da família da parte do teu pai tem asma?	☐	☐
A tua mãe ou alguém da família da parte da tua mãe tem asma?	☐	☐
Tomas remédios para a asma ou as tuas alergias?	☐	☐

	1	2	3
Quantas vezes por dia?	☐	☐	☐

O que é que tomas para a asma ou para as tuas alergias?

_____

Annex VIII – Concentration of the eicosanoids obtained for the urine of asthmatic patients and healthy individuals

Table 2A – Levels (ng mL^{-1}) of 11β-prostaglandin $F_{2\alpha}$ ($11\beta PGF_{2\alpha}$), leukotriene E_4 (LTE_4) and leukotriene B_4 (LTB_4) determined in urine of asthmatic patients and healthy individuals (control group).

Sample	$11\beta PGF_{2\alpha}$[a] (ng mL^{-1})	LTE_4[b] (ng mL^{-1})	LTB_4[c] (ng mL^{-1})
Asthmatics			
1	48.71 ± 1.06	0.92 ± 0.07	1.62 ± 0.07
2	44.62 ± 8.57	2.80 ± 0.10	2.14 ± 0.05
3	47.05 ± 6.78	< LOD[d]	1.27 ± 0.05
4	34.99 ± 2.70	1.60 ± 0.32	1.00 ± 0.01
5	103.65 ± 13.25	3.12 ± 0.25	1.07 ± 0.04
6	310.32 ± 9.59	3.27 ± 0.23	2.64 ± 0.05
7	99.25 ± 8.53	< LOD[d]	< LOD[d]
8	< LOD[d]	< LOD[d]	1.42 ± 0.07
9	292.41 ± 13.51	1.41 ± 0.21	1.88 ± 0.11
10	173.12 ± 21.64	1.81 ± 0.08	2.22 ± 0.07
11	178.50 ± 21.06	0.94 ± 0.07	1.95 ± 0.07
12	69.84 ± 2.06	2.34 ± 0.14	0.90 ± 0.01
13	181.01 ± 38.03	< LOQ[e]	1.81 ± 0.03
14	37.26 ± 1.56	1.44 ± 0.14	0.73 ± 0.04
15	32.39 ± 0.63	1.06 ± 0.22	1.79 ± 0.14
16	212.98 ± 33.85	< LOD[d]	1.31 ± 0.22
17	30.84 ± 0.79	1.70 ± 0.08	2.42 ± 0.21
18	108.05 ± 14.69	3.52 ± 0.42	1.91 ± 0.13
19	56.45 ± 4.28	0.52 ± 0.04	< LOD[d]
20	80.28 ± 2.82	< LOD[d]	0.60 ± 0.02
21	38.83 ± 1.70	< LOD[d]	< LOD[d]
22	< LOD[d]	< LOQ[e]	1.12 ± 0.05
23	170.71 ± 1.25	0.78 ± 0.03	0.77 ± 0.01
24	106.20 ± 2.10	1.18 ± 0.27	2.58 ± 0.04
25	121.05 ± 5.46	0.43 ± 0.06	1.27 ± 0.05
26	302.29 ± 15.99	4.94 ± 0.22	1.65 ± 0.08
27	139.89 ± 5.52	0.42 ± 0.03	1.40 ± 0.04
Mean	**112.96[f]**	**1.27[f]**	**1.39[f]**
Healthy			
1	113.60 ± 5.71	< LOQ[e]	< LOD[d]
2	38.09 ± 0.41	0.58 ± 0.06	1.25 ± 0.16
3	35.82 ± 0.99	0.74 ± 0.13	< LOD[d]
4	32.16 ± 1.00	0.56 ± 0.08	< LOD[d]
5	45.68 ± 1.76	< LOQ[e]	1.29 ± 0.05
6	167.29 ± 17.75	< LOD[d]	1.20 ± 0.08
7	41.84 ± 4.41	1.94 ± 0.19	1.57 ± 0.07
8	47.59 ± 1.88	< LOD[d]	0.76 ± 0.04
9	51.32 ± 1.36	2.31 ± 0.20	0.70 ± 0.04
10	58.79 ± 1.86	1.98 ± 0.26	1.74 ± 0.05
11	50.38 ± 5.26	0.71 ± 0.08	< LOD[d]
12	102.27 ± 7.67	0.47 ± 0.06	0.82 ± 0.05
13	33.79 ± 1.43	< LOQ[e]	1.03 ± 0.07
14	66.70 ± 0.84	1.59 ± 0.13	< LOD[d]
15	38.77 ± 7.32	2.53 ± 0.41	1.46 ± 0.19
16	54.09 ± 6.52	0.57 ± 0.03	1.12 ± 0.10
17	85.38 ± 6.91	1.00 ± 0.17	< LOD[d]
Mean	**62.56[f]**	**0.89[f]**	**0.76[g]**

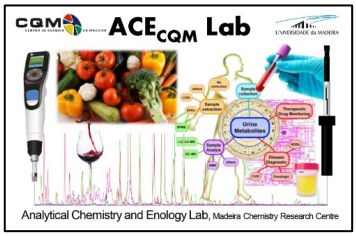
[a] $11\beta PGF_{2\alpha}$ – 11β-Prostaglandin $F_{2\alpha}$

[b] LTE_4 – Leukotriene E_4
[c] LTB_4 – Leukotriene B_4
[d] LOD – limit of detection.
[e] LOQ – limit of quantification.
Means followed by different letters (f and g) for a given parameter are significantly different at $p < 0.001$ (independent samples *t*-test).

UNIVERSIDADE da MADEIRA

CQM CENTRO DE QUÍMICA DA MADEIRA

SESARAM E.P.E.
Serviço de Saúde da RAM E.P.E.



arditi

FCT
Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA CIÊNCIA, TECNOLOGIA E ENSINO SUPERIOR

OPERAÇÃO CQM+
OPERATION CQM+

MADEIRA 14-20 · REGIÃO AUTÓNOMA DA MADEIRA · PORTUGAL 2020 · UNIÃO EUROPEIA Fundos Europeus Estruturais e de Investimento